Microbial Zoonoses

TEXTBOOK

Subhash Chandra Parija
Abhijit Chaudhury *Editors*

Textbook of Parasitic Zoonoses

Springer

Microbial Zoonoses

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Subhash Chandra Parija, Sri Balaji Vidyapeeth, A Deemed-to-be-University, Pondicherry, India

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Subhash Chandra Parija •
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The Supreme Power Who has Willed and given us the Strength to take up this work

To my wife Ms Jyotirmayee Parija for selfless support
my mother Late Smt Nishamani Parija
my father Late Shri Managovinda Parija, and
my sister-in-law Late Smt Satyabhama Parija for their blessings.
Also to my professional colleagues and mentors for their guidance

-**Subhash Chandra Parija**

To my wife Oiendrilla for her support and encouragement
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my father Late Dr Prashanta Kumar Chaudhury for their blessings

-**Abhijit Chaudhury**

Foreword

Research and studies in the field of parasitology was at one time of global quality in India at the School of Tropical Medicine, Calcutta. It was of the same standard as that of London, Liverpool, Hamburg, and Amsterdam schools. It developed in some institutes like Central Drug Research Institute, IICB, some of the ICMR specialized institutes like RMRI, Patna, centres in Bhuvaneshwar, Puducherry, and Madurai, and National Institute of Malaria Research in Delhi. It also emerged in certain academic institutes such as PGI, Chandigarh, where a full-fledged Department of Parasitology was established. There were also centres in JIPMER, Puducherry, Sher-e-Kashmir Institute in Srinagar, Banaras Hindu University, and Aligarh Muslim University. It was also scattered as some specialized centres established in TIFR, National Institute of Immunology, Jaipur University, ICGEB, etc. The Indian Society for Parasitology was also established to foster the subject. However, a good comprehensive compendium was not available particularly, when some of the major programmes deal with this subject in national and global priorities. One of the major victories was elimination of *Dracunculus medinensis*. Big success was observed in the elimination of leishmaniasis and rapid elimination of filariasis, bringing down the malaria burden to one-third as well as significantly bringing down the soil transmitted helminthiasis burden. Vector control also got big fillip as some of the viruses such as dengue, chikungunya, zika, yellow fever, and Japanese B encephalitis also had surges in India. The "One Health" concept has been adopted by WHO where human, animal, and environment are taken as a single entity to address these zoonotic diseases. Against this backdrop, Prof. SC Parija and Prof. Abhijit Chaudhury have come out with a multi-author textbook on Parasitic Zoonoses in which they have tried to put under one cover almost all the parasitic diseases of humans of animal origin.

Professors Parija and Chaudhury have undertaken a daunting task in editing a multi-author textbook with more than 60 chapters, which they have safely steered to its destination with admirable result. This book will definitely occupy a very prominent place among the practising human and animal parasitologists and will serve as an advanced textbook for students. I have full confidence that this book represents yet another important addition to the parasitology literature and fill the vacuum of a good reference book.

This book deals with the latest in these areas and will be useful for students, researchers, and teachers. This book should be revised from time to time so

that it could incorporate major new findings. Wishing this book all the success.

Indian Council for Medical Research (ICMR), N. K. Ganguly
New Delhi, India

Preface

Human interaction with animals in the form of hunting for food started more than 1.5 million years ago when the modern humans appeared. Then in the late Pleistocene era, 15,000 years back, and even before humans have started cultivation, they domesticated the dog. This dependence of humanity on the animals of diverse origin has offset a process of transfer of microorganisms from these animals to humans resulting in infections in the latter. No wonder the history of zoonotic human diseases dates back to antiquity.

Parasitic diseases in humans are a major public health problem in Asian, African, and South American continents, and with measurable presence in Europe, North America, and Australia. The vast majority of the parasitoses are linked directly or indirectly to the animal populations, leading to a scenario that most of the human parasitic diseases are zoonotic in nature. Industrialization and population burden have taken a heavy toll on the natural eco-systems of the globe, where humans are venturing into unexplored animal territories by expanding the cities, destroying the forests, building dams, etc. for their personal use and thus disturbing the flora and fauna of a region. These human activities have resulted in many parasites crossing the boundary into human domain and an increase in emerging and re-emerging parasitic zoonoses caused by so-called strict animal pathogens. Increased global connectivity has further facilitated in spreading these pathogens from their unique ecologic niche to virgin areas with similar environment. Manipulation of animals in the food stock enterprises for better and higher yields by diverse measures has resulted in altered microbiota in these animals. This has contributed to parasites travelling beyond the boundary and posing a threat to mankind. In essence, it is the human activities including the human behaviour that have also contributed immensely to the dissemination of the animal pathogens and zoonotic diseases to a large extent.

A textbook incorporating the latest information where the chapters are a healthy balance of a review article and a standard textbook is the need of the hour. It is envisaged that the *Textbook of Parasitic Zoonoses* will be useful for anybody, cutting across the disciplines and interested in zoonotic parasitic diseases. The book aims to provide necessary understanding of zoonotic parasitic diseases for both undergraduate and postgraduate students in addition to faculties, public health experts, scientists, and administrators across the disciplines of medical sciences, veterinary sciences, public health sciences, and many other allied health sciences.

We wholeheartedly thank all our contributors who have chipped in this project. Our publisher *Springer Nature* has shown a track record of executing the projects undertaken by the chief editor every time with a different flavour. We hope this book will also have an international readership, across all the continents, for those who are interested in knowing and understanding parasitic zoonoses.

Pondicherry, India — Subhash Chandra Parija
Tirupati, India — Abhijit Chaudhury

Acknowledgements

We wish to place on record our sincere thanks to all those who helped us in bringing out this book. The authors and co-authors of various chapters have done a commendable job in submitting the manuscripts in time in spite of their preoccupation with multiple tasks.

Shri M.K. Rajagopalan, the Chancellor of Sri Balaji Vidyapeeth, Pondicherry, and Prof. B Vengamma, Director-cum-Vice Chancellor, Sri Venkateswara Institute of Medical Sciences, Tirupati, have provided the academic ambience and support for us to venture into this task of international publication.

We sincerely thank Prof. S. Pramodhini, Dr. Namratha Bhonsale, and Dr. K. Vanathy, faculty of Shri Mahatma Gandhi Medical College Research Institute (MGMCRI), Pondicherry, Dr Ezhumalai, Consultant, Statistics, Shri Balaji Vidyapeeth; Ms. MP Vani from Information Technology Department at Sri Venkateswara Institute of Medical Sciences, Sri Ram Kumar, IT, Shri Balaji Vidyapeeth for their assistance in the preparation of the manuscript. Subhash Chandra Parija wishes to thank specifically Shri Kailash Chandra Parija, niece Er Kukumina Ray, son-in-law Er Subhasis Ray, nephew Er Rajkumar Parija, daughter-in-law Ms. Smrithi Parija, and daughters Dr. Madhuri Parija and son-in-law Dr. Ajay Halder, Ms. Er Mayuri Parija and son-in-law Er Shailesh Nandan, and grandchildren Shri Harihar, Sri Ram, and Ms Shyama for their support during the preparation of the manuscript.

Abhijit Chaudhury wishes to thank his daughter Dr. Madhulika Chaudhury and son Mr. Shashank Shekhar Chaudhury for their support and encouragement.

Thanks are due to our publisher Springer Nature for extending whole-hearted support to us in carrying out this project.

About the Book

The Genesis

Parasitic infections constitute a huge proportion of infectious diseases worldwide, and for the most part, majority of them are prevalent in resource-poor regions of the world. Except for a few "Poster Child" diseases like malaria and cryptosporidiosis, the bulk of these infections languish in oblivion and their true estimate is almost difficult to ascertain. This had prompted the WHO to take up the issue of "Neglected Tropical Diseases (NTD)" programme targeting about 19 diseases affecting more than 1 billion people worldwide, largely in rural areas of low-income countries. The estimate may not be reflective of the true prevalence since according to one estimate, 880 million children may need medication for soil transmitted helminthic infections alone. If we look at the list of 19 NTDs, it is no surprise that 10 of them are parasitic infections.

Zoonotic infections seem to be emerging and re-emerging in all parts of the world, and since 1940, over 60% of around 400 identified infectious diseases are being zoonotic. Specifically, zoonotic parasitic infections are of interest, considering the shifting interactions between humans and other animals as well as global trade and agriculture. This in turn has given rise to the "*One Health*" concept as a public health discipline within the past decade and adopted by WHO and FAO. *One Health* is an approach that recognizes that the health of people is closely connected to the health of animals and our shared environment. *One Health* is not a new concept, but it has become more important in recent years. This is because many factors have changed interactions between people, animals, plants, and our environment.

Parasitology continues to languish as a sub-discipline of Microbiology as far as Human Medicine is concerned, although this subject is given due importance in Veterinary Medicine. This is an ironical situation, given that parasitic diseases in humans continue to be a scourge in many countries across the world. This is because of the associated morbidity and because the major burden of these diseases is borne by the same countries.

This book aims to provide necessary understanding of the diagnosis, treatment, and control of these diseases to undergraduate and postgraduate students, faculties, public health experts, administrators of medicine, veterinary and allied sciences, and to others who are interested in the management of these parasitic zoonotic infections. In our effort, we were ably supported by

about 60 eminent faculty members who willingly contributed chapters for this mammoth task.

The Content Organization

We have followed a student-friendly approach in organizing the content of this book. The topics chosen for the book are organized under four parts.

Part I, General Parasitology, provides "Background Information on Zoonotic Parasitic Infections". We have included necessary chapters on taxonomy, diagnosis, and anti-parasitic agents. The twenty-first century will be the age of genomics, proteomics, and other *–omics*. Hence, students need to be sensitized to these developments that will facilitate the study of parasitic zoonoses. Therefore, we have also included a few more chapters in the book which highlight the advancements in the study of genomics, proteomics, and immunology of parasitic infections. In the current century, it is envisaged that the conventional study of parasites will be migrating and integrating with technology-based developments such as molecular biology, transcriptomics, and imaging sciences. These need to be integrated with the study of parasitic infections; hence, some of these topics have also been included in the present book. In addition, keeping in mind the public health importance of many of these diseases, two chapters, one on Epidemiology and the other on Prevention and Control, have been incorporated.

Systematic Parasitology, has five parts (II, III, IV, V and VI). Part II includes the chapters on zoonotic protozoal infections while Parts III to VI deals with zoonotic helminthic and arthropod infections. Each of the chapters in Parts II to VI follows a uniform pattern consisting of historical information, taxonomy, biology of the parasite, proteomics, genomics, immunology, pathogenesis, epidemiological features, clinical manifestations in humans and animals, diagnostics, therapy, and prevention and control measures. Part IV deals with chapters on infections caused by Pentastomids, arthropods, and other ectoparasites.

The Value Addition

In addition to other features of a standard book, we have added two unique features in these chapters. One is a typical case study, and the other is a list of unanswered research questions regarding the parasite. Case studies are included to provide a platform to think critically to apply the knowledge gained in clinical practices including diagnostics, therapy, and other application. The list of current research questions regarding the parasite is meant as an impetus for young researchers to study these unexplored areas. Each chapter begins with learning objectives for the benefit of students.

In summary, *Textbook of Parasitic Zoonoses* is written to cater to the diverse needs of readers, either students, faculties, experts, or beginners. We had about 60 contributors of repute, both young and experienced, from

diverse fields of medical, veterinary, public health, and allied health sciences and from different parts of the globe, with a vast experience in their fields which is another strength of the book.

Wish you the best for a good reading of the book!

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Part IV Zoonotic Helminth Infection: Cestode

Part V Zoonotic Helminth Infection: Nematode

Editors and Contributors

About the Editors

Subhash Chandra Parija, MBBS, MD, PhD, DSc, FRCPath, FAMS, FICAI, FABMS, FIMSA, FISCD, FIAAVP, FIATP, FICPath, is currently the Vice Chancellor of Shri Balaji Vidyapeeth, Pondicherry. He was the former Director and also Dean (Research) of the Jawaharlal Institute of Postgraduate Medical Education & Research (JIPMER), Pondicherry. He obtained MBBS (1977) from Utkal University, Cuttack; MD (Microbiology) (1981) from Banaras Hindu University (BHU), Varanasi; and PhD (Microbiology) (1987) from the University of Madras, Chennai. He is one of the very few Medical Microbiologists of India to be conferred DSc (Microbiology), highest degree in research, for his contribution to the field of Medical Parasitology by the University of Madras, Chennai. Prof. Parija was conferred with the Distinguished BHU Alumnus Award in the field of Medical Sciences during the celebration of 150 years anniversary of BHU in the year 2012. Prof. Parija has nearly four decades of teaching and research experience, contributing mainly to the field of infectious diseases, especially parasitology, diagnostics, and public health. He is actively involved in conducting research programmes on immunology, epidemiology, and simple, cost-effective diagnostic tests for parasitic diseases in the community and low resource settings. Prof. Parija and his team have contributed to clinical and diagnostic parasitology. They were the first to demonstrate and detect excreted parasitic antigens in urine for diagnosis of hydatid disease and cysticercosis; to report detection of *Entamoeba histolytica* DNA in the urine and saliva for diagnosis of amoebic liver abscess; document *Entamoeba moshkovskii* from India; evaluate multiplex PCR for simultaneous detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in stool specimens for diagnosis of intestinal amoebiasis; and also document the subtype identification of *Blastocystis* in Pondicherry. Prof. Parija did innovations in stool microscopy techniques by the use of LPCB, KOH, wet mount preparation of stool, thick smear of stool, formalin-acetone method for concentration of stool, etc. for better identification of parasitic diseases. Prof. Parija, author of more than 400 research papers, has supervised more than 85 PhD, MD, and other postgraduate theses as supervisor and co-supervisor. He has two published patents, three copyrights, and one technology transfer. Prof. Parija authored/edited 16 books including the most popular *Textbook of Parasitology*, and the

most recent *Effective Medical Communication, The A, B, C, D, E of It*, published by Springer. Prof. Parija is a Fellow of the Royal College of Pathologists, London, and International Academy of Medical Sciences, New Delhi. He is also a Fellow of many professional bodies of eminence such as the National Academy of Medical Sciences, New Delhi; the Indian College of Pathologists, New Delhi; the Indian Academy of Tropical Parasitology, Pondicherry, and many others. Prof. Parija is the member of the Expert Committee of the Food and Agricultural Organization–World Health Organization (FAO-WHO) for formulating guidelines on food safety for parasites. He has chaired the Committee for Pondicherry Declaration on the Identification and Detection of *Entamoeba histolytica* and transferred the cost-effective diagnostic tests in parasitic diseases to the University of Parendenya, Sri Lanka, under the aegis of Indo-Sri Lanka Joint programme on technology transfer. He has served in the academic, examinations, and research advisory committees of many national and international institutes including the BP Koirala Institute of Health Sciences, Dharan, Nepal; Colombo University, Sri Lanka; College of Medicine & Health Sciences, Sultan Qaboos University, Muscat, Oman; and Faculty of Medicine, University of Malaya, Malaysia. Professor Parija is currently the Editor-in-Chief of the journal *Tropical Parasitology*; Executive Editor of *SBV Journal of Basic, Clinical and Applied Health Science* (JBCAHS), *Annals of SBV, Pondicherry Journal of Nursing, Journal of Scientific Dentistry*; Editor of *Topical Series on HIV/AIDS and Opportunistic Diseases and Co-infection*; and Associate Editor of *BMC Infectious Diseases, BMC Journal of Case Reports (JCR), BMC Research Notes,* etc. in addition to being the member of the editorial board of many journals.Prof. Parija has founded the Indian Academy of Tropical Parasitology, the only such organization in India; started *Tropical Parasitology*, a scientific journal in parasitic diseases; initiated IATP quality assurance programme for parasitic diseases in India; and started the postdoctoral programme in Tropical Parasitology, currently being introduced in many premier medical institutes across the country. He has also founded the Health & Intellectual Property Rights Academy (HIPRA), a unique body, to promote the knowledge, study, and practice of healthcare intellectual property rights of India. Prof. Parija has received more than 26 awards both internationally and nationally including BPKIHS Internal Oration Award (1997) of the BP Koirala Institute of Health Sciences, Nepal, Dr. BC Roy National Award (2003) of the Medical Council of India, Dr. R.V. Rajam Oration Award (2019), and Dr. PN Chuttani Oration Award (2007) of the National Academy of Medical Sciences, Prof. BK Aikat Oration Award (1998) and Major General Saheb Singh Sokhey Award (1992) of the Indian Council of Medical Research, Dr. Subramaniam Memorial Oration Award (2015) of the Indian Association of Biomedical Scientists, Sri SM Ismail Oration Award (2005) of the Indian Association for Development of Veterinary Parasitology, Dr. BP Pandey Memorial Oration Award (1998) of the Indian Society for Parasitology, Dr. SC Agarwal Oration Award (2001) of the Indian Association of Medical Microbiologists, Dr. BP Pandey Memorial Oration Award (1998) of the Indian Society for parasitology, etc.Prof. Parija has contributed immensely

towards institution building and medical education and mentored undergraduates, postgraduates, PhD scholars, and young faculty to pursue their interest in research and career in parasitic diseases of public health importance. He conceptualized, procured the site for, and started the new campus of JIPMER at Karaikal in the year 2017.

Abhijit Chaudhury, MBBS, MD, DNB, D(ABMM), is presently working as Professor in the Department of Microbiology at Sri Venkateswara Institute of Medical Sciences and its affiliated Sri Padmavathy Medical College for Women at Tirupati, Andhra Pradesh, India. He started his career in Microbiology at Himalayan Institute of Medical Sciences, Dehradun, and then moved to Manipal College of Medical Sciences, Pokhara, Nepal, and finally from 1998, he has been associated with the present institute. He also had short stints as a faculty of Microbiology at Medical University of St Eustatius, Netherlands Antilles; SSR Medical College, Mauritius; and Oman Medical College, Sohar, Oman, in the intervening years. His research interests include pathogenesis of protozoan parasites, mycobacterial genomics, and biofilm infections. He was a member of the Task Force for Curriculum Development for Laboratory Technology courses by the Ministry of Health and Family Welfare, Government of India, and a reviewer for Antibiotic Treatment Guidelines published by the Government of West Bengal. He has served as referee for a number of national and international journals like *Indian Journal of Medical Research*, *Indian Journal of Medical Microbiology*, *BMC Microbiology*, *Journal of Medical Microbiology*, *Frontiers in Microbiology*, *Microbial Pathogenesis*, and *FEMS Immunology and Medical Microbiology*. He has more than 25 years of teaching experience in Medical Microbiology for undergraduate and postgraduate students and has supervised or co-supervised the MSc, MD, and PhD work of a number of students. He has been instrumental in designing horizontal and vertical integrated teaching modules for MBBS students much before the introduction of Competency-Based Medical Education by the Medical Council of India. He has also published nearly 90 research articles in peer-reviewed national and international journals and authored one book chapter. He is also a member of scientific societies like Indian Association of Medical Microbiology and Indian Academy of Tropical Parasitology and assessor for National Medical Council (formerly Medical Council of India) and National Assessment and Accreditation Council, India. At present he is the Chief Managing Editor of *Tropical Parasitology* journal which is the official publication of the Indian Academy of Tropical Parasitology.

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Part I

General Parasitology

Parasite Taxonomy

Subhash Chandra Parija and Abhijit Chaudhury

Learning Objectives

1. To have a broad overview of the existing classification of parasites.
2. To understand the basis for the classification.
3. To have a knowledge about the modern methods for taxonomy and the associated changes in classification of some parasites.

Introduction

Ever since the first classification of living beings into two kingdoms—Animalia and Plantae by Carl Linnaeus in 1758—new information and discoveries have resulted in increasing complexity and complications in designing a proper classification. The earlier classification systems relied heavily on the morphological aspects of the organisms. The advent of ultrastructural details, their enzymatic pattern and genetic makeup have been instrumental in the re-classification of many of these parasites. Recent advancements in gene sequencing and other methodologies have found that some earlier phylogenetic classifications do not necessarily fall in line with the evolutionary past. Hence, new changes and modifications are necessary as new discoveries come to light. There is a need to understand the taxonomical classification of parasites from two points of view: the traditional and the modern. While most scientists are familiar with the older and conventional classification, the modern-day system using more sophisticated data has classified and re-classified the existing parasites, and new nomenclature has even been assigned to them. This has created understandable confusion among the various stakeholders. Thus a compromise is necessary between the current evolutionary thinking and the more practical need for a system of nomenclature which will allow scientists from diverse backgrounds to effectively communicate with each other and retrieve relevant information from archival and historical data.

The Evolution of Classification Systemics

The broad division of all living beings into two kingdoms, Animalia and Plantae, in 1758 by Linnaeus, marked the advent of taxonomical classification. The discovery of numerous unicellular organisms with the invention of the microscope prompted scientists like Haeckel in 1876, credited with the creation of a third kingdom, Protista, to include these life forms. Subsequently, four

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kingdoms were proposed by Copeland in 1949 (Animalia, Plantae, Protoctista and Mychota). The removal of fungi from the plant kingdom necessitated the addition of the fifth kingdom of fungi. Jahn and Jahn in 1949 modified further the kingdoms which formed the basis of a five-kingdom classification of Whittaker in 1969. This classification included Monera (prokaryotes), Animalia, Plantae, Fungi and Protista. Corliss (1994) proposed six kingdoms in the empire Eukaryota retaining the old Plantae, Fungi and Animalia and introducing three kingdoms of unicellular organisms of Archezoa, Protozoa and Chromista.

Taxonomy of Protozoal Parasites

The unicellular eukaryotic organisms have been given various names: Protozoa, Protista or Protoctista. Each name has its own proponents and followers. Protozoa and Protista are the favourites among parasitologists and protozoologists, respectively. When additional kingdoms were introduced, the status of Protozoa rose to the level of the kingdom. The Protozoa was first classified by Goldfuss in 1818 into three groups: amoebae, flagellates and ciliates based on their organs of locomotion. Subsequently, the sporozoans were included in the kingdom by Butschli in 1883. Since then, numerous classification systems and re-classifications have been suggested.

Cavalier-Smith (2003) Classification of the Kingdom Protozoa

The kingdom Protozoa as proposed by Cavalier-Smith (2003) is based on certain traits which distinguish them from other unicellular living organisms. The classification proposed by Cavalier-Smith suggests that the kingdom Protozoa includes 11 phyla of which only a few are pathogens in humans and animals. The kingdom Protozoa includes more than 200,000 protozoa species, of which only about 10,000 (0.5%) are parasites, with or without any pathogenic potential. Phyla Amoebozoa, Trichozoa, Percolozoa, Euglenozoa, Miozoa and Ciliophora are the only phyla of 11 phyla in the kingdom Protozoa that contain potentially pathogenic species for humans and animals (Table 1):

1. **Amoebozoa:** These include protozoa that have pseudopodia as locomotory organs or are motile by protoplasmic flow. Flagella, if present, are restricted to one particular life stage. They reproduce asexually by fission; sexual reproduction is associated with free living amoebas. Mitochondrial cristae tubular or mitochondria and peroxisomes are absent.
2. **Euglenozoa:** Protozoa included in the group have flagella, often with the presence of paraxial rod. They also have discoidal mitochondrial cristae and cortical microtubules and show persistence of nucleoli during meiotic division.
3. **Percolozoa:** Percolozoa have heterotrophic flagella or amoeboflagella and discoid mitochondrial cristae. They commonly alternate between a flagellate phase with pellicle and a main non-ciliate trophic amoeboid phase.
4. **Trichozoa:** These protozoa are flagellates or, rarely, amoebae consisting of hydrogenosomes and prominent Golgi dictyosomes. They exhibit closed mitosis with extra-nuclear mitotic spindle.
5. **Miozoa:** The Miozoa consists of protozoa which commonly or ancestrally feed by the process of myzocytosis. These protozoa therefore pierce the cell wall or cell membrane of the host with a conoid or feeding pipe and suck out the cellular contents.
6. **Ciliophora:** These protozoa are parasites of digestive tracts. They have cilia and cortical alveoli and, typically, have two types of nuclei (*heterokaryotic*). The Ciliophora protozoa may exhibit sexual phenomenon of *conjugation* or *autogamy* and *cytogamy* or asexual reproduction by transverse fission. Contractile vacuoles are present.

Table 1 Revised detailed classification of pathogenic protozoan parasites (After Cavalier Smith, 2003)

Kingdom: Protozoa

A. Subkingdom: Sarcomastigota

Phylum: **Amoebozoa**
Subphylum 1: Protamoebae
Class 4: Variosea: *Acanthamoeba*, *Balmuthia*
Subphylum 2: Archamoeba
Class: Archamoeba: *Entamoeba*, *Endolimax*.

B. Subkingdom: Biciliata

Infra-kingdom: Excavata

Phylum 2: Metamonada
Subphylum: **Trichozoa**
Superclass 1: Parabasalia
Class 1: Trichomonadea: *Trichomonas*, *Lophomonas*
Superclass 3: Eopharyngea
Class 1: Trepomonadea:
Subclass 1: Diplozoa: *Giardia*
Class 2: Retortamonadea: *Retortamonas*, *Chilomastix*
Superphylum 1: Discicristata

Phylum 1: **Percolozoa**
Class 1: Heterolobosea: *Naegleria*
Phylum 2: **Euglenozoa**
Subphylum 2: Saccostoma
Class 1: Kinetoplastea: *Trypanosoma*, *Leishmania*.

Infra-kingdom: Alveolata

Phylum 1: **Miozoa**
Subphylum 3: Apicomplexa
Infraphylum: Sporozoa
Class 1: Coccidea: *Toxoplasma*, *Cryptosporidium*
Class 2: Hematozoa: *Plasmodium*, *Babesia*.
Phylum 2: **Ciliophora**
Subphylum 2: Intramacornucleata
Class 2: Litostomatea: *Balantidium*

Table 2 Utilitarian classification of pathogenic protozoa (After Corliss, 1994)

Kingdom	Phylum	Class	Order	Agent
Archezoa	Metamonada	Trepomonada	Diplomonadida	*Giardia*
			Enteromonadida	*Enteromonas*
		Retortamonada	Retortamonadida	*Retortamonas*, *Chilomastix*
	Microspora	Microsporea	Microsporida	*Encephalitozoon*, *Enterocytozoon*, *Nosema*, *Septata*
Protozoa	Percolozoa	Heterolobosea	Schizopyrenida	*Naegleria*
	Parabasalia	Trichomonadia	Trichomonadida	*Trichomonas*
	Euglenozoa	Kinetoplastidea	Trypanosomatida	*Trypanosoma*, *Leishmania*
	Ciliophora	Litostomatea	Vestibuliferida	*Balantidium*
	Apicomplexa	Coccidea	Eimerida	*Cryptosporidium*, *Cyclospora*, *Toxoplasma*, *Isospora*, *Sarcocystis*
		Haematozoa	Haemosporida	*Plasmodium*
			Piroplasmida	*Babesia*

Corliss (1994) Classification of the Kingdom Protozoa

Classification by Corliss (1994) is another simplified system of classification. This classification encompasses both conventional and molecular characteristics of parasites. This system also retains the older names of parasites for the sake of simplicity and familiarity. This system of classification is highly useful for medical and veterinary parasitologists and is of practical importance (Table 2).

As per the classification by Corliss (1994), pathogenic potential protozoal species that can cause infections in humans and animals are included in the following phyla:

1. **Metamonada:** These protozoa are parasites of the intestinal tract. They have two or more flagella and contain hydrogenosomes instead of mitochondria.
2. **Microspora:** These are unicellular spore-like structures containing one or two nuclei with sporoplasm and a polar filament. They lack mitochondria and peroxisomes but have 70S ribosome.
3. **Parabasalia:** These protozoa have multiple flagella. They have parabasal fibres which arise at the kinetosomes. The parabasal apparatus is analogous to the Golgi apparatus. They lack mitochondria.
4. **Apicomplexa:** The protozoa belonging to this phylum have a unique structure known as the apical complex. The complex comprises a polar ring, micronemes, rhoptries, conoid and subpellicular tubules. They have cortical alveoli and represent the sporozoans described in the old classifications of protozoa.

Taxonomy of Helminthic Parasites

Classification of helminths into cestodes, trematodes and nematodes is a working classification of convenience, more familiar among parasitologists in the field of medical and veterinary sciences.

A zoological system of classification of helminths includes the subkingdom Bilateria in the Animalia kingdom that consists of helminthic parasites. The infra-kingdom 1 (*Ecdysozoa)* comprises the nematodes (Table 3), while the infra-kingdom 2 (*Platyzoa*) contains the trematodes and cestodes (Table 4):

1. **Nematodes:** Nematodes are typically bilaterally symmetrical and are elongated with tapering ends. They possess a body cavity or *pseudocoel*. The digestive system

Table 3 Classification of pathogenic Nemathelminth parasites

Phylum	Infraphylum	Class	Superfamily	Family	Members
Nemathelminthes	Nematoda	Adenophora (Aphasmidea)	Trichinelloidea	Trichinellidae	*Trichinella spiralis*
				Trichuridae	*Trichuris trichiura*
		Secernentea (Phasmidea)	Ancylostomatoidea	Ancylostomatidae	*Ancylostoma duodenale*, *Necator americanus*
			Ascaridoidea	Ascarididae	*Ascaris*, *Toxocara*
				Anisakidae	*Anisakis*
			Dracunculoidea	Dracunculidae	*Dracunculus*
			Filarioidea	Onchocercidae	*Wuchereria*, *Brugia*, *Onchocerca*, *Dirofilaria*, *Mansonella streptocerca*
			Gnathostomatoidea	Gnathostomatidae	*Gnathostoma*
			Metastrongyloidea	Angiostrongyloidae	*Angiostrongylus*
			Oxyuroidea	Oxyuridae	*Enterobius*
			Rhabditoidea	Strongyloididae	*Strongyloides*
			Spiruroidea	Gongylonematidae	*Gongylonema*
			Strongyloidea	Chabertidae	*Oesophagostomum*, *Ternidens*
				Sygamidae	*Mammomonogamus*
			Thelazioidea	Thelaziidae	*Thelazia*
			Trichostrongyloidea	Trichostrongyloidae	*Trichostrongylus*

Table 4 Classification of pathogenic Platyhelminthes

Phylum	Class	Order	Family	Members
Platyhelminthes	Digenea	Strigeida	Diplostomadae	*Diplostomum*
			Schistosomatidae	*Schistosoma*
			Clinostomatidae	*Clinostomum*
		Echinostomatida	Echinostomatidae	*Echinostoma*
			Fasciolidae	*Fasciola* *Fasciolopsis*
			Zygocotilidae	*Gastrodiscoides hominis* *Watsonius watsoni*
		Plagiorchiida	Dicrocoeliidae	*Dicrocoelium dendriticum*
			Heterophyidae	*Heterophyes*, *Metagonimus*
			Opisthorchiidae	*Opisthorchis (Clonorchis)*
			Lecithodendriidae	*Phaneropsolus*
			Paragonimidae	*Paragonimus*
			Plagiorchiidae	*Plagiorchis*
			Troglotrematidae	*Nanophyetus salmincola*
	Cestoidea	Pseudophyllidea	Diphyllobothridae	*Diphyllobothrium*, *Spirometra*, *Sparganum*
		Cyclophyllidea	Anoplocephalidae	*Bertiella*
			Davaineidae	*Raillietina*
			Dipylidiidae	*Dipylidium caninum*
			Hymenolepididae	*Hymenolepis* (*Rodentolepis*) *nana*, *Hymenolepis diminuta*
			Mesocestoididae	*Mesocestoides*
			Taeniidae	*Taenia*, *Echinococcus*, *Multiceps*

comprises the mouth, pharynx and anal opening. The digestive canal is tri-radiate. The body does not possess cilia or flagella but has a variety of *sensilla* as sensory organ. The worms are dioecious with separate male and female adults. Females are normally larger and mostly oviparous. The ventral vulva represents the opening of the female reproductive system, while in the male, it opens into a cloaca along with the digestive system.

2. **Platyhelminthes:** They are called flatworms since they have a dorso-ventrally flattened bilaterally symmetrical body. They do not have a body cavity. The body is covered with tegument. Most of the body is made up of parenchyma and muscle fibres can be found in parenchyma. The digestive system is a blind sac-like structure with a mouth at the anterior end. The flame cells represent the excretory system of the worms. Most members are monoecious and can fertilize their own eggs.

Platyhelminthes are classified into two groups, trematodes and cestodes:

1. **Trematodes:** They are hermaphrodite worms. They are also known as flukes and have a leaf-like body and two suckers, one at the anterior and another at the posterior end. Trematodes have a digestive system. They require definitive hosts harbouring the adult stage and two intermediate hosts harbouring the larval stages of miracidium, sporocysts and cercaria.
2. **Cestodes:** The cestodes have three embryonic layers: ectoderm, mesoderm and endoderm. The head or scolex present at the anterior end of the body helps in attachment of the cestode to tissue of the host. The body or *strobila* is segmented and is unique to these parasites. The *strobila* consists of a linear series of male and female reproductive organ systems, and the surrounding area is known as the segment or *proglottides*. New proglottides or segments are found at the anterior end, while gravid proglottides are found at the posterior end. The gravid proglottides contain branched uterine structures filled with eggs. They lack a digestive tract and absorb all nutrition from the external covering or tegument with high metabolic activity.

Modern Methods for Classification of Parasites

While the nineteenth century and the first half of the previous century relied almost exclusively on light and later on electron microscopy to classify parasites, newer techniques were gradually introduced to study the relationship between these life forms at the molecular level. The need for these techniques has arisen due to multiple reasons (Fig. 1).

One of the first such methodologies to be applied was to study the *isoenzyme profiles*. This was very useful to distinguish between closely related organisms, and the classical example was to differentiate pathogenic and non-pathogenic forms of the *Entamoeba histolytica*. It was similarly used for *Toxoplasma gondii* and to identify the subspecies of *Trypanosoma brucei.* In recent years this technique has also been used for phylogenetic classification of *Plasmodium falciparum* and *Cryptosporidium hominis*. Subsequently, the new *DNA and RNA technological advances* overshadowed all other methods, and they now remain the most commonly used methodology for systemic classification, particularly for resolving taxonomical and phylogenetic controversies and problem-solving. Historically, the small subunit of ribosomal RNA was first utilized to create a phylogenetic tree in the 1980s. At present the 16S and 18S small nuclear RNAs and DNA probes are extensively used in taxonomy works, and they are particularly useful to find the evolutionary distance between the strains and create phylogenetic trees. *Molecular karyotyping* is another method which helps in determining the chromosomal size differences. It has been employed for the agents of cutaneous leishmaniasis and helped in geographical grouping of the strains. The study of *whole genome sequencing* may help in assigning some atypical or unclassified members of a genus

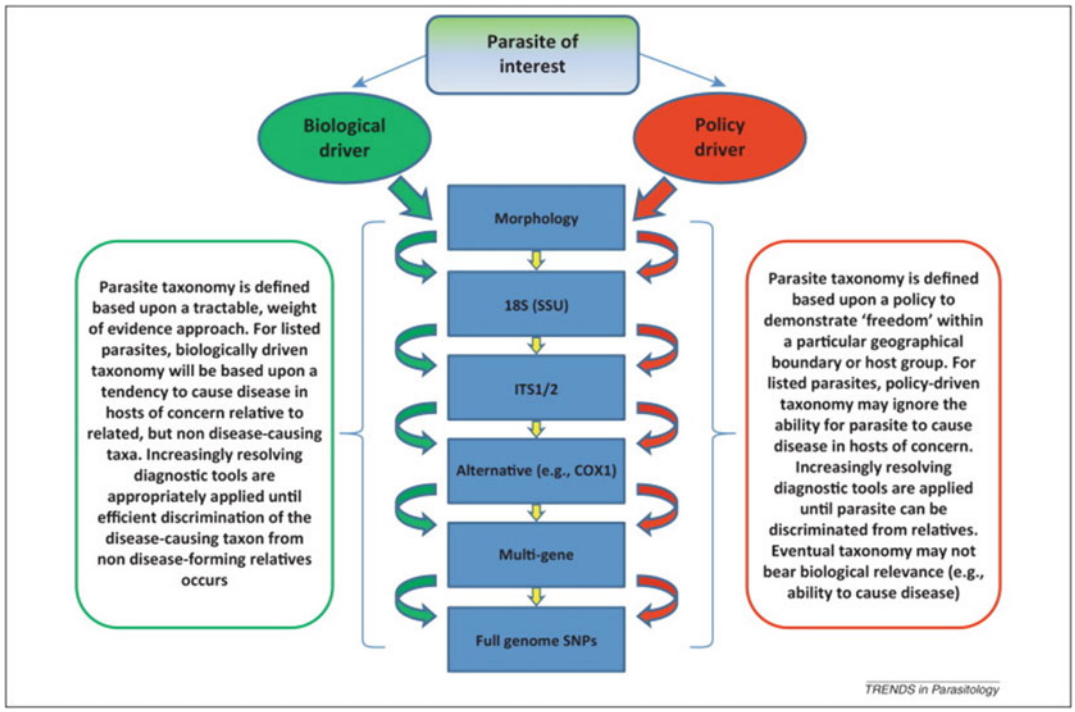

Fig. 1 The 'diagnostic cascade'. The driver to circumscribe a particular parasite taxon may be driven by 'biological' or 'policy' reasoning. (From: Stentiford G, Feist S, Stone DM, Peeler E, Bass, D. Policy, phylogeny, and the parasite. Trends in parasitology. 2014. 30. https://doi.org/10.1016/j.pt.2014.04.004)

to novel subspecies or sub-genus levels. In addition to these, the emerging field of *proteomics* may open up new corridors in the classification of organisms. Genomic studies are useful to determine the evolutionary trends as well as giving indications about the level of genetic differences, but they do not take into account post-transcriptional regulation of protein expression and cannot determine the degree of cross-reactivity between parasite species. It is possible that proteomic comparisons can give a better indication of phenotypic differences between different parasites.

The above mentioned newer technologies are gradually changing the way we look at these parasites and bringing up new and useful information. Further refinements in the taxonomy of parasites are expected in the next few decades, which may result in re-classification of existing parasites and creation of new classes or genera of these organisms.

Case Study

Taxonomical Position of Microspora

The Microspora has traditionally been considered as protozoans, but research findings in the last decade have found otherwise. In the fungal zygomycetes group, the *sex* locus is a syntenic gene cluster that governs sexual reproduction and comprises a high mobility group (HMG) gene, flanked by a triose phosphate transporter and a RNA helicase gene. The microsporidian genomes harbour a *sex*-related locus with the same genes in the same order. Moreover, genome-wide analysis of synteny reveals multiple other loci common to microsporidia and zygomycetes. These findings support the hypothesis that microsporidia are true fungi that descended from a zygomycete ancestor and suggest the microsporidia may have a genetically controlled sexual cycle. On the basis of these findings, Microspora is no longer considered a protozoan parasite but is designated as a fungus.

1. Give one or two examples where similar re-classification of parasites has been made.
2. Describe the methods available for identifying a new parasite which shows some similarity with a known parasite.
3. Define a hybrid parasite. Name one common parasite which exhibits this feature.

Research Questions

1. Like microsporidium, are there other protozoa which do not belong to the parasite group, but belong to fungi or some hitherto unknown group?
2. Is it possible to separate some algal forms, euglenids and dinoflagellates from protozoa which have totally different biology?
3. How can one create a more refined taxonomy of helminths based on the newer methods of classification?

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Host–Parasite Relationship and Clinical Manifestations

Alladi Mohan and Kanchi Mitra Bhargav

Learning Objectives

1. To understand the effect of parasite infections on the host.
2. To know about the organ systems affected by human parasitic infections.
3. To gain knowledge about the clinical manifestations of important parasitic infections.

Introduction

Parasitic diseases are considered to be a major cause of morbidity and mortality, especially in developing countries. These diseases frequently affect humans living in places with inadequate sanitation and in close contact with domestic animals, livestock and infectious vectors and those with certain food habits. Malaria, once considered a major tropical disease, has now assumed global importance because of increased international travel (prior to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) coronavirus disease (COVID-19) pandemic). Three enteric protozoa, *Entamoeba*, *Cryptosporidium* and *Giardia*, account for a major proportion of diarrhoeal disease worldwide. In 2020, globally, there were an estimated 241 million cases and 627,000 deaths due to malaria. Several parasitic diseases have been included in the World Health Organization (WHO) list of "neglected tropical diseases" (Table 1) because these diseases affect the health of millions of individuals, especially those who are impoverished. This chapter provides an overview of the current understanding of the host–parasite relationship and the spectrum of clinical manifestations of certain commonly observed parasitic diseases in humans.

Definitions

In zoology, "the living together in intimate association or close union of two dissimilar organisms" has been broadly defined as *symbiosis*. A *parasite* is an organism that lives in or upon and thrives by acquiring nutrients from its *host*, either temporarily or permanently. The *host* harbours the parasite and provides nourishment and shelter for it. The smaller of the associating pair of animals is regarded as the parasite, and the larger is considered to be the *host*. A *commensal* is different from a parasite in that it does not feed on the host's tissues. Parasitism is considered to be *antagonistic symbiosis*. *Invasion* or *introduction* is the term used to describe human-aided spread of species to new areas.

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Table 1 The World Health Organization list of parasitic infections included among the "neglected tropical diseases"

Chagas disease
Human African trypanosomiasis
Leishmaniasis
Taeniasis and cysticercosis
Dracunculiasis (guinea-worm disease)
Echinococcosis
Foodborne trematodiases
Lymphatic filariasis
Onchocerciasis (river blindness)
Schistosomiasis
Soil-transmitted helminthiases
Scabies and other ectoparasites

While etymologically the term "parasite" encompasses a wide range of organisms, it is commonly used to denote protozoa and helminths. Parasites can be classified as (1) e*ctoparasites* (inhabit only the host body surface without penetrating the tissue, e.g. lice, mites, ticks) and (2) endoparasites (live within the body of the host and cause infection, e.g. protozoa, helminths). Endoparasites can be *obligate parasites (*cannot exist without a host, e.g. *Toxoplasma gondii*, *Plasmodium* spp.) or *facultative parasites* (live either as parasitic or free-living forms, e.g. *Naegleria fowleri*). *Accidental parasites* infect an unusual host (e.g. *Echinococcus granulosus). Aberrant (wandering) parasites* are those which infect a host in which their further development is not possible (e.g. *Toxocara canis* infection in humans).

The adult parasite lives and undergoes sexual reproduction in the definitive host (e.g. humans for roundworm infection and mosquito in malaria are definitive hosts). The larval stage of the parasite lives or asexual multiplication takes place in the *intermediate host*; these may be one or more for certain parasites. In *paratenic hosts* the larval stage of the parasite remains viable without further development. *Reservoir hosts* harbour the parasite and act as an important continuing source of infection. The host where a parasite is not usually found is an accidental host (humans for *E. granulosus*). In a *transport host*, there would not be any growth and reproduction of the parasite, but these hosts can transmit infection to others.

Colonisation is considered to be the process by which a species spreads to new areas successfully and is established within the local environment. It refers to growth occurring in or on body sites exposed to the environment, without causing any infection. *Infestation* is the term that denotes parasitisation with ectoparasites.

A parasite causing disease in a host is called a *pathogen*. The parasite may survive inside or on the surface of the host. The entry, growth and multiplication of the parasite in the body of the host is called *infection*. The interaction between the parasite and the host's immune system results in disease. Many factors, such as infecting dose, virulence of the pathogen and host's immunity, play major roles in the process of infection.

Primary pathogens infect healthy hosts; opportunistic pathogens infect immunocompromised hosts. Parasites can be classified in several ways, based on duration (permanent or temporary), degree of dependence (facultative or obligatory), position (ectoparasite or endoparasite) and size (micro-parasites or macro-parasites).

A carrier is a person who is infested with a parasite but has any symptoms. Carriers (who are usually asymptomatic) are considered to be a vital source of spreading infection. Clinically, parasitic diseases in humans can be chronic or acute.

Sources of Infection

The sources of infection include contaminated soil and water, food, vectors (biological/mechanical vectors) and sometimes the environment.

Modes of Transmission

Parasites can be transmitted to humans by animals (zoonotic disease), insects and other vectors (vector-borne) or water (water-borne). The commonly encountered modes of transmission include vectors, oral, skin transmission and direct transmission. Sometimes vertical and iatrogenic transmission can occur. A significant number of human parasitic diseases are zoonotic diseases and are transmitted from vertebrate animals to humans. Host suitability is essential for the survival of parasites. While some parasites can flourish in a wide range of host environments, many other parasites can survive only in specific host species. A properly orchestrated inflammatory response in the host results in elimination of the parasites, mitigation of inflammation and initiation of tissue repair and healing.

Many of the parasites have learned to counteract the host immune system by developing adaptive mechanisms. For attaching and entering cells of the host, parasites are equipped with tools such as secretary molecules and surface proteins. These are considered to be essential for the entry and endurance of the parasite in the host. *Pathogenicity* refers to disease-causing ability; virulence is disease-causing ability in quantitative terms. *Infectiveness* refers to invasiveness and disease-causing ability.

Arthropods transmit disease by biting, regurgitation, abrasion, contamination or a combination of all of the above. The route through which the parasite enters the host is referred to as the *portal of entry*. The gastrointestinal tract, respiratory tract, skin and urogenital tract are the usual portals of entry. Many parasites have a specific route of entry and it serves as a precondition for causation of the disease.

Clinico-pathological Correlations

Effect of Parasite on the Host

Several changes are caused by the parasite in the infected host. Physical and physiological properties play a role in the antigenicity of the parasite. The current research focuses on the enzymatic pathways, metabolites and chemical composition of parasites. The ability to escape host immunity is significantly variable among parasites. Protozoa multiply and survive inside the phagocytes and host cells, while many multicellular forms cannot multiply within the host. Both cellular and humoral immunity play predominant roles in restricting parasite entry and in their elimination.

Macrophages, different factors of serum, natural killer cells (NK) and other immune cells can inflict injury to parasites. Nutrition is an important factor for robust immunity, especially for cell-mediated immunity. It can further affect lymphocyte count and can alter T-cell subgroups significantly. Other defence mechanisms such as antibody secretion of the mucosa, complement activity and ability of phagocytes to kill parasites are lessened. Changes such as these in host resistance determine the end outcome in parasitic infections.

Effect of Host on Parasite

The immune status and general constitution of the host significantly influence the host–parasite relationship. The parasite undergoes several modifications (*parasitic adaptations*) to survive in the hostile host environment and also has several specific effects on it.

The age of the host also appears to influence the pathogenesis of parasitic infections. It is known that human schistosomes usually infect young persons, and persons aged over 30 years are seldom infected on exposure to the parasite. The type of nutritive material consumed by parasites affects their development. A diet consisting of milk, because it is deficient in p-aminobenzoic acid (PABA) that is essential for parasite growth, adversely affects intestinal helminths and protozoa. A high-protein diet inhibits the development of many intestinal protozoa. A low-protein diet seems to favour the manifestations of symptoms of amebiasis. Host hormones have been shown to have a direct effect on the growth and sexual maturity of parasites.

Several protozoal and helminthic infections do not confer long-lasting immunity to reinfection. However, while they are still in the host's body,

parasites seem to stimulate resistance to hyper-infection (called *premunition*). Variations in host specificity and parasite density also have a role to play in causation of the disease.

Interactions with the Host Intestinal Microbiota

The term intestinal bacterial microbiota encompasses a complex community of bacteria comprising at least several hundred species which form a asymbiotic relationship that influences human physiology and disease progression.

Enteric protozoa are usually transmitted through the faecal-oral route. In the human host, the intestine is densely populated with commensal bacteria with which the protozoal parasites directly interact. Further, protozoa living in the tissues or blood of human hosts are also affected by the interaction between the gut microflora, host metabolism and immune system. It has been observed that changes in the composition of the intestinal microbiota may enhance resistance to parasite infection at intestinal mucosa. This is postulated to be due to mechanisms like decreased virulence or parasite adherence. Alterations in the microbiota may also alter systemic immunity to parasites by effects on granulopoiesis or adaptive immunity. It is likely that hitherto unclear mechanisms enabling microbiota-mediated protection could be the reason for clinical variability and may help in treatment of parasitic protozoan infections.

Clinical Manifestations

Epidemiologically, disease outbreaks can be *sporadic*, *endemic* (a disease that is present permanently in a region or population), *epidemic* (a disease that affects a large number of humans within a community, population or region) or *pandemic* (disease occurring all over the world) in nature.

Spectrum of Clinical Manifestations

The host–parasite interaction basis of some of the common parasitic diseases is presented below. The clinical manifestations have been mentioned system-wise, but it should be noted that many parasites can affect multiple systems as part of systemic infection or they may affect organ systems in an accidental or aberrant manner, different from their original niche. Table 2 summarises the list of parasites affecting different organ systems. The clinical manifestations can be conveniently grouped together under the following headings:

1. **Gastrointestinal (GI) tract**: The GI tract is the site of infection for the majority of parasites, particularly the helminths. It also happens that a parasite may inhabit the GI tract but may travel to other areas and produce clinical manifestations in another system. Since these infections are transmitted by the faecal-oral route, it is logical to believe that they are common in resource-poor settings where proper sanitation and safe drinking water may not be available. While this is indeed so, in recent years these infections are establishing a foothold in high-income countries due to a number of factors. These include increased travel to endemic countries, a higher number of immunocompromised subjects due to infections like HIV or procedures like transplantations, consumption of raw or partially cooked food as ethnic delicacies, and immigration from endemic resource-poor areas of the world:
   (a) **Diarrhoea**, **Dysentery and Enteritis**: These conditions commonly manifest as abdominal pain or cramps, distension and diarrhoea with passage of watery stool or dysentery with passage of blood and mucus. Protozoan parasites like *Cryptosporidium* and *Giardia* commonly cause diarrhoea, while dysentery is the hallmark of *Entamoeba histolytica* and *Balantidium coli* infections. Worldwide, cryptosporidiosis is documented to be the most common parasitic infection among HIV-seropositive individuals.
   (b) **Invasive Infection**: *B. coli* and *E. histolytica* can cause invasive lesions and, in untreated cases, may progress to development of frank ulcers. Some zoonotic helminths like *Anisakis* may burrow into the intestinal mucosa causing severe

Table 2 Parasites affecting different organ systems of the body

Body system	Protozoa	Nematode	Cestode	Trematode
Gastrointestinal tract	*Entamoeba histolytica*, *Giardia*, *Balantidium coli*, *Cryptosporidium*, *Cyclospora*, *Isospora*	*Ascaris lumbricoides*, *Enterobius vermicularis*, *Ancylostoma*, *Necator americanus*, *Trichuris trichiura*, *Capillaria*, *Trichostrongylus*, *Strongyloides stercoralis*	*Diphyllobothrium latum*, *Taenia saginata* and *solium*, *Hymenolepis nana*	*Fasciolopsis buski*, *Schistosoma mansoni*, *Schistosoma japonicum*
Respiratory system	*Entamoeba histolytica*, *Toxoplasma gondii*, *Cryptosporidium*	*Ascaris lumbricoides*, *Strongyloides stercoralis*, *hookworm*, *Dirofilaria* spp., *Toxocara* spp., lymphatic filariasis (tropical pulmonary eosinophilia)	*Echinococcus granulosus*	*Paragonimus westermani* and other species, *Schistosoma* spp.
Central nervous system	*Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Entamoeba histolytica*, *Leishmania*, *Toxoplasma gondii*	*Gnathostoma Angiostrongylus Toxocara Strongyloides Baylisascaris Dracunculus, Onchocerca volvulus*	*Taenia solium (Cysticerci)*, *Echinococcus granulosus*, *Sparganum larva*	*Schistosoma*, *Paragonimus*
Circulatory and lymphatic systems	*Plasmodium* spp., *Babesia*, *Toxoplasma*, *Leishmania* spp., *Trypanosoma cruzi*, *Trypanosoma brucei*	*Wuchereria bancrofti*, *Brugia* spp., *Mansonella* spp.		*Schistosoma*
Cardiovascular system	*Trypanosoma cruzi*, *Trypanosoma brucei*, *Entamoeba histolytica*, *Toxoplasma gondii*, *Naegleria fowleri*, *Sarcocystis* spp.	*Trichinella* spp., *Dirofilaria* spp., visceral larva migrans	*Echinococcus granulosus*, *Taenia solium*	–
Liver and biliary system	*Entamoeba histolytica*, *Plasmodium*, *Babesia*, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania*, *Toxoplasma gondii*, *Cryptosporidium*, *Cystoisospora*	*Toxocara*, *Capillaria*, *Strongyloides*, *Ascaris*	*Echinococcus*	*Schistosoma*, *Fasciola*, *Opisthorchis*, *Clonorchis*
Genitourinary system	–	*Wuchereria bancrofti Onchocerca volvulus Dioctophyme renale*	*Echinococcus*, *Taenia solium*	*Schistosoma*
Skin, soft tissue and muscle	*Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania*, *Entamoeba histolytica*	*Gnathostoma*, *hookworm*, *Trichinella*, *Dirofilaria*, *Onchocerca*, *Loa loa*, *Mansonella*, *cutaneous larva migrans*	*Echinococcus*, *Taenia solium*, *Sparganum larva*	*Schistosoma*
Eye	*Acanthamoeba*, *Giardia*, *Trypanosoma cruzi*, *Leishmania*, *Plasmodium falciparum*, *Toxoplasma gondii*	*Angiostrongylus*, *W. bancrofti*, *Brugia*, *Dirofilaria*, *Loa loa*, *Onchocerca*, *Toxocara*, *Thelazia*, *Baylisascaris*, *Trichinella*	*Taenia solium*, *Echinococcus*	*Schistosoma*, *Fasciola*

abdominal pain. Sometimes there may be intestinal perforation and the worms may come to lie in the abdominal cavity. The helminths which have an indirect life cycle return to the intestine after their passage through the lungs, and the larval forms penetrate the intestinal walls.

(c) **Mechanical Obstructions**: *Ascaris* infection is notorious for causing a number of obstructive features like blockage

of the biliary duct, which may result in severe pain and vomiting. Pancreatic duct obstruction may lead to acute pancreatitis. Heavy infection with *Ascaris* is also associated with complete intestinal obstruction, particularly in children. This parasite, like *Anisakis*, can cause intestinal intussusceptions. Entry of some helminths like *Enterobius vermicularis*, *Taenia solium* or *T. saginata* and *Ascaris* into the appendix can lead to acute appendicitis.

(d) **Depletion of Nutrients**: Severe and uncontrolled diarrhoea by some protozoan parasites may lead to water and electrolyte loss from the body, which may lead to electrolyte imbalance. *Diphyllobothrium latum* competes with the host in the absorption of vitamin B_{12}, which may lead to pernicious anaemia in the host in long-standing cases. Many parasites take up the various nutritional elements from food, depriving the host, which causes malnutrition. Hookworm is noted for causing blood loss from the intestines and is an established cause of iron deficiency anaemia in resource-limited endemic regions of the world. It has been reported that 500 hookworms can result in loss of 250 cc of blood by parasite feeding.

2. **Respiratory Tract**: Some parasites have a predilection for the respiratory system, and clinically the lesions produced by them may be confused with tuberculosis or malignancies. These parasitic lung diseases can be cured successfully by appropriate medical or surgical therapies. In the lungs there may be focal lesions or sometimes diffuse involvement. Clinically, focal lung lesions can be divided into cystic lung lesions, coin lesions and consolidation or pleural effusion. Diffuse lung disease may manifest as transient pulmonary infiltrates or alveolar/interstitial lung changes. Paragonimiasis is a common parasitic disease in certain parts of the world, the signs and symptoms of which closely resemble those of tuberculosis. Transpulmonary passage of larval forms of *Ascaris* may manifest as Loffler's pneumonia.
3. **Central Nervous System**: Many parasites are capable of infecting the central nervous system (CNS). Cysticercosis, toxoplasmosis, malaria, African trypanosomiasis, schistosomiasis, angiostrongyliasis, echinococcosis, etc. are important conditions which may affect the CNS. Encephalitis or encephalopathy or intracerebral location of the parasite may trigger seizures or epileptic manifestations, and neurocysticercosis (Fig. 1) and malaria are the most important aetiologies for these forms of the disease. Eosinophilic meningoencephalitis is a typical form of parasitic infection of the CNS and is most often caused by helminthic parasites.
4. **Circulatory and Lymphatic Systems**: Parasites causing malaria, babesiosis, leishmaniasis and African or American trypanosomiasis are found in blood circulation. Apart from these parasites, developmental forms of *Toxoplasma* and *Schistosoma* can also remain in circulation for variable periods of time. Systemic manifestations like chills, myalgia, fever, headache, etc. are common with these parasitic infections. The chronic stage of these parasitoses may manifest with other features with or without the systemic signs or symptoms. The lymphatic system is the habitat for the filarial nematodes belonging to *Wuchereria*, *Brugia* and *Mansonella*, which cause lymphatic filariasis (Fig. 2). Lymphangitis, lymphoedema and lymphatic obstruction in the final stages leading to elephantiasis of the affected part are the cardinal features of filariasis.
5. **Cardiovascular System**: Several parasites are capable of infecting the heart. Myocarditis or pericarditis is principally caused by *Trypanosoma cruzi*, *T. brucei*, *Toxoplasma gondii*, *T. solium* and *Trichinella spiralis*. Pericardial involvement may be seen in amebiasis and echinococcosis. Apart from these, rare cases of cardiac manifestations have been reported with *Naegleria fowleri* infection and in schistosomiasis, sarcocystosis and zoonotic filariasis and in cases of visceral larva migrans.

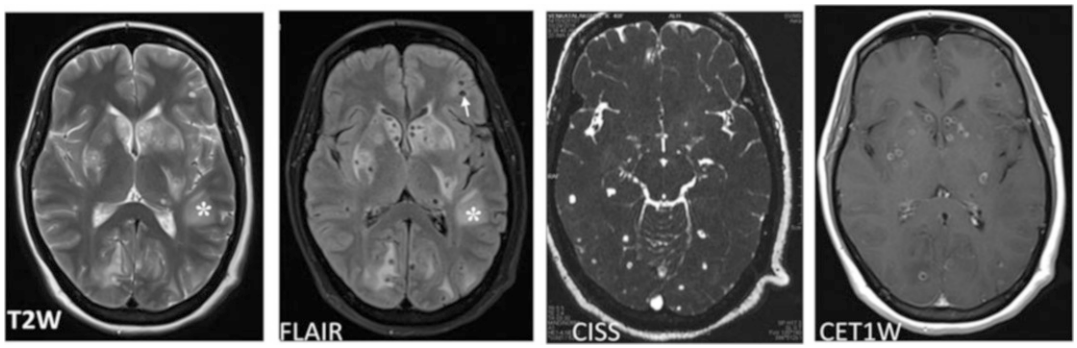

Fig. 1 Magnetic resonance imaging head, axial images showing multiple tiny ring-enhancing lesions in bilateral cerebral hemispheres with eccentric nodule suggestive of scolex (arrow) and peri-lesional oedema (asterisk) suggestive of neurocysticercosis. *T2W*: T2-weighted; *FLAIR*: fluid-attenuated inversion recovery; *CISS*: constructive interference in steady state; *CET1W*: contrast-enhanced T1-weighted. (*Kind courtesy: Professor B. Vijayalakshmi Devi, Department of Radiodiagnosis, Sri Venkateswara Institute of Medical Sciences, Tirupati*)

6. **Liver and Biliary System**: Several parasites can infect the liver or biliary tree, either in larval form or in adult stages. Hepatic involvement can manifest as liver abscess in the case of amebiasis, cyst formation in hydatid disease or hepatitis as in schistosomiasis. Biliary tree infection may cause cholecystitis, biliary tree obstruction, recurrent cholangitis, strictures and sometimes cholangiocarcinoma. Parasites which preferentially infect the biliary system

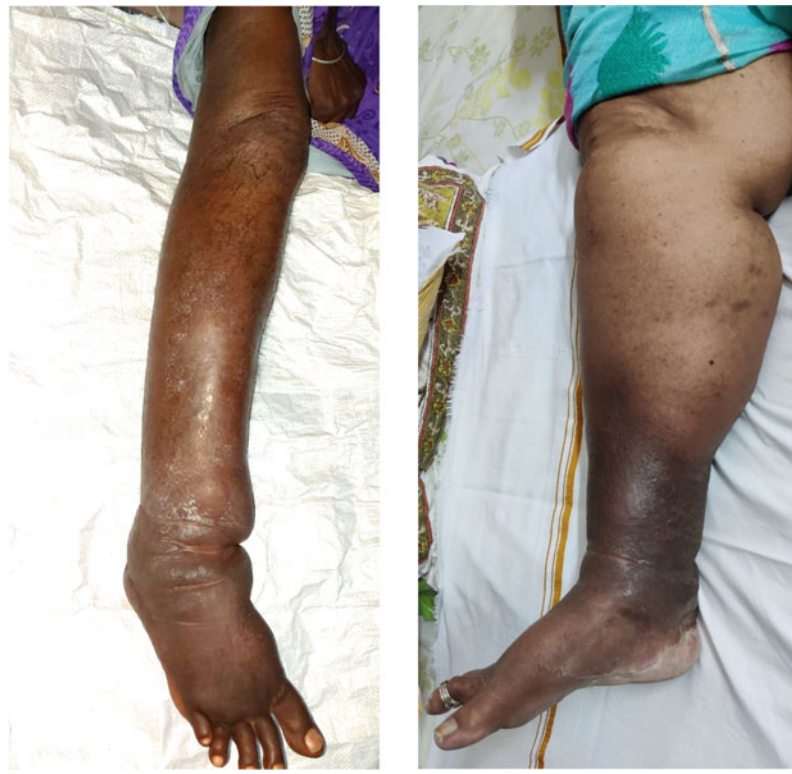

Fig. 2 Clinical photographs showing lymphoedema and elephantiasis in two different patients with filariasis. (*Kind courtesy: Professor B. Srihari Rao, Department of General Surgery, Sri Venkateswara Institute of Medical Sciences, Tirupati)*

include *Fasciola hepatica*, *Clonorchis sinensis* and *Opisthorchis*. The reticuloendothelial system of the liver may be involved in leishmaniasis and malaria.

7. **Genitourinary System**: The most common parasitic diseases affecting the genitourinary system in humans are helminthic, which include schistosomiasis, hydatid disease and filariasis. The protozoans which can infect the system are *Trichomonas vaginalis*, *Plasmodium falciparum* and *Entamoeba histolytica*. The clinical manifestations can be varied depending on the nature of the parasite and its location. Thus while bladder involvement with haematuria due to egg granuloma is a cardinal feature of *Schistosoma haematobium* infection, hydatid disease results in cyst formation in the kidneys or bladder and presents as flank pain or abdominal mass. Chyluria in filariasis and black water fever in falciparum malaria are also well-known phenomena.
8. **Skin, Soft Tissue and Muscle**: A fleeting skin lesion in the form of vesicular eruption may occur in African and American forms of trypanosomiasis at the site of the arthropod bite. However, it is cutaneous and mucocutaneous forms of leishmaniasis which cause definitive lesions on the skin, which may heal with disfiguration. Visceral leishmaniasis similarly can cause post-kala-azar dermal leishmaniasis. Among the helminthic parasites, cercarial dermatitis in schistosomiasis is well recognised. Among cestodes, *T. solium*, *E. granulosus* and *Sparganum* larvae may form subcutaneous cysts. Various zoonotic nematodes can infect humans and cause subcutaneous lesions. Nematode species infecting the skin include *Gnathostoma* spp. Various filarial species like *Loa loa*, *Onchocerca volvulus*, *Mansonella streptocerca* and *Dirofilaria* spp. cause swellings or nodules. Some like hookworm and *Strongyloides* which infect through the skin produce popular pruritic skin lesions at the point of entry. Cutaneous larva migrans and guinea worm infections produce typical cutaneous lesions. The larval forms of *T. spiralis* and other *Trichinella* species inhabit the muscle tissues and produce asymptomatic infections. *T. solium* cysticerci, *T. cruzi* and *O. volvulus* infections can also involve the muscles.
9. **Eye Infections**: A large number of protozoans and helminths can cause eye infection, which may sometimes be severe enough to cause blindness. Among the protozoan parasites, *Acanthamoeba* and *Toxoplasma* are well-known examples which can cause keratitis and uveitis, respectively. Palpebral and periorbital oedema is a hallmark of *T. cruzi* infection. Apart from these, other protozoan parasites like *P. falciparum* and *Leishmania* spp. can cause rare ocular manifestations. Of the helminth parasites, river blindness caused by *O. volvulus* and *Loa loa* eye infections is common in endemic areas. Infrequent involvement by other worms like *Toxocara*, *Angiostrongylus* and *Schistosoma* has also been recorded.

Case Study

A 25-year-old adult from a remote hill area in Mizoram, India, presented with chronic cough and history of passage of rust-coloured sputum for 6 months and dyspnoea. The patient developed severe pleuritic pain and was hospitalised. Chest X-ray showed bilateral pleural effusion which was drained, which relieved the pain and dyspnoea. The pleural fluid and sputum were examined for acid-fast bacilli (AFB) but were reported negative. As the patient had a positive Mantoux test, he was treated for tuberculosis with standard regimen for 6 months. The cough and expectorations persisted, and it was considered a drug-resistant *Mycobacterium tuberculosis* strain, and second line of anti-tuberculosis therapy was initiated but symptoms persisted. He was readmitted for further investigations. Auscultation of the chest revealed diffuse rales on both sides. The chest X-ray showed mixed opacities at the upper lobe of the right lung and small nodules in one lung base with interstitial thickening. A CT scan showed several nodules and patchy frosted glass appearance. Sputum examination for

3 consecutive days showed abundant operculated eggs but negative for AFB. Praziquantel was administered for 3 days, and the cough resolved in 2 weeks' time, and at follow-up after 6 months the patient was keeping well, and repeat examination of sputum was negative for AFB and any eggs.

This case illustrates the importance of one parasitic disease which can mimic tuberculosis particularly in areas where both tuberculosis and this condition are endemic. Misdiagnosis can lead to improper treatment, which may aggravate the actual condition and result in increased morbidity or sometimes mortality.

1. What are the possible parasites which can cause this infection?
2. Based on geographic region, what is the most probable aetiology?
3. What is the mode of transmission of this parasite to humans?
4. What are the precautions to be taken to avoid this infection?

Research Questions

1. What are the roles of host and parasite factors in varied clinical manifestations? How can this knowledge help in better management of parasitoses?
2. How do we enrich evidence-based recommendations for management of parasitic infections, which is lacking for most of these infections?
3. Can the antibody detection tests available distinguish between past and new infections?

Further Readings

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Parasite Immunology

Abhijit Chaudhury

Learning Objectives

1. To understand the immune response of the host against parasites and host immunity.
2. To make the reader aware of the importance of immune evasion by parasites and establishment of chronic infection.
3. To know about the immunoregulation in helminth infections.

Introduction

In 1879, Heinrich Anton de Bary, the German doctor turned botanist and mycologist, stated that "Any two organisms living in close association, commonly one living in or on the body of the other, are symbiotic, as contrasted with free living." The nature of interaction between the symbionts varies considerably, and one such interaction leads to parasitism, i.e., one species, the parasite, lives at the expense of the other, the host, and frequently causes some degree of injury or harm to the host. The parasite, after coming in contact with the host and being a foreign invader, encounters the host's defense system. Whether the host is susceptible or resistant to the infection depends on a complex interplay between the host's immune system and the parasite's ability to combat or evade it. The innate immune response and the adaptive mechanism are equally important in determining the outcome, but one type of adaptive response (humoral or cell mediated) may be predominant over the other. In general, protozoan parasites are frequently intracellular and hence the cell-mediated response plays a prominent role, while for the larval or adult forms of the helminth parasites which are large enough to be extracellular, the antibody response predominates. In spite of these responses, many parasites tend to establish a chronic infection for long-term transmission. This strategy may be facilitated by various mechanisms like immune evasion, immunoregulation, or immunomodulation, which in turn also suppresses or minimizes the immunopathological damage to the host.

Innate Immune Responses

Pathogenic organisms have molecular structures which are shared among similar organisms and are needed for infecting the host. These structures, which are absent in mammalian cells, are termed pathogen-associated molecular patterns (PAMPs). These patterns are recognized

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by pattern recognition receptors (PRRs) present in host cells at all levels (cell membrane, cytoplasm, and endosomes). A number of PAMPs have been described in parasites as well as corresponding PRRs in the host. The best studied PRR is the Toll-like receptors (TLRs), a total of 10 of which have been identified in mammalian cells. A number of PAMPs have been found in protozoan parasites, which include glycophosphatidylinositol and phosphoglycans present in trypanosomes, *Leishmania*, *Toxoplasma*, and *Plasmodium falciparum*. These molecules stimulate TLR2 and also TLR4 to upregulate nitrogen oxide synthase production and synthesis of pro-inflammatory cytokines. In addition, parasite nucleic acids also function as ligands for recognition of TLRs. Thus, TLR9 recognizes unmethylated CpG motifs present in protozoan DNA. The profilin proteins of *Toxoplasma* and *Cryptosporidium* trigger IL12 production in murine dendritic cells due to stimulation of TLR11, although this TLR is absent in mammalian cells. In addition to TLRs, other PRRs have been identified which are classical human receptors. These include mannose-binding lectins which bind to lipophosphoglycan of *Leishmania*, *P. falciparum*, and *Trypanosoma cruzi* and pentraxin which binds to sporozoites of malaria parasites. A few other PRRs like cytosolic DNA sensors, NOD-like receptors, and RIG-1-like receptors have been identified, but studies on these receptors are still rare and controversial.

Helminth parasites also express ligands for TLRs, but their role is not clearly elucidated. Certain PAMPs like ES-62, a glycoprotein of filarial worms and lipophosphatidylserine moieties of *Schistosoma* membrane, have been described which can trigger TLR4 or TLR2. Eggs of *Schistosoma* can also trigger TLR3 in dendritic cells.

Cellular Effectors of Innate Immune Response: A number of cell types take an active part in the innate response and form the backbone of this type of immunity:

1. **Macrophages and Granulocytes:** Phagocytosis by macrophages and granulocytes like neutrophils, eosinophils, and basophils plays an important role in innate immune response for protozoan parasites. Activation of the oxidative metabolism and generation of reactive oxygen species for NADPH sets the stage for intracellular killing of phagocytosed parasites. On the other hand, parasites have evolved a number of strategies to avoid or withstand these assaults which increase their chance of survival within these cells. These include, among others, inhibition of respiratory burst by certain parasite molecules, opsonic entry through receptors which do not activate NADPH oxidase, and ability to withstand or escape from the acidified, hydrolytic environment of phagolysosomes. In contrast, helminths, which are too large to be phagocytosed, can be killed by macrophages after activation of adaptive response. Only the eosinophils play some limited role in innate response to helminthic larva by releasing granules containing membrane-damaging enzymes and other proteins.
   Normally, following an antigenic stimulus, the monocytes differentiate into mature macrophages and dendritic cells. Two types of macrophages have been described. The M1 or classically activated macrophages are induced by IFN-γ and microbial products and can kill intracellular pathogens by endocytosis, production of nitric oxide, and synthesis of reactive oxygen intermediates. The second type or M2 (alternatively activated macrophages, AAMs) cells get differentiated in response to IL4, IL13, and some other cytokines and are typically associated with TH2 adaptive immune response and tissue repair seen in helminthic infections. Dendritic cells, which are specialized macrophages, have a dual role to play: as classical macrophages in innate response and also priming of the immune system for the ensuing adaptive response.
2. **Innate Lymphoid Cells (ILCs):** This is a growing family of immune cells that mirror the phenotypes and functions of T-cells of adaptive response. But in contrast to these T-cells, the ILCs do not express antigen receptors or clonal selection when stimulated. Instead, they react to the antigens to produce

various cytokines which direct the immune response needed for the parasite challenge. The natural killer (NK) cells can be considered the innate counterparts of cytotoxic CD8+ T-cells, whereas the ILC1, ILC2, and ILC3 may represent the innate components of TH1, TH2, and TH17 cells, respectively. Tissue signals in the form of IL12, IL15, or IL18 stimulate ILC1 which in turn produce effector cytokines like IFN-γ and TNF-α and help in macrophage activation with generation of reactive oxygen intermediates. Type 2 ILCs are stimulated by IL25, IL33, and TSLP in response to helminthic infections and in turn produce various effector molecules like IL4, IL5, and IL13 which take part in M2 activation and mucus production, along with tissue repair. Survival of ILC2 in the intestine and the lungs is controlled by IL9, a cytokine which also enhances TH2 response. Lastly, the ILC3 plays an important role in bacterial infections and helps in the phagocytic process.

3. **Natural Killer (NK) Cells:** These cells are particularly important for the innate defense against intracellular protozoan parasites. They become activated in response to infections by *Leishmania*, *Toxoplasma*, and *P. falciparum* and also by the excretory-secretory proteins of hookworm. Activation of NK cells occurs as a consequence of PRR-mediated activation of DCs and is both contact dependent and cytokine driven (IL12, IL18). Both mechanisms induce the production of IFN-γ. This cytokine serves as multiple effector for both innate and adaptive responses. Thus it activates macrophages and neutrophils and also helps in transformation of TH1 cells, thereby playing an important role in protozoan infections. The inhibitory action of IL4, IL10, and TGF-β on NK cell activation corresponds to the relatively unimportant role of these cells in helminthic infections where TH2 response predominates. However, the increase in NK cell population in some helminthic infections suggests a role since a few helminths are capable of producing both TH1 and Th2 responses due to the presence of different developmental stages during infection in the host.
   Regulation of NK cell activity is carried out by IL10 and other cytokines which have a downregulatory effect on IFN-γ production or by direct suppression of NK cell activity. This is useful in protecting the host from excessive tissue damage by IFN-γ or TNF-α.
4. **Natural Killer T (NKT)-Cells:** These cells help in rapid cytokine response. They recognize glycolipids in association with CD1d molecules, and these cells have been proposed to be the early sources of TH1 and Th2 cytokines. They express restricted T-cell receptors of limited diversity, and their role in innate response remains controversial. However, they may initiate the adaptive immune response.
5. **γ-δ T-Cells:** These T-cells have T-cell receptors (TCR) made up of γ- and δ-chains in contrast to the more common α- and β-chains. They are found predominantly in gut mucosa and have fewer antigen receptors. These cells are part of innate response since they release cytokines like IFN-γ and TNF-α which can damage infected cells. They also form a bridge between innate and adaptive response by acting as antigen-presenting cells, and they also have regulatory functions. They may contribute to tissue damage by heightened immune response due to the release of IL17. For helminthic infections, various attributes of these cells have been mentioned for different parasites, but the definitive role played by these cells remains unclear.

Apart from the various types of cells mentioned above, for intestinal helminths the first barrier which they encounter is the secreted mucus. There is marked goblet cell hyperplasia noted in such infections, and the secreted mucus gel consists of high molecular weight glycosylated glycoproteins, and Muc2 is the predominant molecule. This mucus production is under the control of both innate and adaptive host response. Type 2 cytokines, particularly IL4, IL13, and IL22 secreted by ILC2 as well as

CD4+ T-cells, are potent inducers of mucin production and resultant goblet cell hyperplasia. This mucin accelerates the expulsion of the helminths from the intestine.

Adaptive Immune Response

Adaptive immune response is primarily mediated by T- and B-lymphocytes with initial priming by various cells of innate immune response. The T-cells are mainly of four types: T-helper cells (CD4+ T-cells), cytotoxic T-cells (CD8+ T-cells, T_c), T17 cells, and T-regulatory cells (T_{REG}). Presentation of the antigen by antigen-presenting cells results in the differentiation of TH1 and Th2 subsets. It is now well established that TH1 response is elicited in infections caused by intracellular protozoan parasites, while the extracellular helminthic infections result in the differentiation of the TH2 subset. However, TH response may vary with the particular type of parasite and its developmental stage.

Adaptive Response to Protozoan Parasites

1. **TH1 Response:** This is mediated by a set of cytokines, chief among which is IFN-γ. The protective role of this cytokine and of TH1 has been conclusively shown in mouse models of *Leishmania* infection. The C57BL/6 mice strains which produce IFN-γ are resistant to *Leishmania* infections, while those which cannot produce it are susceptible to infection. The IFN-γ produced by CD4+ TH1 cells binds to specific receptors on macrophages and causes their activation with the production of antiparasitic molecules. In addition, IFN-γ increases MHC-I expression to help in the recognition and killing by CTLs, together with MHC-II expression to promote antigen presentation to CD4+ T-cells. The TH1 response is important in protection against pre-erythrocytic stage of *Plasmodium* apart from protection against *Leishmania* and *Toxoplasma* infections.
2. **Cytotoxic T-Cells (CTLs, CD8+ T-cells):** These cells function both in the recognition and killing of target cells. As part of adaptive response, they display the necessary specificity, and following antigen stimulus, they start producing cytotoxic granules. The killing mechanism is somewhat nonspecific and involves three types of cytotoxic molecules:
   (a) **Perforins:** It is a 66 KDal molecule which can produce pores or holes in the target cell membrane.
   (b) **Granzymes:** They exist as pro-enzymes and are cleaved by cathepsin. Their entry into cells is facilitated by the pores induced by perforins. Once inside the cells, they can induce apoptosis.
   (c) **Granulysin:** It helps the granzyme to kill the parasite inside the cells by a process similar to apoptosis.

   Whatever may be the mechanism, even if the intracellular parasites are not killed by the above processes, their release from the destroyed cells can lead to killing by activated macrophages. The CTL plays a pivotal protective role by destroying hepatocytes infected with the sporozoites of malaria parasites. It is also important in protection against *Leishmania*, *Toxoplasma*, and *T. cruzi* infections. In *Leishmania* infections, these cells have a dual role to play. On the one hand, they have a protective role in *Leishmania donovani*, *Leishmania major*, and *Leishmania infantum* infections. On the other hand, overproduction of IL10-producing CTLs has been observed in disseminated cutaneous leishmaniasis as well as in post-kala-azar dermal leishmaniasis, pointing to its involvement in disease dissemination. CTLs have also been implicated in tissue destruction and disease progression in mucocutaneous leishmaniasis.
3. **TH2 Response and Role of Antibodies:** All protozoan infection elicits an antibody response, but the role of humoral immunity in protection has not been demonstrated, except for a few selected instances. Thus, for

Trypanosoma brucei, which is an extracellular protozoan parasite, IgG plays an important role in control of infection. The antibodies have also been described to play a role in direct lysis of *T. cruzi* or complement-mediated destruction of plasmodial gametocytes. Antibodies can also facilitate macrophage function by binding with F_c receptors and effective phagocytosis of *Toxoplasma gondii* or RBCs infected with malaria parasites. These antibodies can also prevent the entry of the parasites into target cells by neutralizing certain antigens of the parasite necessary for penetration. This has been demonstrated in *T. gondii* and *P. falciparum.* Thus, it appears that humoral immunity does play a part in containment of protozoan parasites, but by itself it may be less efficient in clearing the infections.

Adaptive Response to Helminth Parasites

The helminths are larger in size compared to protozoan parasites and are extracellular in nature, and hence in such infections, TH2 response predominates over TH1 response. A number of hypotheses have been advanced to explain this phenomenon. It has been shown that helminths exhibit a relatively low number of TLR ligands, leading to a poor production of IL12 by dendritic cells, an interleukin essential for TH1 differentiation. Additionally, the excretory-secretory antigens of helminths may suppress IL12 production and in turn may upregulate cytokines like IL25 and IL33, which enhances TH2 differentiation. Whatever may be the mechanism, the TH2 cells start producing various cytokines like IL3, 4, 5, 9, 10, and 13 which also activate other cells like eosinophils, mast cells, and basophils, along with IgE production by B-cells. Thus a concerted mechanism comes into play to eliminate the helminthic parasite from the body.

Dendritic cells act as classical antigen-presenting cells (APCs) in the body. Apart from this, it has been found that ILCs and basophils can also act as APCs. In the intestine, mucin containing the parasite antigens is taken up by dendritic cells. The induction of highly polarized CD4+ TH2 cell response with the release of a plethora of cytokines promotes immunity through multiple mechanisms and effector cells:

1. **Mast Cells**: IL3 and IL9 produced by TH2 cells act synergistically and cause accumulation of mast cells in the mucosa of the small intestine. The mast cells prevent the adhesion and penetration of parasite into the mucous membrane by releasing chondroitin sulfate. These mast cells also express high-affinity IgE receptors.
2. **Eosinophils**: Elevated eosinophil levels are common in helminthic infections, but their exact role is somewhat controversial. Circulating eosinophils are attracted to the site of helminthic infection by IL4 and IL13 as well as by chemokines. Degranulation or activation of eosinophils occurs under the influence of various cytokines as well as immunoglobulins. In vitro studies have shown parasite destruction by molecules of eosinophilic granules. This has been demonstrated for *Schistosoma mansoni*, *Strongyloides stercoralis*, and *Trichuris muris*, but no such effect could be demonstrated in vivo in animal models. In *Trichinella spiralis*, eosinophils may actually promote infection.
3. **Antibody Response**: IL4 released by TH2 cells is a promoter for immunoglobulin class switching to IgE, which is the prototype immunoglobulin seen in helminthic infections. However, its role in host protection remains unclear, and it is surmised that most of the IgE may not be parasite specific and it may also be a part of parasite evasion strategy. In some cases, IgE contributes to intestinal anaphylaxis due to mast cell degranulation. This can lead to a rapid elimination of the larval stage of the parasite due to intestinal physiology and chemistry of the gut epithelium. In some cases, IgA may neutralize the secreted

metabolic enzymes of the parasite and thus interfere with the feeding of the worm.

In experimental animals and also in natural host animals, the following immune mechanisms have been observed which can restrict helminthic, particularly nematodal infections:

1. **Breed resistance:** It has been seen that individual Merino lambs may be classified as responders and nonresponders on the basis of their immunological response to infection with *Trichostrongylus colubriformis* and these differences are genetically transferable.
2. **Age Resistance:** In older age, the nematodes either fail to develop or get arrested in larval stages in the tissues. *Strongyloides* infections of ruminants and horses are most commonly seen in very young animals and conversely in some parasites such as *Anaplasma*; young cattle are more resistant to infection than older cattle. The reason for this age resistance is unknown. Unlike sheep and cattle, goats do not develop age-related immunity. *Trichostrongylus* spp. stimulate a slower immune response and are therefore sometimes seen in older livestock.
3. As exemplified in infections of the rat with the trichostrongyloid nematode *Nippostrongylus brasiliensis*, the adults may be stunted in size, and in some cases these adult worms are killed and expelled automatically from the animal.
4. Sometimes immunological unresponsiveness is seen in ruminants with gastrointestinal infections. The mechanism is not fully understood. It has been agreed upon that in these animals luminal immunity is due to TH2 type of response. There are increased gut mast cells and gut receptors for worm-specific IgE antibodies. These sensitized mast cells produce vasoactive amines that lead to increased mucus production and capillary leakage. These changes can lead to decreased oxygen tension in the gut, thus leading to detachment and expulsion of the worms. This local gut response by immune cells varies greatly with parasites. For example, mast cell response is required to expel *T. spiralis*, but it is not required in the case of *Nippostrongylus brasiliensis* infection.

Role of T17 Cells in Helminth Infections

The naive T-cells can differentiate into another subset known as TH17 as a result of antigen recognition in the presence of TGF-β and IL16. These TH17 cells produce IL17, which is a pro-inflammatory cytokine. It helps in recruitment of granulocytes and release of other pro-inflammatory cytokines. IL17 may also be produced by cells primarily involved in innate response like NK cells and γδ-T-cells. By promoting inflammation, the TH17 cells contribute to various pathologies associated with helminthic infections, including tissue damage. They may also promote intestinal hypermotility.

T-Regulatory Cells and Immunoregulation

A noteworthy feature of most helminthic infections is their long life span (sometimes many years) and persistence, but causing minimal harm or any life-threatening pathological consequence. This feature is due to a complex interplay of immune evasion and regulation of host immunity. The chronicity of infection causing persistent dominant TH2 response induces the expansion of natural as well as parasite-induced regulatory T-cells (T_{REG}). T_{REG} cells are a distinct population of T-lymphocytes which has the ability to suppress the function of other lymphocytes. Thus they can exert this effect on CD4+ CD25- T-cells, CD8+ T-cells, as well as B-cells. This subset can be identified by the expression of CD4, CD25-, and FOXP3. By its suppression effect, these cells can exert a profound state of immune tolerance in the host. The same response causes an immunoglobulin class switching in B-cells to IgG_4. In effect, the helminth enters into a niche with low parasite

antigen-specific lymphocyte proliferation, higher antigen-specific IgG_4/IgE ratio, and increased levels of regulatory cytokines IL10 and TGF-β. These are the characteristics of an asymptomatic chronic helminthic infection. The complex interplay and the roles played by different cells are depicted in Fig. 1.

In summary, helminths are very complex organisms phenotypically as well as genetically. Due to their physical size, they cannot be ingested by phagocytic cells or destroyed by classic cytotoxic T-cells. The immune cells usually deploy type 2 immune responses or the allergy-type immune responses against the helminths. These responses are characterized by increase in the concentrations of interleukin (IL)4 and other Th2-type cytokines, such as IL5, IL9, IL13, and IL21. There is an increased recruitment and activation of effector cells, such as eosinophils, basophils, and mast cells which can produce various cytokines. In these parasitic infections, innate and acquired components of an active immune system constantly communicate with each other. T-cell signals increase and modify the function of effector B-cell, which in turn induces antibody response.

Immune Evasion by Parasites

Immune evasion is a strategy adopted by various microorganisms including protozoans and helminths to survive in a host in spite of effective immune response. The mechanisms involve one or more of the following strategies:

1. **Antigenic Variation:** Strains of parasite can be distinguished by the presence of immunodominant antigens, and strain-specific immune response defines the parasite population. A loss or gain or alteration in a particular immunodominant antigen group due to the corresponding loss/gain/change of one of the polypeptides or polysaccharide antigen is defined as antigenic variation. Hence, although the adaptive immune response may be effective against the original infective serotype, it becomes ineffective against the same strain displaying the new antigenic variant. Many parasites including malaria parasites, giardia, and agent of African trypanosomiasis undergo antigenic variation by changing the expression of their variant antigen molecules, collectively known as variant specific surface groups (VSG). A parasite may contain a large number of VSG genes but only one will be expressed at a time. Electron microscopy has shown that the VSG form a dense layer on the parasite surface and contain the immunodominant antigen. With the increase in the level of antibody in the host, a small fraction of the antigen population switches to produce a new coat of VSG with a new antigenic character no longer recognized by the circulating antibodies.
2. **Immunosuppression:** The phenomenon of parasite-induced immunosuppression was first described almost 60 years ago when high prevalence of malaria was co-related with low incidence of autoimmune diseases, which led to the foundation of the hygiene hypothesis. In helminthic infections, there is an inability of effector T-cells to proliferate and to secrete pro-inflammatory cytokines, an effect called immunological tolerance. These infections are also characterized by elevated IgG_4 levels and corresponding IL10 production, which is a down-modulatory cytokine. Helminth parasite can induce TGF-β receptor production, which results in generation of T_{REG} cells and suppression of dendritic cells and macrophages and T-cell activation, all of which have an overall immunosuppressive effect. In addition, parasite molecules can also modulate CD4+ T-cell differentiation, B-cell isotype switching, and B-regulatory cell induction and thus can produce a milieu for survival of the immune-shy parasite.
3. **Molecular Mimicry:** Many parasites display some antigens which resemble a host molecule which confers a survival benefit for the parasite. The antigenic resemblance helps in non-recognition of parasite antigen by the host, confusing it as a self-antigen. Sometimes some of these antigens may mimic host hormone receptors or the hormone itself, resulting

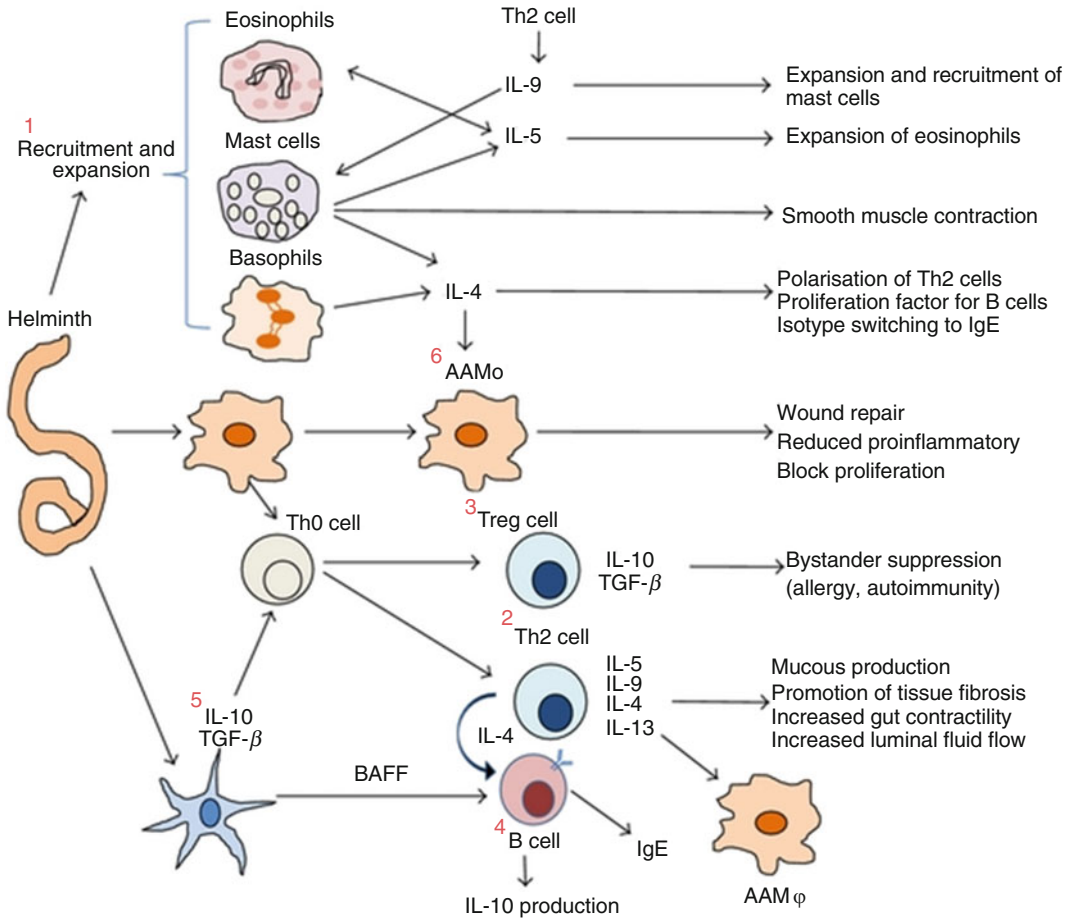

Fig. 1 Helminth infections are strong inducers of a Th2-type immune response. These infections are characterized by the expansion and activation of eosinophils, basophils, and mast cells (1). Their upregulation due to high levels of immunoglobulin E (IgE) and the proliferation of T-cells that secrete IL4, IL5, IL9, and IL13 is part of the host immune response against the parasite (2). However, helminth infections tend to be long-lived and largely asymptomatic because they are sustained through a parasite-induced immunomodulatory network, in particular through activation of regulatory T-cells (3) and systemically elevated levels of IL10 produced by B-regulatory cells (4). They are additionally affected by the expression of the regulatory cytokines IL10 and TGF-β, produced by regulatory dendritic cells (5) and alternatively activated M (AAM) (6). (From: Salazar-Castañon VH, Legorreta-Herrera M, Rodriguez-Sosa M. Helminth parasites alter protection against *Plasmodium* infection. *Biomed Res Int*. 2014;2014: 913696. doi: https://doi.org/10.1155/2014/913696)

in either a response to hormonal signals or sending the signals. This ability of the parasite to mimic host molecules can be the outcome of either transfer (acquisition of host molecule by the parasite) or convergence (evolution of the mimic molecule). The genomic era has opened up a vista where direct comparison of host and parasite proteins and their sequences can be studied and the molecular mimicry candidate proteins or macromolecules can be directly predicted.

Table 1 shows the various immune evasion strategies employed by parasites.

Table 1 Different mechanisms of immune evasion by parasites

SN	Mechanism	Specific mechanism	Parasite
1.	Anatomical seclusion	Intracellular location of parasite	Malaria parasite (RBC) *Leishmania* (macrophages) *Trichinella* (muscle cells)
2.	Antigenic variation	Different antigens in various life stages, variable surface glycoproteins	Malaria parasite, *Trypanosoma*
3.	Size	Large size of parasites	All helminths
4.	Coating with host protein	Blood group antigens and MHC class I and II molecules on the parasite tegument	*Schistosoma*
5.	Molecular mimicry	Fibronectin cell receptors	*Trypanosoma cruzi*
6.	Immunosuppression	Binding of β-integrin CR3 by parasite protein causing neutrophil dysfunction	Hookworm
7.	Parasite enzymes	Glutathione peroxidase and superoxide dismutase cause resistance to antibody-dependent cellular cytotoxicity	Filarial worms

Conclusion

The host-parasite interaction is a highly complex phenomenon and becomes more complicated in helminthic parasites because of the size and myriad of constituent macromolecules. The immune response to parasites is an intricate and interrelated process where there is a large overlap of natural and adaptive immune responses. In addition, parasites have evolved numerous strategies to evade the host's immune onslaught, which has a direct bearing on immunity to parasites and its long-term survival. The present age of genomics, proteomics, and other *-omics* has opened up a floodgate of information concerning various parasites which is expected to elucidate this complex interplay and address many unanswered questions.

Case Study

Lipophosphoglycan (LPG) is an important component of *Leishmania* envelope and has a significant effect on impairment of macrophage function by various mechanisms like cytokine cleavage, prevention of phagolysosome maturation, and activation of negative regulatory factors. Thus it plays an important role in survival of the parasite inside the macrophages. In an experimental mouse model, LPG induced an increased production of IFN-γ and TNF-α by producing reactive nitrogen intermediates and a killing effect on *L. major*. LPG along with BCG has been shown to raise TH1 immune response in mice as well as hamster models. Thus LPG is an important target for the future vaccine development for visceral leishmaniasis.

1. What are the various candidate *Leishmania* vaccines which have entered Phase 1 or 2 of vaccine trials?
2. What is a therapeutic vaccine?
3. Name the parasite vaccine which has shown the most promise to date. What is its composition?

Research Questions

1. What are the PAMPs which are important in different helminth parasites?
2. What is the exact role, if any, of eosinophils in parasitic infections?
3. What is the efficacy of therapeutic worm infection in the treatment of autoimmune diseases and metabolic disorders?

Further Readings

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Parasitic Genomics

Sumeeta Khurana and Parakriti Gupta

Learning Objectives

1. To know the tools used for genomic studies and the targets.
2. To have an understanding about the application of genetic studies in parasitology.

Introduction

Parasites are unique among all the microorganisms as most have complex life cycles involving one or more hosts, are difficult to culture in the laboratory, and lack suitable experimental models. The advent of novel approaches to genetic study and manipulation has promoted the research on parasites. International parasitic genome networks are now established and have resulted in an exponential increase in genomic data for parasites. All this data is stored in databases and can be accessed online and used for structural and functional analyses. Availability of genomic data has altered the way infectious diseases are studied. However, this mammoth big data serves no purpose unless it is interpreted as gene annotation and error correction are still exhaustive and critical. Prediction of the gene function is still a major challenge. Lately, *metagenomics* is making possible analysis of the relationship of complex microbial communities, especially those which cannot be cultured.

Beginning of Parasitic Genomic Era

Decrypting of the whole genome of most parasites has been successful owing to their smaller genome size of approximately 10–270 megabases (Mb) (Table 1). However, parasitic genomes vary in size, nucleotide composition, content, polymorphism, and repetitive sequences, all of which affect the feasibility and application of sequencing strategies. The groundbreaking landmark was witnessed in 2002, when the genomic sequence of protozoon *Plasmodium falciparum* was published as a product of international collaborative determinations. Subsequently, genomes of *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania major* and now many more parasites have been decoded. Helminths have a much larger genome compared to protozoa but very small when compared to mammals. However, they contain almost the same number of genes as humans. However, in nematodes, genes are gained and lost frequently, and there is horizontal gene transfer from bacteria, fungi, amoeba, or endosymbionts. The free-

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S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
https://doi.org/10.1007/978-981-16-7204-0_4

Table 1 Genomic sizes of common parasites of humans and animals

Species	Host	Genome size (in Mb)
Giardia duodenalis	Humans	12.6
Entamoeba histolytica	Humans	24
Plasmodium falciparum	Humans	22.8
Ancylostoma caninum	Dogs	344
Ascaris lumbricoides	Humans	230
Brugia malayi	Humans	96
Onchocerca volvulus	Humans	150
Trichinella spiralis	Humans, pigs	63
Trichinella muris	Mouse	96
Echinococcus multilocularis, *Echinococcus granulosus*	Humans, rodents	150
Taenia solium	Humans	270
Schistosoma mansoni	Humans	390
Schistosoma japonicum	Humans	400

living nematode, *Caenorhabditis elegans*, is the first and one of the best-studied metazoan parasites whose genome has been fully sequenced. In recent years, the draft genomic sequences of many other parasites have been made available.

The molecular techniques available for decrypting the genome of parasites include Sanger's sequencing (Fig. 1), microsatellite markers, microarray, Luminex (multianalyte profiling), random amplification of polymorphic DNA or arbitrarily primed PCR, restriction fragment length polymorphism, amplified fragment length polymorphism, whole-genome sequencing, RNA interference, and bioinformatics approaches (Table 2). The relatively new next-generation sequencing technology has enabled analysis of genomic sequence with comparative genomics, functional genomics, transcriptomics, metabolomics, proteomics, and epigenetics.

The data obtained from sequencing studies is interpreted in three categories: (1) complete or nearly complete genomic sequences as *contigs* of series of overlapping DNA sequences, (2) genome-survey sequence tags (GSS)

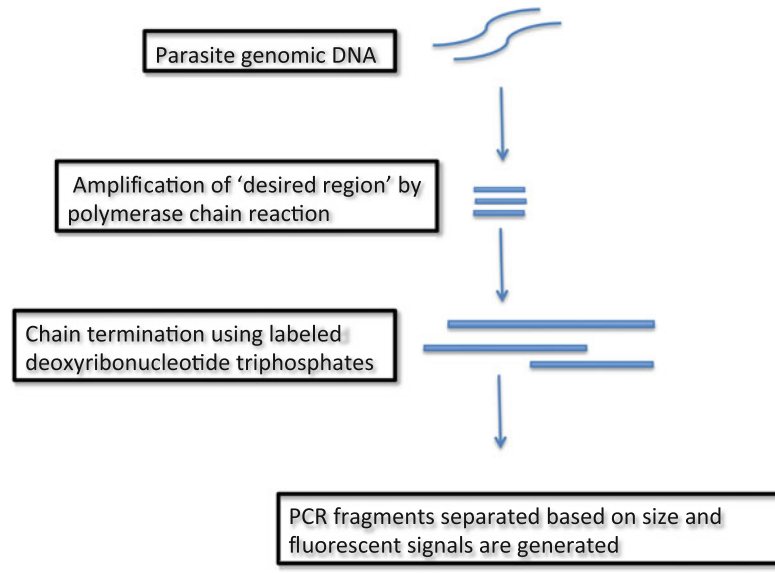

Fig. 1 Flowchart depicting Sanger sequencing in parasites

Table 2 Molecular techniques available for decrypting genome of parasites

Molecular techniques	Examples of parasites
Sanger sequencing	*Leishmania*, *Plasmodium*, *Echinococcus*, and *Trypanosoma*
Microsatellite markers	*Plasmodium*, *Ascaris*
Microarray	*Plasmodium*, *Toxoplasma*, and *Trypanosoma*
Multianalyte profiling	*Plasmodium*, *Cryptosporidium*, and *Leishmania*
Random amplification of polymorphic DNA	*Plasmodium*, *Leishmania*, *Echinococcus*, and *Trypanosoma*
Restriction fragment length polymorphism	*Cryptosporidium*
Amplified fragment length polymorphism	*Cryptosporidium* and *Leishmania*
RNA interference	*Plasmodium*, *Giardia*, and *Entamoeba*
Whole-genome sequencing	*Plasmodium*
Bioinformatic approaches	*Plasmodium*

generated after skimming of the genomic sequences, and (3) expressed sequence tags (ESTs) generated from the respective mRNA expressed in different stages of the parasitic life cycle. All these data are stored in databases and can be accessed online at their own sites, but most of the data is available via *GenBank* and by web-based *BLASTSearch*. These data can be used for structural and functional analyses. The Institute for Genomic Research (TIGR), founded in 1992, located in Maryland, USA, is involved in the sequencing of the genomes of prokaryotic and eukaryotic organisms and post-sequence analysis. One of the main objectives for generating the sequence data is to identify genes related to parasite evolution, development, metabolism, pathogenicity, immune evasion, diagnostic markers, etc.

Application in Parasitology

Genomics has manifold applications in parasitology (Table 3), some of which are discussed here.

Parasitic Diagnosis

Conventionally, diagnosis of parasitic infections has relied upon microscopic demonstration of different life stages of parasites. However, these techniques are often insensitive to warrant exploration of newer, more sensitive modalities. Factors responsible for failure of conventional techniques include lower parasite numbers in comparison to bacteria and viruses and presence of morphologically similar nonpathogens. Parasites are usually not cultured routinely due to lack of expertise and facility to culture, longer turnaround time, and parasite fastidiousness to grow. Nucleic acid amplification technologies, especially polymerase chain reaction (PCR) and its modifications, are increasingly being used to facilitate parasitic diagnosis, especially in the developed centers. For example, amebiasis is routinely diagnosed in fecal samples or liver pus by PCR as it can differentiate *Entamoeba histolytica* from the nonpathogenic morphologically identical *Entamoeba dispar* with excellent sensitivity and specificity. Similarly, kinetoplast DNA, 18S, and ITS regions are used as targets for identification of *Leishmania* species.

Unlike direct pathogen identification, serology is frequently employed for diagnosis of infectious diseases. However, it has a number of limitations. Firstly, serological response, especially specific antibodies, can only be detected in serum after a lapse of a few days to weeks of infection. Sometimes distinction between current and past infection is not possible as antibodies can persist for a period of months to years. Moreover, sensitivity of serological tests varies with the organism and host immune system and may be unreliable in immunocompromised conditions. One such example of appropriate use of technology is diagnosis of toxoplasmosis during pregnancy which was conventionally diagnosed by serology. PCR on amniotic fluid has become the new gold standard for detection of maternally transmitted toxoplasma infection to the fetus.

Table 3 Applications of genomic studies in parasites

Entity	Examples of parasites
Parasitic diagnosis	*Entamoeba* spp.
Parasite discovery	*Plasmodium knowlesi*
Host–parasite interactions	*Brugia malayi* and *Wolbachia*
Host susceptibility studies	*Trypanosoma*, *Schistosoma*, and *Plasmodium*
Molecular mimicry and antigenic variation	*Plasmodium* and *Trypanosoma*
CRISPR-Cas studies	*Anopheles*, *Plasmodium*, *Trypanosoma*, and *Leishmania*
Epidemiological tracking	*Giardia*
Drug discovery and resistance	*Plasmodium*
Vaccine development	*Toxoplasma*, *Echinococcus*

Pathogen Discovery

In addition to diagnosis, many new parasites infecting humans have been discovered recently and are the result of application of genomic technologies like whole-genome sequencing, metagenomics, etc. For example, *Plasmodium knowlesi*, which was initially misidentified as *Plasmodium malariae*, could be discovered only by sequencing and has now been established as the fifth human malaria species originating from monkeys. In addition, many new parasites are being discovered owing to genomic technologies, e.g., *Bertiella*, *Taenia asiatica*, etc.

Host–Parasite Interactions

Apart from studying the genomic characterization of parasites on their own, extensive research has also been carried out in scrutinizing host–parasite interactions. A landmark study described the prototypic endosymbiotic interaction between *Brugia malayi* and *Wolbachia* after whole-genome sequencing of the *B. malayi* genome was deciphered. *Wolbachia* as a bacterial endosymbiont is required for filarial multiplication, and lipopolysaccharide present in the bacterial cell wall also acts as one of the most potent virulence factors for the parasite, inducing a profound inflammatory reaction. This discovery was subsequently sustained by a highly effective therapeutic response to tetracycline that acts on *Wolbachia* and not *B. malayi*, which resulted in dramatic decline in filarial burden and is currently under research as a potential vaccine candidate. *Plasmodium* pathogenesis is increasingly being explored utilizing genomics, proteomics, and transcriptomics. For example, several Toll-like receptors, e.g., TLR-2 and TIR-domain carrying adaptor molecule 2, have been associated with difference in clinical severity based on genomic studies.

Host Susceptibility Studies

Parasites successfully subsist in the host, which can be attributed to very specific genetic adaptations. Thus, detection of such genes which are important for this adaptation can be utilized to understand disease and propose a cure. Genomic studies are the key to establishing the parasite–host–microbe relationship by analyzing the helminth-induced changes in human gut using transcriptional repeats following parasitic infection. This can be further exploited to delineate the role of gut microbiota in parasitic infections and in selecting novel targets for limiting infections, novel drugs, and vaccines. Furthermore, host susceptibility, severity, and mortality of infection are also exposed by these extensive genetic studies.

It is a long-established fact that patients with sickle cell anemia and thalassemia and those with absence of glycoprotein receptors are resistant to malarial infection. Similarly, other host cell receptors like CD234 have been associated with susceptibility to infection. CD234 is essential for *Plasmodium vivax* entry into red blood cells, and

FY mutation deleting this CD234 provides protection from malaria. Individuals with ENU mutation in ankyrin-1, a significant membrane protein, with erythropoietic protoporphyria with decreased levels of ferrochelatase are noted to be resistant to cerebral malaria. In contrast, patients with mdr1a mutation in P-glycoprotein, CD36 deficiency, apobec3b deficiency, and metallobetalactamase-2 deficiency are more susceptible to cerebral malaria. Some mutations making the host susceptible or resistant to infections have also been noted in other parasites e.g, *nramp* 1 mutation and *lpl* gene mutation in *Leishmania*, single nucleotide polymorphisms in STAT-3 in *Entamoeba*, and *apo2–1* mutation in *Trypanosoma* increase susceptibility of host to infection.

Molecular Mimicry and Antigenic Variation

Genomic approaches have been utilized by researchers to identify the phenomenon of molecular mimicry in *Plasmodium* species, exhibited by members of the KIR family. Many genes have a high level of identity with the molecular domains of CD99, an immunoregulatory protein present on the membrane of lymphocytes and T cells. Furthermore, transcriptomic studies have shown the sharing of *P. falciparum* genes to short sequence motifs upstream of ATGs. Site-directed mutagenesis validated their role in promoter activity, especially AP2 in the ookinete stage. However, transcription of all the genes including the antigenic variation is further regulated at chromosomal level. Since the antigenic variation is not present in all parasites, researchers conducted extensive studies to disclose the basis and correlation between the antigenic variation of *Plasmodium* spp. and *Trypanosoma* spp. Epigenetic and chromatin immunoprecipitation studies unearthed the role of histone enzymes, histone methylase, and deacetylase in switching and antigenic variation.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Associated (CRISPR-Cas) in Parasites

Though the parasitic genome was decoded quite late compared to other microbes, CRISPR-Cas technology has been exploited in *Plasmodium*, *Leishmania*, *Trypanosoma*, platyhelminths, as well as vectors like *Anopheles* as a genome-editing tool. In *Plasmodium*, gene expression can be modulated even in the absence of genome editing via usage of CRISPR interference or activation (CRISPRi/a). The role of CRISPR-Cas in *Anopheles* is reasonably promising in the field of *gene drive*, which refers to the genetic systems in which a specific trait is selectively transmitted between the populations in a selfish manner defying the normal Mendelian inheritance. The main goal of this gene drive is to negatively control mosquito fertility by use of genetic modifications. CRISPR-Cas is exploited in identification of genes involved in infectious process; subsequently, gene libraries are formulated and can be exploited in the generation of immunogenic or non-virulent or nonpathogenic parasites serving as candidates for drugs or vaccines.

Epidemiological Tracking

Newer whole-genome sequencing is now a major tool to discriminate between closely related strains and track real-time evolution of disease-associated clonal isolates and plays an important role in epidemiological investigations. The commonly employed methods used to discriminate the strain relatedness in such scenarios include RFLP, AFLP, RAPD, and PFGE.

Drug Discovery

Primarily through genomic technology, novel promising targets are identified, and appropriate expansion of these functional genes is

accomplished, followed by assay for high-throughput sequencing. The genome can provide information about the biochemical pathways likely to be involved for a proposed drug or treatment. Thus drug discovery has been able to be expedited tremendously compared to conventional approaches.

Drug Resistance

One of the strongest examples in this scenario is the artemisinin resistance in malarial parasite *P. falciparum*, in which kelch 13 mutations revealed by genomic data could help in framing a tiered approach, focusing on the areas with emergence of resistance. Malaria GEN is one such approach to containing the spread of resistance by enhancing the global research based on sequencing of a large number of samples for chloroquine and pyrimethamine resistance lineages and *Anopheles* vector. Another added advantage of this strategy is exacting the molecular basis of resistance using expression profiling. Screening using microarray and serial analysis of gene expression is of immense importance in localizing and predicting the role of a particular gene product at the right time for the right patient in the right place as an immunological target. Resistance studies have also been conducted to analyze the selection pressure and spread of resistance genes as in *Plasmodium* and *Leishmania*.

Vaccine Development

Very few vaccines are available for parasitic infections like toxoplasma in sheep, echinococcosis in animals, and malaria in humans. Completed genomes will provide the source material for vaccine development. Genomic *chinks in the armor* of parasites screened by expression of libraries with immune sera help to identify the candidate antigens, inciting an immune response and the cryptic ones that fail to elicit immune response and evade the immune system. Other strategies for vaccine development include mRNA-based techniques, differential display, and serial analysis of gene expression. Expressed sequence tags (ESTs) are identified in clusters followed by generation of their consensus sequences that enables the rapid assembly of data. Candidate antigens that can activate Toll-like receptors are also being identified.

Conclusion

Thinking beyond the role of genomic analysis, functional manipulations and editing is the need of the hour to analyze the information obtained after sequencing and to scrutinize the resulting hypothesis. Though maintenance of parasites is challenging, transgenesis and CRISPR-Cas experiments have already been achieved in some parasites. The choice of an appropriate parasitic model being edited, utilizing older and newer molecular tools, can help us comprehend the basic biology of these parasites, simultaneously deciphering the ability to manipulate and control the human illnesses caused by them and exploiting an interactive and collaborative team of molecular parasitologist, epidemiologist, infectious disease physician, and computational and data analyst.

Case Study

The application of genomic studies has been elegantly brought out in one report regarding the population genetic analysis of Guinea worms (*Dracunculus medinensis*) collected from Chad, Africa. There was an apparent reemergence of human Guinea worm disease in Chad after an almost 10-year absence of reported cases. It also coincided with a recent finding of high prevalence of Guinea worm infection in dogs in Chad. To determine whether worms from human and non-human hosts were, in fact, the same species, the worms were collected from both human and non-human hosts. Genetic variation was measured in these worms using sequence variation of mitochondrial DNA genes and repeat number polymorphism at 23 nuclear microsatellite loci. It was found that Guinea worms collected from

nonhuman hosts were *D. medinensis* and that the same population of worms infects both humans and dogs in Chad. This genetic data and the epidemiological evidence suggest that transmission in the Chadian context is currently being maintained by canine hosts.

1. Name some other zoonotic parasites for which similar kinds of study have been conducted.
2. List the common techniques by which strain relatedness among parasites is determined.
3. Name the parasites for which genetic studies have been done to determine drug resistance.
4. Enumerate the important target genes which have been identified and used as molecular markers for identification and quantification of *Leishmania* in clinical samples.

Research Questions

1. What are the fundamental cellular mechanisms involved in host–parasite interactions?
2. What is the molecular basis of response to anti-parasitic drugs and vaccines?
3. What is the genetic basis of emergence and reemergence of parasitic infections?

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Parasite Proteomics

Tejan Lodhiya, Dania Devassy, and Raju Mukherjee

Learning Objectives

1. To have a knowledge about the various methods to study proteomics.
2. To know about the application of proteomics in parasite biology.

Introduction

A large part of the global public health burden of infectious diseases is contributed by tuberculosis, HIV/AIDS, influenza, and malaria. Neglected tropical diseases, including the parasitic diseases of zoonotic origin that primarily affect the poorest of the people living in the tropical and subtropical regions, often get ignored, and the situation is expected to remain so, post COVID-19 outbreak. On the other hand, destruction of biodiversity reserves as a result of industrialization and extensive agriculture has increased our interaction with the wild, thereby enhancing the spillover of parasites to new hosts. Furthermore, limited funding and resource allocation has resulted in inadequate knowledge of pathophysiology and the intricacies of the host–parasite interaction, which is pivotal for designing successful chemotherapy and vaccine strategies. This chapter summarizes the major discoveries and few success stories using a proteomics approach and also highlights how the use of this technology has elevated the understanding of host–pathogen interactions and how one can use this information to design effective therapeutics.

Expression of different genes of an organism is a dynamic process and is highly responsive to environmental cues, including exposure to chemicals, stressors, and growth conditions, and this is entirely translated into an altered proteome. Their identification enables the understanding of the tightly regulated pathways and suggests new modalities of interventions during infections. Efforts were made to identify the conditional modulation of genes, leading to increased stability of a given phenotype. Before the introduction of proteomics, studies of gene expression relied on Northern blotting and Western blotting techniques, which, however, could reveal the gene expression status of only a handful of genes at mRNA and protein level, respectively, making them impractical to get any system-wide insight. The development of cDNA microarrays was a breakthrough as it permitted measuring expression of thousands of genes simultaneously, from the relative abundance of mRNAs. However, these hybridization-based approaches had several limitations like false positives due to cross-hybridization, dependence on the availability of genome sequence, and limited detection

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range. Development of more sensitive deep sequencing methods like RNA-Seq in the last decade has completely altered our understanding of the versatility and complexity of transcriptomes.

If transcriptomics can measure gene expression, then why do proteomics? Though transcriptomics analysis enables researchers to capture the global gene expression status, it has some inherent limitations. The measured mRNAs have varying stability and translational efficiency, which impact the rate of translation. Once synthesized, proteins differ in their turnover rates and are sometimes regulated by posttranslational modifications (PTMs). In addition, the temporal changes in protein activities due to physical and functional intermolecular interactions determine the cellular phenotype. Given the ambiguity between the levels of mRNAs and protein activity, the mere abundance of mRNAs is misleading in suggesting a protein's functional status. Hence, the need for techniques that can directly identify and quantify functional proteins has emerged.

Tools for Proteomic Analysis

Marc Wilkins and colleagues first introduced the term *proteomics* in the early 1990s, which refers to the study of global analysis of proteins (gene products) in an organism. Currently, proteomics has become routine in many laboratories worldwide and is a comprehensive technique to reveal biological mysteries. The availability of multidimensional peptide separation techniques, high-resolution mass spectrometers, and sequence databases has helped to map the proteome of several organisms. Improved tandem mass spectrometry techniques and better computational tools have paved the way for rapid identification and quantification of almost all expressed proteins.

This chapter discusses various strategies available for proteomic analysis. It fundamentally uses two approaches: "bottom-up," which analyzes short peptides to identify the proteome, and "top-down," which directly analyzes intact proteins.

Bottom-Up Proteomics

This is also called shotgun proteomics (Fig. 1), where a mixture of proteins from the cells (parasite/host) or cellular compartment of interest is directly digested in a *gel-free method*, with the help of proteolytic enzymes that specifically cleave a polypeptide chain at particular amino acids. Digestion with trypsin yields many thousands of peptides with an arginine or lysine at their C-terminal, which can overload a mass spectrometer if analyzed together. Therefore, the peptide mixture thus generated is usually separated using different liquid chromatography (LC) techniques before its mass can be measured. As the peptides elute out from the column, they are nebulized and ionized in the presence of a high voltage via electrospray ionization. A mass spectrometer then analyzes the ionized peptides at a high resolution to record their mass-to-charge (m/z) ratio in a form of MS spectrum. Once in the gas phase, the most abundant peptides are further sequentially isolated and collided with neutral gases like nitrogen, helium, and argon. This increase in energy of the charged peptide results in cleavage along the peptide bonds generating fragment ions, by the mechanism of collision-induced dissociation (CID), which is recorded as a fragment ion spectrum. However, this high-energy fragmentation leads to a loss of PTMs on the amino acid side chain, so another complementary technique involving radical mediated transfer of electrons to large multiply charged peptides facilitating cleavage at the backbone N-Cα bonds is preferred. Thus, the parent peptide's mass and abundance information is inferred from the MS or MS1 spectrum, while the peptide sequence information is deduced from a fragment ion spectrum or MS2 spectra. Both, when combined, help decipher the precursor peptide sequence and, thereby, identify the corresponding protein.

However, to perform this process of peptide identification by matching with the protein sequence for all the detected peptides requires computation power and a search engine. The search engine aims to identify the protein of

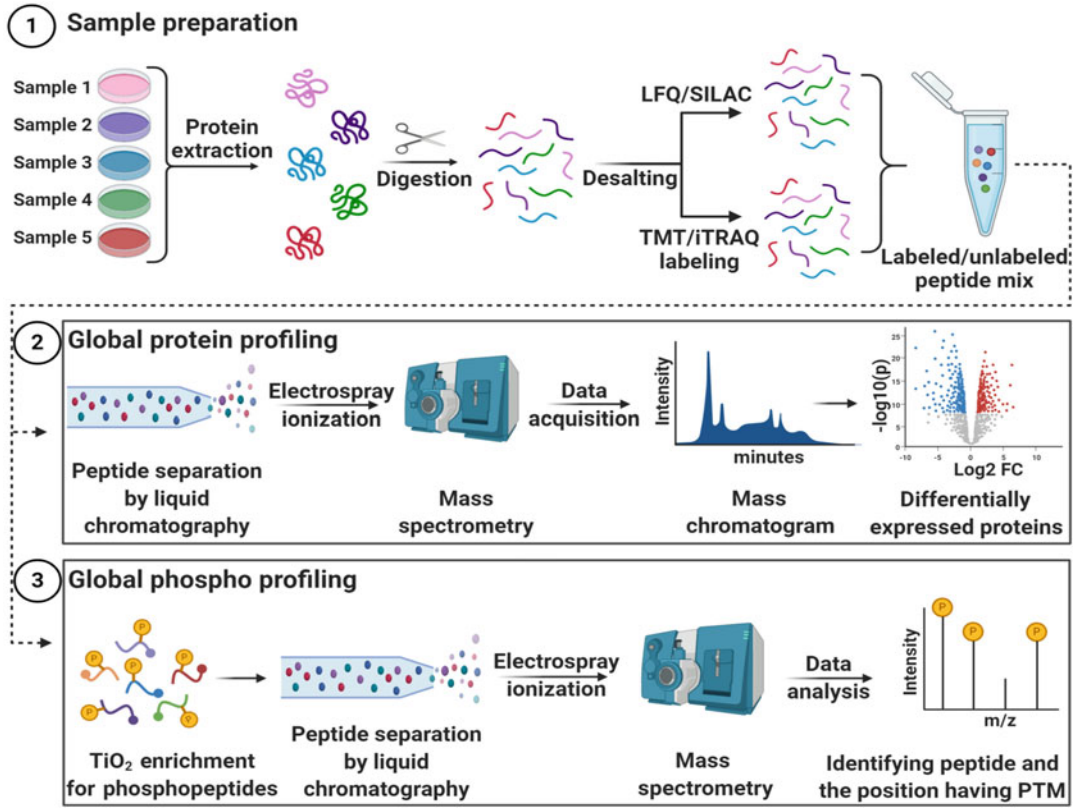

Fig. 1 A typical workflow for shotgun proteomics. Step 1 shows the sample preparation protocol and its variants SILAC and LFQ do not require any in vitro labeling of peptides, whereas in TMT/iTRAQ-based approaches, peptides are labeled post digestion. The digested peptides are then analyzed for discovery (Step 2) proteomics; purified peptides are separated in a nanoLC and then introduced to the mass spectrometer. For phosphoprotein profile (Step 3), fractionated peptides undergo an extra round of enrichment for phosphopeptides, which are then analyzed to identify the phosphorylated amino acids

interest using a reference sequence. Numerous database search engines are available and they rely on the protein sequence databases like the *UniProt*. The search engines theoretically digest each protein from the database and generate all possible peptide precursors. These peptides are then theoretically fragmented into smaller peptides. This in silico processing of the database essentially mimics the experimental enzymatic digestion and mass spectrometric fragmentation of peptides. The search engines then match the experimentally acquired MS1 and MS2 spectra with the theoretical list to decipher the peptide sequence by a process termed peptide-spectrum match (PSM). The PSM, when performed for all the tryptic peptides, provides a qualitative identity of the proteome. Some of the frequently used search engines are listed in Table 1. Since the proteolysis is not always complete and the fragmentation of the tryptic peptide is not equally efficient at every amino acid, one can take the help of available statistical parameters to lower the false discovery rate. Each search engine is more suitable for certain experiments, so their use should depend upon the need of the experiment.

In contrast, in the *gel-based method*, proteins are first separated through one- or two-dimensional gel electrophoresis (2D-PAGE). Differentially visible protein spots

Table 1 Commonly used proteomics databases and search engines used in discovery proteomics

Database and search engines	Description	Website
BioGRID	Database for protein–protein interactions, genetic interaction, chemical associations, and PTMs	https://thebiogrid.org/
PRIDE	Proteomics identification database for MS-based proteomics data, as well as PTMs	https://www.ebi.ac.uk/pride/
PeptideAtlas	Collection of peptides identified from tandem mass spectrometry proteomics experiments	http://www.peptideatlas.org/
Proteopedia	Encyclopedia of structural and functional information about protein, RNA, DNA, and their assemblies and interactions with small molecules	http://proteopedia.org/
Mascot	Search engines for identification, characterization, and quantitation of proteins using MS1 and MS2 data	www.matrixscience.com
Andromeda	Search engine for peptides based on probabilistic scoring model and integrated with MaxQuant	https://maxquant.org/
SpectraST	Spectral library-based search engine	http://tools.proteomecenter.org
X!Hunter	Search engine based on pattern matching from a spectral library	https://thegpm.org/HUNTER/index.html
Lutefisk	De novo peptide sequencing tool	https://bio.tools/lutefisk
PEAKS	De novo sequencing tool and database search engine for PTM detection and quantification	https://www.bioinfor.com/peaks-studio/

from the gel are excised and digested enzymatically using "in-gel digestion" procedures. In comparison to the gel-free approach, the gel-based method has a disadvantage of lower sensitivity. Low-abundance proteins are often not visualized on a gel and, hence, are not identified. It must also be mentioned that a successful proteomics experiment is highly dependent on an effective chromatographic separation, which is often overlooked. Several factors, including the number of peptides loaded on the column, the mobile-phase gradient, the flow rate, and the chemistry of the analytical column, are optimized depending on the complexity of the sample.

Quantitative Proteomics

Shotgun proteomics allows for identifying thousands of proteins. Nevertheless, quantitative information of these proteins is equally important and crucial to improve our understanding of the global protein dynamics and complex molecular networks. Thus, it is essential to have rapid, reliable, and reproducible methods for protein quantification. Both *relative* and *absolute* quantification are performed in MS-based proteomics. In the case of relative quantification, labeling of proteins/peptides with isotopes or isobaric tags and label-free strategies are employed.

In SILAC (stable isotope labeling with amino acids in cell culture), cells are grown in heavy (^{13}C and ^{15}N) labeled arginine- and lysine-containing medium. After incorporation, proteins from both labeled and unlabeled samples are mixed early in sample preparation, thus reducing procedural errors during proteolysis. The difference in the intensities of labeled and unlabeled peptides reveals the differential abundance of the corresponding proteins. In a TMT (tandem mass tag) and iTRAQ (isobaric tags for relative and absolute quantitation) experiment, labeling is performed on peptides post digestion. These tagged peptides are then pooled in equal amounts and analyzed together. All of the above methods allow for multiplexing of the sample in a single analysis. In comparison, in a label-free quantification (LFQ) experiment, which is easy and cost-effective, all biological and technical replicate samples are analyzed in a sequence. The relative abundance of a protein in different samples is calculated based on the identified peak intensities or spectral counts. All of the above differential proteomics approaches have been extensively

employed in studies on cancer, diabetes, and other metabolic disorders.

Notably, it is also possible to have an absolute quantification of all the proteins. In this case, synthetic peptides of known concentrations are analyzed to obtain a standard curve for comparison with the detected peptide intensities. Labeled standard peptides can also be mixed with the digested peptides, and the ratio of their peak intensities reveals their abundance. Some of the targeted proteomics methods for absolute quantification include multiple reaction monitoring (MRM) and selected reaction monitoring (SRM). These monitoring systems are routinely performed in pharmaceutical research for measuring drug metabolites and in pharmacokinetic studies on plasma samples.

Top-Down Proteomics

With the availability of advanced fragmentation options in modern mass spectrometers, it is now possible to obtain sequence information on intact proteins without the requirement for proteolytic digestion. The top-down approach is thus suitable for identifying protein isoforms (splicing variants) and PTMs. An increasing number of proteins with a mass range as large as 200 kDa can be isolated and fragmented through top-down proteomics, allowing near-complete coverage. However, due to the limitations in chromatography techniques that can separate complex mixtures of intact proteins, the top-down method has mostly been used to identify the purified proteins.

Emerging Proteomics Strategies

Once a set of proteins of interest has been identified through shotgun proteomics, they can be selectively measured in a complex mixture using a more focused data-independent acquisition (DIA) method. It is inspired by the methods used for protein quantification where specific peptides are measured, ignoring the global protein expression. DIA is an advancement over discovery proteomics methods, which chooses only the most abundant peptides for acquiring fragment spectra and was more dependent on MS1 data. In principle, DIA offers fragmentation and detection of all the peptides regardless of their abundance by using a moving m/z selection window from the MS1 spectrum. Sequential window acquisition of all theoretical fragment ion spectra (SWATH) methods use this approach in a high-speed mass spectrometer. Combining targeted quantitation with discovery proteomics is ideal for identifying the disease-specific biomarkers. Body fluids like saliva, urine, plasma, serum, and CSF have been successfully used for the diagnosis and prognosis of diseases including cancers, diabetes, kidney diseases, autoimmune disorders, and cardiovascular diseases. These methods, being noninvasive in nature, are cost-effective and have the potential to minimize diagnosis time. The critical task is to identify early biomarkers that are specific, chemically stable, and widely observed in the affected pathological condition and quantifiable.

Applications of Proteomics in Parasite Biology

The past decade has witnessed a massive surge in the use of proteomics technologies, resulting in studies analyzing high-throughput proteomics data to address critical questions in pathophysiology in parasitic infections. This chapter discusses some of the critical findings on human parasites, including *Plasmodium*, *Leishmania*, *Toxoplasma*, and helminths, where proteomics has played a critical role.

Malaria

The *Plasmodium* parasite's life cycle is complex and multistage and includes two hosts: humans and the *Anopheles* mosquito. While it is challenging to study the parasite within mosquitoes, research on human cells has been productive. Two significant studies that characterized the *Plasmodium* proteome across life cycle stages

were groundbreaking for malarial research. They reported that the gene clusters encoding co-expressed proteins were prevalent across the entire *Plasmodium falciparum* genome. Further, transcriptomics and proteomics comparison revealed that several mRNAs are regulated by translational repression, at least in the gametocyte stage. This would not have been possible to realize without the data from differential proteomics. Since the merozoites and sporozoites are not enclosed by host cells, their surface proteins can be targeted by antibody-based interventions. Several merozoite surface proteins were identified using proteomics and can be taken forward for producing recombinant antigens. Similar vaccine candidates are being aimed to inhibit the liver stage of the parasites.

Mosquito salivary glands are the sites of malarial parasites during the sporozoite development. The first proteomic study on the salivary gland of the vector, *Anopheles culicifacies*, cataloged the proteins and reported the D7 family to be the prominent group. These proteins, unique to the dipteran family, play a role in hematophagy and may be involved in parasite transmission. It was also observed that antiplatelet aggregation proteins and anti-inflammatory proteins help in blood feeding. Proteins related to autophagy and blood-feeding mechanisms were upregulated in the salivary glands of the blood-fed vectors in comparison to the blood-starved mosquitoes. A detailed study of these differentially expressed proteins can help us better understand feeding behavior and parasite transmission, providing new methods of parasite and vector control.

Toxoplasmosis

The complex life cycle of *Toxoplasma* involves distinct tissue-specific sexual and asexual developmental stages. One of the significant efforts was to decipher the proteome profiles of three distinct infective stages of *Toxoplasma gondii* by iTRAQ-based quantitative proteomics. Gene Ontology enrichment analysis revealed that the ribosomal proteins were upregulated in the cyst and oocyst stages, allowing parasite survival in adverse environmental conditions. In comparison, pathway enrichment analyses of the tachyzoite stage revealed that energy-generating metabolic proteins and growth-promoting proteins were upregulated at this stage, suggesting active replication. Interestingly, virulence factors were upregulated only in the oocyte stage and are consistent with gene expression read-out. The study helped unravel a pool of proteins that determine how the parasite crosses the different developmental barriers in different conditions.

A similar study focused on the bradyzoite stage in vivo and integrated both transcriptomics and proteomics approaches to understand the deep biology involved in this stage, where the parasite is encapsulated in a cyst and hides from the host. One of the significant findings from this study was the presence of a novel stage-specific isoform of sporoAMA1 protein, which can act as a bradyzoite-specific marker. Another interesting observation was the presence of two host transporters among the parasite proteins, which were speculated to be playing a role in nutrient uptake and evading the activation of the host's immune system. These important findings generate many open questions that should be pursued to get complete insight into the infection.

While vaccine development still remains a challenge, the exosomes derived from the *T. gondii* are known to cause immune modulations and disease protection in the host. In the tachyzoite stage, parasites secrete exosomes and ectosomes, differing in size and morphology. Because the vesicles have the same composition as that of the parasite's secretome, its characterization can be a great leap to vaccine development. A differential proteomic analysis of the exosome, ectosome, and secretome led to the identification and quantification of different unique proteins present in these pools. The exclusive ones can be used as a differential marker for the pool; for example, MIC3 was present only in the ectosomes. An elegant study on the mitochondrial proteome discovered the components of the cytochrome *c* oxidase complex. The study also revealed the novelty and high degree of divergence of these proteins from their eukaryotic

counterparts, thus creating a new window for novel therapeutics.

Leishmaniasis

Over 20 *Leishmania* species infect humans, and their intensity and symptoms vary with the species involved. The parasite uses host macrophages to evade immune recognition. In vitro study using label-free quantification (LFQ) on three different species from different geographical locations helped understand the protein alterations in macrophages upon infection. In order to neutralize the macrophage's intracellular defenses, the parasites upregulate several proteins that inhibit cell death and apoptosis, leading to persistent infection. Many of these proteins could be potential drug targets.

One of the most severe problems in the treatment of leishmaniasis is the relapse of the infection and the emergence of drug resistance. Proteomics strategies were employed to understand the molecular mechanisms leading to this phenomenon and identify novel targets. A recent study reported the parasite's adaptation to amphotericin B-induced stress by upregulating enzymes of the mitochondrial oxidative phosphorylation pathway, which in turn may contribute to the relapse. Another important finding was the overexpression of the flagellar proteins. These indicated a better adaptation to stress conditions because they help in sensing the extracellular environment. Proteome characterization of the released extracellular vesicles played a pivotal role in assigning biomarkers unique to the drug-resistant parasites. A subgroup of nine proteins, including histone 3, core histone-like transcriptional factors, and ribosomal proteins, was found to be enriched in the drug-resistant strains.

Helminthiasis

Fasciola hepatica follows a circuitous migratory route from the host's intestine to the liver. During this journey with layovers at multiple host tissues, the parasite communicates with diverse host macromolecules. A study aimed to understand the secretome of the parasite at distinct developmental stages identified large numbers of proteases as primary virulence factors followed by antioxidants. Secretome proteomics also revealed that extracellular vesicles are taken up by the host cells through interactions between the vesicle surface proteins such as myoferlin and endocytic recycling proteins and the host membrane. Preventing or disrupting this interaction, this study suggests, may help to intervene with host–parasite communication.

Integrative Proteomics Is the Future

Applications of proteomics are not just restricted to identifying and quantifying proteins from given samples. Advanced mass spectrometry techniques have allowed a comprehensive understanding of the complex biological systems.

Proteomics data can be broadly classified into three types: discovery proteomics, structural proteomics, and interaction proteomics. Discovery proteomics generates a list of a large number of differentially expressed proteins. These proteins that are functionally annotated and enriched using bioinformatic tools like Gene Ontology are employed to identify the most enriched biological processes. This helps in narrowing down the search to the most affected pathways/proteins that are validated through qPCR or biochemical assays. Further, with the resolution of modern mass spectrometers reaching the order of 10^5, accurate identification of small molecules has become routine. For instance, it is now possible to decipher how the metabolism of the host changes upon parasite infection and how the parasite's metabolism changes upon drug treatment. Enrichment of metabolic pathways from the proteomics dataset can also be performed and integrated with the host or parasite metabolomics dataset to get a greater insight into the pathogenesis.

Additionally, it is now understood that proteins do not work in a silo. Each protein is directly or indirectly associated with many other proteins, and together they determine the

direction and magnitude of the phenotype. Such spatiotemporal protein–protein interactions also mediate the dynamic networks that aim to optimize cellular physiology under the influence of the local environment. Cross-linking mass spectrometry (XL-MS) using different types of chemistry appears promising in tethering specific interaction and capturing the global interactome. Importantly, not all interactions are physical; some are functional. Several proteins do not physically associate with each other, but rather a group of proteins together govern the given biological process. Protein–protein interaction databases such as MINT, BioGRID, and STRING use protein topological information along with its abundance to construct the interaction network. Such analyses can help decipher the regulatory proteins like scaffold proteins, kinases, and phosphatases that act as molecular drivers for a given phenotype. Proteomics also has a great potential in deciphering the structural elements in proteins, which are difficult to capture using conventional methods of macromolecular crystallography and biomolecular NMR. Hydrogen/deuterium exchange mass spectrometry, oxidative footprinting, and ion mobility separation have allowed the determination of protein structure.

With the rapid growth in next-generation sequencing, whole-genome mapping of SNPs and transposon sequencing have become possible. Identifying the SNPs is vital to understand drug resistance in parasites. The transposon mutagenesis technique will be useful to identify the conditionally essential genes required for virulence, drug resistance, and in vivo growth of parasites. In summary, an integrative approach of using multiple proteomic techniques together with transcriptomics, metabolomics, and network analysis is required for getting a deeper fundamental insight into the processes and the mechanism of host–parasite interaction (Fig. 2). These, when clubbed with the knowledge of immunology and skills in medicinal chemistry, may strengthen effort in controlling parasitic infections to increase equity in life in the tropics.

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Case Studies

Sporozoites of the malarial parasites infect the hepatocytes to generate more merozoites, which are then released into the bloodstream. Given the necessity of the liver stage development in initiating the infection, several vaccine candidates can be developed to mitigate the parasites within the liver stage. Similarly, the proteins present on the merozoites are crucial for the survival of parasites in the extracellular environment and in mediating the infection of the new red blood cells (RBCs). Several merozoite surface proteins (MSPs) such as GPI-anchored proteins, 6-Cys family proteins, and rhoptry proteins were identified and are being developed as recombinant antigen-based vaccines. Antibody production against MSP-119 and MSP-3 showed the strongest correlation with the lower incidence of malaria and protection. The MSP-Fu24, a fusion protein containing the conserved regions of MSP-119 and MSP-3, offers considerable potential for MSP-based vaccine. Glycoproteins thrombospondin-related adhesion protein (TRAP) and circumsporozoite protein (CSP) are two potential vaccine candidates from the sporozoite. Vaccine candidate apical membrane antigen 1 (AMA1), one of the surface-exposed proteins in the sporozoites, is undergoing clinical trials. PIESP1 and PIESP2 are the two new surface markers on RBCs infected with *P. falciparum*, while in the case of cerebral malaria, the mature parasite-infected erythrocyte surface antigen (MESA) and *P. falciparum* antigen 332 (Pf332) are the important biomarkers.

In *T. gondii*, a study aimed at identifying the proteome of the mitochondrial matrix was groundbreaking. It found that 22 of 27 previously characterized proteins were localized to the mitochondria. TgApiCox25 was shown to be a part of the COX complex required for parasite growth and oxygen consumption. Since 40–50% of the parasites' mitochondrial proteins do not

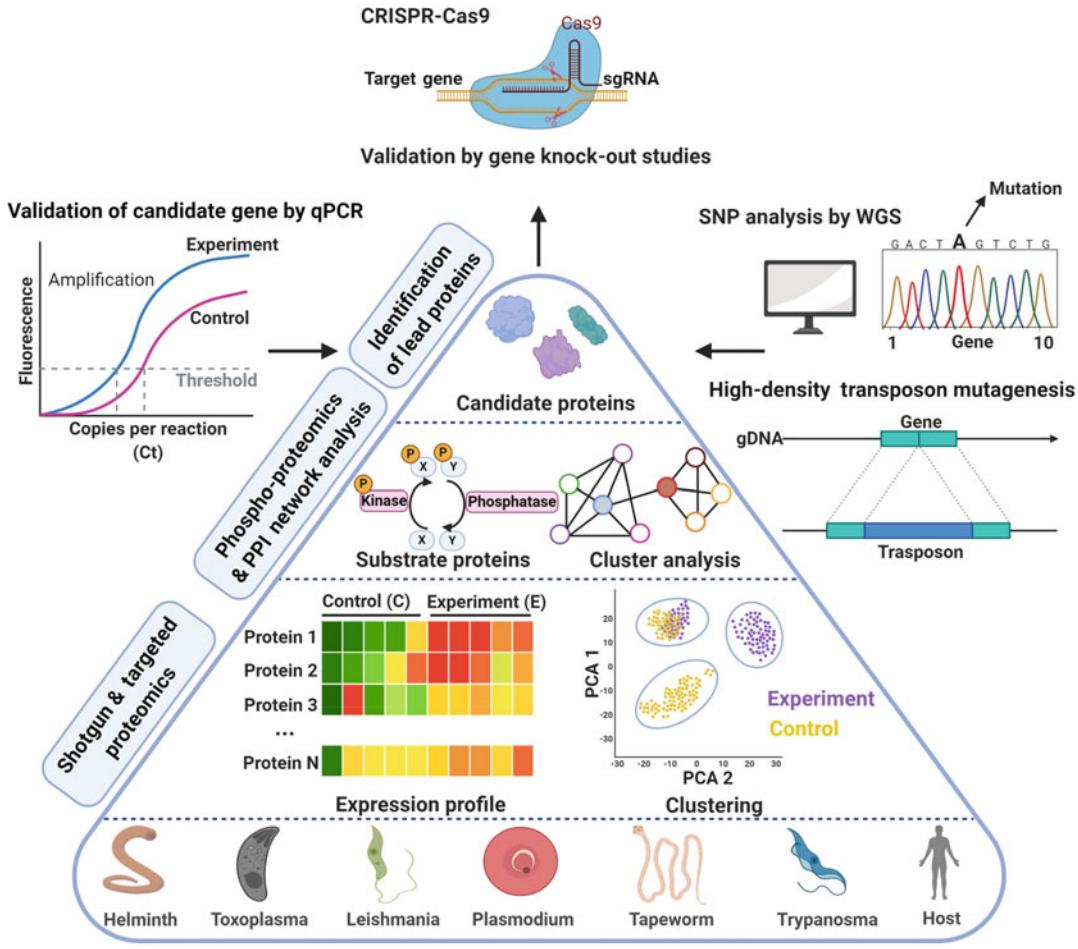

Fig. 2 Integrative proteomics through combined approaches of "bottom-up" and "targeted" proteomics reveals system-wide alterations in the proteome and helps to identify the most impactful protein cluster through interaction analysis

have orthologs in humans, these are being pursued as potential drug targets.

Post-kala-azar dermal leishmaniasis (PKDL) is a condition that precedes visceral leishmaniasis (VL). One of the major concerns here is the difficulty in the detection of lesions in macular (MAC) PKDL patients due to low parasitic load. Here again, proteomic profiling using mass spectrometry helped to identify the glycoproteins plasminogen and vitronectin as biomarkers specific for MAC PKDL among the silent carriers. Immuno-chromatographic assays based on these glycoproteins can be developed in the future to efficiently diagnose the condition. The *maoc* family dehydratase-like protein (Ld-mao1) and peptidyl-prolyl cis-trans isomerase/rotamase (Ld-ppi1) are the two validated protein biomarkers for *Leishmania donovani*. These together can help in the development of a highly sensitive and specific multiplexed test to detect either *Leishmania infantum* or *L. donovani* VL. *Leishmania infantum* iron superoxide dismutase 1 (Li-isd1), tryparedoxin 1 (Li-txn1), and nuclear transport factor 2 (Li-ntf2) are a few of the

antigens found in the urine of VL patients and were used to generate antibodies for serodiagnosis. Multiplexed assay of these could detect 90% of the cases with 100% specificity.

In schistosomiasis, the parasite eggs are primarily involved in pathology and in stimulating antigenic and granulomatous responses in the host. It is critical to understand the components of the egg and its secretome, which are potential vaccine candidates. A comparative proteomic analysis of the differentially expressed proteins in mature and immature eggs identified protease inhibitors and proteins involved in energy metabolism and stress, as enriched in mature eggs. Importantly, an ELISA kit developed based on schistosome SjSP-13 protein marker (rSP13-ELISA) showed a substantial advantage over other diagnostic methods. In another study, the schistosome tegument proteins phosphoglycerate mutase and UV excision repair protein RAD23 homolog B were evaluated for their potential as markers for serological tests. Compared to the soluble egg antigen-based ELISA that is currently in use, these proteins showed higher specificity and sensitivity and less cross-reactivity.

1. What are the malaria vaccines which are in the pipeline and which vaccine has shown maximum promise?
2. What are the antigens which have been used for developing antibody detection tests for visceral leishmaniasis, and which one is being used in commercial tests?
3. Which serological tests are currently in use for schistosomiasis, and which protein antigens are used in these tests?

Research Questions

1. What are the interactions of proteins in terms of activities, modifications, and localization between host and parasite?
2. How are the posttranslational modifications of proteins used by parasites to regulate their own function together with interacting with the host's immune system?
3. What are the different proteomes in different life stages of the parasite in human and other hosts and in drug-resistant parasites?

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Epidemiology of Parasitic Infections

Vijaya Lakshmi Nag and Jitu Mani Kalita

Learning Objectives

1. To have an idea about the types of parasites and their hosts.
2. To review the epidemiological characteristics including transmission and geographic distribution and burden of parasitic infections.
3. To know about the basic principles of prevention and control of parasitic infections.

similar characteristics associated with the disease conditions, especially in people from low socioeconomic strata of the society. Social, geographical, economic, and political factors contribute to those conditions. Human behavior has an important role in the epidemiology of emerging or re-emerging parasitic diseases. Changes in demography and environmental alteration, climate change, technology, and land use favor the emergence and spread of parasitic diseases. This chapter outlines the generic concepts of the three cornerstones of epidemiology, namely, the agent, the host, and the environment. The global burden of infection and the general principles of surveillance, prevention, and control have also been described.

Introduction

The word *epidemiology* comes from the Greek words *epi*, meaning on or upon; *demos*, meaning people; and *logos*, meaning study. In the context of parasitic diseases, epidemiology is the study of any parasitic disease and disease-causing agent at the population level. The patterns of distribution and prevalence of the disease and the factors responsible for these patterns are the key points of epidemiological studies. The prevention and control of parasitic disease also constitute important components of epidemiology. Parasitic diseases have biological diversity and have

The Symbiosis

The coexistence of two living beings in close proximity has been described as symbiosis. The nature of interaction between these two living beings may differ, which may or may not be beneficial for one of them. Thus, a symbiotic relationship can be classified into three types as mentioned below:

1. **Mutualism**: It is an obligatory relationship, since neither of the partners can survive without the other. In this type of relationship, both the partners benefit from each other. This type of relationship is more common in nature and can be demonstrated, for example, between

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leeches and their gut bacteria and between termites and their intestinal flagellate protozoan partners. Filarial nematodes like *Wuchereria bancrofti* harbour the bacteria of the genus *Wolbachia*. Although the nature of metabolic dependence between the nematode and bacteria is not exactly known, it has been demonstrated that treatment with tetracyclines not only kills the bacteria but also causes simultaneous death of the nematode parasite.

2. **Commensalism**: It is a type of relationship in which one partner benefits but the other partner is neither benefitted nor harmed. Humans and animals are populated with a large number of bacteria as well as several protozoans which behave as commensals. For example, *Entamoeba gingivalis* lives in the mouth and feeds on food particles and dead cells without harming the human partner.
3. **Parasitism**: In this relationship, the parasite lives at the expense of the other who is termed the *host*. This kind of partnership is harmful for one partner. The *parasite* can inflict mechanical injury to the host, which in turn causes inflammatory and/or immune damage to the tissue; or else the parasite can deprive the host of essential nutrients.

The Parasite

Parasites are broadly divided into *endoparasites* and *ectoparasites*, depending on whether they reside inside the host or on the surface of the host. They are classified as follows:

1. **Obligate Parasite**: Obligate parasites are those which cannot exist without a host. In contrast to free-living parasites which can exist in nature without depending on any host, an obligate parasite lives in the host to complete its life cycle. During some stage of their life cycle, they may behave as free-living entities in water or soil, but they cannot survive for long outside a living host. The majority of pathogenic parasites are obligate parasites such as malaria parasites, *Toxoplasma*, and various helminths.
2. **Facultative Parasite**: Facultative parasites have both parasitic and free-living existence depending on the situation. Usually, these parasites are free living in nature, but if they gain access to the body, they cause harmful effects in the infected host. The free-living amoebae such as *Naegleria fowleri* or *Acanthamoeba* spp. or free-living nematode *Micronema* are a few examples of such parasites.
3. **Accidental/Incidental Parasite**: Parasites that infect an unusual host are called accidental parasites. These parasites enter the body of an unusual host, which is different from the normal host. In this unusual host, the parasite can develop to some extent, but complete development of the parasite is not possible. For example, *Echinococcus granulosus* causes hydatid cyst in humans, which is not the natural host for the parasite.
4. **Aberrant Parasites**: They are also called wandering parasites, and when they enter a host that is different from their natural host, they reach a site where they cannot live or develop further. *Toxocara canis* is a natural parasite of dogs, but when it enters the human body, its further development stops.

The Host

A host is a living being which harbours the parasite and provides shelter and nutrition. Hosts may be humans, animals, birds, or insects. Hosts, on the basis of their part in the life cycle of the parasite, are classified into the following groups:

1. **Definitive Host**: Hosts in which parasites reproduce sexually or which harbor the most highly developed form of the parasite or adult stage are called definitive hosts. Humans, animals, and even arthropods can act as definitive hosts. For example, humans are the

definitive hosts for many helminths including *Ascaris* or hookworm, while mosquitoes are the definitive hosts for malaria parasites. In many infections transmitted from animals, vertebrates such as dogs, cats, cattle, etc. act as definitive hosts.

2. **Intermediate Host**: Hosts which harbor the larval forms or in which the parasites replicate asexually are called intermediate hosts. The larval developments of some of the parasites in their life cycle are completed in two different hosts; these are then referred to as first and second intermediate hosts. For example, snails are the first intermediate hosts and cray fish and freshwater crabs are the second intermediate hosts for *Paragonimus westermani*. Humans also serve as intermediate hosts, as seen with malaria parasites.
3. **Paratenic/Transport Host**: A host in which the parasite does not undergo any development but the larval form remains viable is called a paratenic or transport host. Such hosts may act as a bridge between the definitive and the intermediate host and help in transporting or transmitting the parasites. A paratenic host is more of an ecological than a physiological phenomenon. Under extreme environmental conditions, the transmission of parasites may be facilitated by these paratenic hosts. For example, freshwater shrimp, flatworms, and frogs act as paratenic hosts for *Angiostrongylus cantonensis*.
4. **Accidental Host:** Hosts in which a parasite is not normally found but where the parasite may undergo some developmental changes are defined as accidental hosts. These are normally dead-end hosts and further transmissions to other hosts do not take place. For example, humans are the accidental hosts for cattle eye worm (*Thelazia gulosa*).
5. **Reservoir Host:** These Hosts which harbor a parasite for a long time but do not suffer from any disease and serve as the source of infections are called reservoir hosts. For example, dogs and other canines are the reservoir hosts for *Leishmania infantum*.

Parasitic Zoonoses

The term **zoonosis** refers to an infection that is transmissible under natural conditions from animals to humans. Parasitic zoonoses are of four types:

1. **Direct zoonoses**: This is characterized by direct transmission of parasites from animals to humans. *Cryptosporidium parvum*, *Toxoplasma gondii*, *Hymenolepis nana*, and *Trichinella spiralis* are a few examples of such parasites.
2. **Meta-zoonoses**: This is characterized by transmission of parasites to humans, mediated by invertebrate intermediate hosts. *Babesia bovis*, *Plasmodium spp*., and *Clonorchis sinensis* are examples of parasites which cause meta-zoonoses.
3. **Cyclo-zoonoses**: This is characterized by transmission of parasites to humans mediated by the vertebrate intermediate hosts. Examples include *Echinococcus granulosus*, *Taenia* spp., and *Sparganum* spp.
4. **Sapro-zoonoses**: Human infections are transmitted from the soil or water and include *Ancylostoma caninum*, *Ascaris suum*, *Capillaria hepatica*, and *Trichuris vulpis*.

Sources of Infections

The source of an infection is the origin from which the infective form of the parasite enters the host. For human infections, the source may be animate (e.g., humans, animals, birds, crustaceans) or inanimate (air, water, or soil).

Humans

Humans do not act as the most important source of zoonotic parasitic infections, in contrast to other infectious disease. Human-to-human parasitic infection, known as anthroponoses, occurs in certain parasitic infections such as mother-to-fetus infection in toxoplasmosis or autoinfections seen in pinworm disease or in strongyloidosis.

Animals

Humans acquire zoonotic parasitic infections transmitted from animals in various ways. They may get the infection by consumption of meat from infected animals or from intermediate hosts or by biological vectors such as mosquitoes which can transfer the infective forms of the parasite to humans. Cattle, dogs, cats, pigs, and fish are some of the most important animal sources of infection. Pigs remain the most important source of *Balantidium coli*, *Taenia solium*, *Trichinella* spp., etc. Human infections associated with eating walrus meat or polar bear meat infected with *Trichinella nativa* have been observed in the Arctic region.

Wild animals like antelopes, bears, elephants, etc. may be sources of certain zoonotic parasitic infections (e.g., *Trypanosoma evansi*, *Cryptosporidium* spp., *Trichinella* spp., gastrointestinal *Strongyloides*). *Trichinella papuae* has been implicated in outbreaks of human trichinellosis in Thailand after eating wild boar meat. Fish and crabs, particularly undercooked, are important sources of clonorchiasis and paragonimiasis, respectively, in humans.

Arthropod Vectors

Arthropods act as intermediate hosts, as well as definitive hosts, to transfer parasitic infections human-to-human, animal-to-human, and animal-to-animal. Most of them are true or biological vectors inside which the parasite undergoes some multiplication or developmental changes. Sandflies transfer promastigotes of *L. donovani* through human-to-human anthroponotic infection in the Indian subcontinent, while *Anopheles* mosquitoes transmit *Plasmodium knowlesi* from monkeys to humans. Vectors like houseflies act as mechanical vectors in transferring the agent of amebiasis from human fecal materials to foodstuffs.

Water and Soil

Water and soil, which may get contaminated with human or animal excreta due to poor sanitation, may act as sources for human infections. For example, larval forms of hookworm in the soil or cercariae of schistosomes in water penetrate skin of the host, causing infections. Similarly, ingestion of water contaminated with infected Cyclops containing *Dracunculus medinensis* larvae may result in dracunculiasis. Water is also the main source for free-living amoebae such as *Naegleria fowleri*, causing a serious, often fatal, infection such as amoebic meningoencephalitis in humans. Recreational water (water used for swimming and other activities) illnesses are diseases that are transmitted by swallowing, breathing, or having contact with contaminated water from swimming pools, hot tubs, lakes, rivers, or the ocean. Diarrhea caused by *Cryptosporidium* and *Giardia intestinalis* is an example of such parasites transmitted by contaminated recreational water.

Transmission of Infections

Parasites may be transmitted in a variety of manners, and Table 1 shows the modes of transmission of important parasites. The following is a brief description of the types of transmission:

1. **Food and water transmission:** Numerous parasites are transmitted by a variety of foodstuffs which may include raw or undercooked fish, crabs, and molluscs (*Paragonimus* spp., *Clonorchis* spp., *Diphyllobothrium* spp., *Anisakis* spp., etc.), undercooked meat or meat products (*Toxoplasma* spp., *Taenia* spp., etc.), raw aquatic plants such as watercress, and raw vegetables (*Fasciolopsis* spp., *Fasciola* spp., etc.) that have been infected by the parasite or water contaminated by human or animal feces (*Cryptosporidium* spp., *Giardia* spp., *Echinococcus* spp., etc.).

Table 1 Some important parasites and their route of transmission

Food- and waterborne zoonotic parasites	
Name of parasite	Route of transmission
Entamoeba histolytica	Ingestion
Giardia intestinalis	Ingestion
Balantidium coli	Ingestion
Sarcocystis spp.	Ingestion
Toxoplasma gondii	Ingestion
Cryptosporidium spp.	Ingestion
Microsporidia spp.	Ingestion, inhalation
Naegleria spp.	Ingestion
Fasciolopsis buski	Ingestion
Echinostoma ilocanum	Ingestion
Heterophyes heterophyes	Ingestion
Metagonimus yokogawai	Ingestion
Gastrodiscoides hominis	Ingestion
Taenia solium	Ingestion
Taenia saginata	Ingestion
Echinococcus granulosus	Ingestion
Echinococcus multilocularis	Ingestion
Diphyllobothrium latum	Ingestion
Spargonia spp.	Ingestion
Ascaris spp.	Ingestion
Strongyloides spp.	Skin penetration
Ancylostoma braziliense	Skin penetration
Toxocara spp.	Ingestion
Trichinella spp.	Ingestion
Vector-borne zoonotic parasite	
Leishmania spp.	Sandfly bite
Trypanosoma brucei	Bite of tsetse fly
Trypanosoma cruzi	Reduviid bug
Zoonotic *Plasmodium* spp.	Mosquito bite
Babesia spp.	Tick bite
Dirofilaria spp.	Mosquito bite
Congenital	
Toxoplasma gondii	Transplacental

2. **Vector Transmission:** Vector-borne transmission of parasitic diseases takes place when the parasite enters the host through the saliva of the insect during a blood meal (malaria) or from parasites in the feces of the insect that defecates immediately after a blood meal (Chagas disease). Table 2 lists important vector-borne parasitic infections.
3. **Cutaneous Transmission:** The larvae of certain helminthic parasites are capable of invading the intact skin and can cause infection in distant parts of the body. For example, the infective larvae of hookworm or *Strongyloides* and cercariae of schistosomes larvae enter through skin but lodge in the intestines and other parts of the infected host.
4. **Iatrogenic and Vertical Transmission:** *Babesia* spp., *Plasmodium* spp., *Trypanosoma cruzi*, etc. during their life cycle are found in the blood during the acute phase of illness. These parasites may be transmitted through blood transfusion, if blood samples are not screened for these parasites before transfusing the blood or blood products. Vertical transmission of parasites from mother to fetus is rare but is an important complication of toxoplasmosis if the mother is infected during pregnancy.

Table 2 Important vector-borne parasitic infections

Disease	Parasite	Insect (vector)
African trypanosomiasis (sleeping sickness)	*Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*	Tsetse flies
Babesiosis	*Babesia microti* and other species	*Babesia microti: Ixodes* (hard-bodied) ticks
Chagas disease	*Trypanosoma cruzi*	Triatomine ("kissing") bugs
Leishmaniasis	*Leishmania* spp.	Phlebotomine sandflies
Malaria	*Plasmodium* spp.	*Anopheles* mosquitoes

Geographical Distribution of Parasites

Geographic distribution of human parasites follows a regular pattern in which a latitudinal gradient of pathogen diversity is seen with low latitudes characterized by high species diversity of human pathogens. This may be surprising since, generally, tropical areas are considered as favoring the prevalence of parasitic diseases (Fig. 1). Various factors may have a bearing on the parasite diversity in a geographic area like age of colonization by humans and population densities. The Palaearctic and Oriental regions were colonized much earlier and reached high population densities, which explains both the diversity and the higher burden of parasites.

Parasitic infections are found worldwide but certain parasites are restricted to particular geographic areas and ecological niches. The distributions of African and American trypanosomiasis are prime examples of restricted occurrences of a particular parasite. The distribution of the causative vectors partly explains this limited prevalence. *Plasmodium knowlesi* is another parasite which has been seen only in Malaysia and in a few neighboring Southeast Asian countries coinciding with the presence of *Macaca fascicularis* and *Macaca nemestrina* monkey species and mosquitoes of the *Anopheles leucosphyrus* group.

Burden of Disease

The global burden of parasitic diseases is estimated to be 96 million disability-adjusted life years in 2015. There has been a decrease in prevalence of a few parasitic infections such as ascariasis, lymphatic filariasis, etc. since 1990, but some other parasitic infections such as leishmaniasis have increased due to conflict and collapsed health systems such as in Syria. The majority of these diseases are closely linked to poverty, especially in rural areas, but urbanization is also facilitating in spread of the disease. Intestinal protozoa are among the most common protozoal infections and top the list with a global burden of 500 million, followed by malaria (228 m), Chagas disease (7.6 m), leishmaniasis (1 m), toxoplasmosis (200,000), and African sleeping sickness (10,000). Except for toxoplasmosis and intestinal protozoal infections, which have global distribution, other parasitic infections are found mostly in Asia, South America, and sub-Saharan Africa in populations living in poverty and in rural areas. Helminthic infections affect almost 1.5–1.7 billion people worldwide, mostly in rural areas of resource-poor parts of the world. Soil-transmitted helminths (1 billion), schistosomiasis (240–400 m), filariasis (160–200 m), and foodborne trematode infections (85 m) are the more important infections in these groups of populations worldwide, although they are mostly prevalent in the continents of Asia, South America, and Africa.

Prevention and Control

Control and prevention of parasitic infections is complex but is essentially based on a multidisciplinary approach. These activities aim to reduce the burden of parasites in the community. These include various strategies including the management of ecology and the environment of the

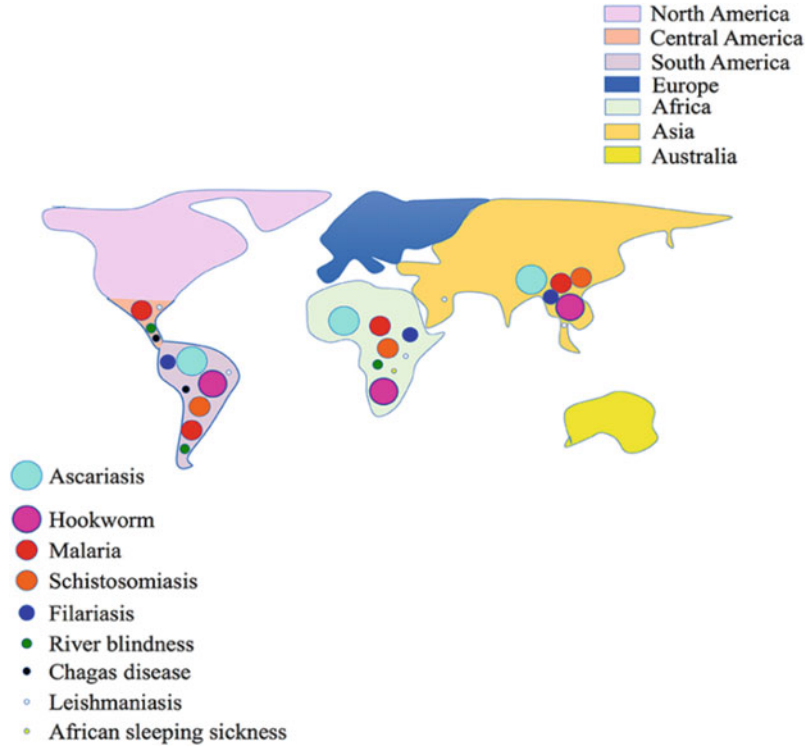

Fig 1 Distribution of important parasitic diseases (Source: Cao B and Guiton P (2018) Important Human Parasites of the Tropics. Front. Young Minds. 6: 58. doi: https://doi.org/10.3389/frym.2018.00058)

region to decrease the parasite load and to halt the transmission risks, and education and behavioral changes of the population at risk, to ensure the success and sustenance of the control and prevention programs.

The various measures can be summarized as follows:

1. **Reduction of Parasite Burden**: Industrialization of pig production with screening measures has considerably reduced the level of *T. spiralis* infection in many European countries, although the practice of organic farming and also the high level of infection in wild boars may reintroduce trichinellosis in these communities. Mass drug treatment in a community has also proven to be of value in reducing the quantum of parasites in a given community.
2. **Animal Reservoir and Vector Measures**: One of the time-tested methods for preventing animal and human infection is education of pet owners and regular deworming of dogs and cats. Health education of pet owners on preventive measures such as personal hygiene, clearing up pet feces regularly, and minimizing exposure of children and pregnant women to the pets and contaminated environment is important. Anthelminthic treatments are most effective when they are initiated early for treatment of young pets. Insecticide-impregnated nets and dog culling have been recommended for control of zoonotic visceral leishmaniasis in a community.
3. **Better Diagnostic Methods:** Availability of economical, rapid, and point-of-care diagnostic tests, such as card test for visceral leishmaniasis and American trypanosomiasis, that are used by minimally trained personnel at the field level facilitates in surveillance and implementation of control measures against parasitic diseases.
4. **Environmental and Ecological Measures:** Geographical information systems, remote sensing, and geostatistics have added new dimensions to the study of the ecology and spatial distribution of parasites, which are key

factors in the control and preventive measures for these parasites. They have been successfully used for schistosomiasis control and have potential for application in areas endemic for a single or multiple parasitic infections.

5. **Human Behavioral and Educational Measures:** An increase in the prevalence of trichinellosis in certain European countries due to the recent increased consumption of raw horse meat is one example that advocates for change in human behavior by initiating several educational activities. The practice of consumption of raw or undercooked fish has been associated with increased incidence of clonorchiasis, opisthorchiasis, metorchiasis, and anisakiasis infection. Both public health personnel and veterinary professionals therefore play a key role in public education for change in human behavior to prevent zoonotic infections.
6. **Financial Resources and International Cooperation:** Effective public health initiatives depend on availability of adequate financial resources for programme implementation and sustenance. The World Health Organization (WHO) has been advocating for implementing integrated disease control programs with primary health care to contain infectious diseases including parasitic diseases. These integrated programs, while operational and successful in developed countries, are not so successful in resource-challenged countries with a higher prevalence of parasitic diseases. Therefore, the involvement of international agencies and institutions, such as the WHO and the Food and Agriculture Organization (FAO), together with the commitment of policymakers, scientists, and field workers is essential for the sustainable control and prevention of parasitic infections.

Case Study

A 37-year-old man attended emergency with complaints of multiple convulsions. CT scan of the brain showed numerous small cystic lesions in both hemispheres. No abnormality was observed in CSF cytology and biochemistry. The patient tested negative for HIV. The patient was a strict vegetarian and gave a history of eating vegetable salad frequently.

Questions

1. What are the parasitic diseases transmitted by consumption of raw vegetables?
2. What precautions should be taken at individual level to prevent this type of infection?
3. What are the preventive measures needed to stop the transmission of such infections?

Research Questions

1. What are reasons for the lack of knowledge regarding the source of infection and control measures for uncommon parasites like *Mammomonogamus*?
2. Is this the right time to relook at the reservoir hosts like dogs for guinea worm disease given the sporadic cases which are being reported from areas where the disease has been eradicated?
3. Is the Indian form of visceral leishmaniasis really an anthroponosis? Is there any reservoir host for *L. donovani*?

Further Readings

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Diagnosis of Parasitic Zoonoses

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Learning Objectives

1. To emphasize the importance of appropriate sample collection.
2. To know the various modalities of diagnosis including the importance of microscopy.
3. To have an idea about the rising importance of molecular methods for species identification and molecular epidemiology.

Introduction

Zoonotic parasites destroy the health of both humans and animals either directly or indirectly, which ultimately affects a country's socioeconomic conditions. The majority of the well-known parasitic diseases caused by protozoans, cestodes, trematodes, helminths and pentastomids are zoonotic. These zoonotic parasites cause several diseases which have diverse clinical manifestation ranging from asymptomatic to symptomatic and acute to chronic, depending upon burden of parasitic infection, immune status of individuals and other co-morbid factors. Recently, parasitic zoonoses, especially cryptosporidiosis, leishmaniasis and toxoplasmosis, have been recognized as critical human infections mainly because of their ability to produce disease in HIV-infected and immunocompromised individuals. An early and rapid diagnosis of parasitic zoonoses, both in humans and animals, prevents the morbidity and mortality due to these diseases to a great extent. This chapter discusses different diagnostic approaches that can be useful for early and accurate diagnosis of zoonotic parasitic infections.

Collection of Specimens

Specimens for diagnosis of parasitic infections include stool, blood, urogenital samples, duodenal fluid, sputum, perianal swab, aspirated materials, cerebrospinal fluid (CSF), biopsy materials and intact or part of a worm (proglottides).

1. **Stool specimen:** To demonstrate intestinal parasites in the stool, it is prudent to defer collection for 5–10 days if the patient intakes mineral oil, bismuth, tetracycline group of antibiotics, antimalarial drugs and antidiarrhoeal agents. At least 7 days after barium sulphate and 10–14 days after tetracycline might be reasonable as these substances have detrimental effects on recovery of intestinal protozoa.

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Specimens ideally are collected in clean, wide-mouth plastic or waxed cardboard containers with a tight-fitting lid to avoid accidental spillage and to maintain moisture inside the specimen. Biosafety must be maintained while handling stool specimens, as fresh stool samples have potential infectious risk. Proper labelling with clear mentioning of date and time of collection is necessary.

The number of specimens both pre-treatment and post-treatment to be examined depends on many factors. These include severity of infection, presence/absence of symptoms, expected frequency of intermittent shedding, quality of sample and sensitivity/specificity of the tests performed. A single stool specimen from a symptomatic patient is often sufficient to diagnose infection. Ideally, three specimens (two from normal movements and one after non-oil-based cathartic) should be examined unless the patient has diarrhoea. In suspected intestinal amoebiasis, though six specimens ideally yield a 90% probability of detection, it is rarely contemplated due to cost factors. If a series of stool specimen testing is planned, for three samples it should be done within 10 days and within 2 weeks in case of six samples with reasonable spacing in between. Post-treatment specimens can be examined 3–4 weeks post-therapy for protozoan infection and 5–6 weeks for helminthic infestation.

Microscopy of fresh stool is necessary for the demonstration of trophozoites, which tend to disintegrate with time. Liquid samples should be tested within 30 min of the passage of stool. Soft and semi-formed samples require testing within an hour. Timing is not so critical in case of formed stool containing protozoal cysts and helminthic ova but preferably should be completed within 24 h. Most helminthic ova and larvae, coccidian oocysts and microsporidial spores survive for an extended time period. After collection, specimens should neither be frozen nor kept at room temperature and may be kept at 4 °C, but for a short time. However, in an anticipated delay, stool specimens need to be preserved to maintain protozoal morphology as well as arresting helminthic development. Preservation will result in loss of trophozoite motility but that outweighs the yield of intact morphology. Different preservation methods are in use and also by avoiding mercury-containing chemicals due to hazardous disposal issues (Table 1).

2. **Blood specimen:** Blood specimens for making smears are collected from capillaries, by fingertip prick with a sterile needle. After a finger prick, blood is allowed to flow freely but not squeezed out. Thick and thin blood smears are made on clean, grease-free slides. Anticoagulated (e.g. EDTA) venous blood samples are collected by the usual phlebotomy procedure. To prepare a thick blood smear, two to three drops of fresh blood are added on the slide, and with the corner of another slide, the drops are mixed in a circular motion to spread over a 2 cm diameter. Continuous stirring for 30 s prevents fibrin strand formation unless the blood is anticoagulated. The slide is air-dried. Thin blood films are prepared exactly like the blood film for leukocyte differential count, the film occupying the central area with thin, feathered end and free margins on both sides. Some prefer to make both the thick and thin smears on a single slide for a single patient. To prepare buffy coat film, anticoagulated venous blood is centrifuged, the buffy coat is formed in between the plasma, and the packed red cells are collected for smear preparation.
3. **Duodenal fluid:** Duodenal fluid is collected endoscopically and sent to the laboratory without any preservative. The specimen is centrifuged (500 × *g* for 10 min) and examined as a wet mount. *Entero-test* or duodenal capsule test is another method where a gelatine capsule containing coiled nylon yarn is administered orally keeping one end of the yarn fixed to the patient's face. Stomach acid dissolves the gelatine letting the string rest in the duodenum and bile-stained mucus cling to it. After 4 h, the string is retrieved along

Table 1 Preservatives used for the stool sample

	Preservative	Advantages	Disadvantages
Mercury-containing preservatives	Polyvinyl alcohol (PVA)	Allows permanent stain and concentration techniques Good preservation of trophozoites and cysts Long shelf life Can be shipped	Difficult to prepare Turns white and gelatinous upon refrigeration Hinders concentration of *Trichuris trichiura* ova and *Giardia lamblia* cysts Alters morphology of *Strongyloides* larvae Interferes with immunoassays
	Schaudinn's fluid	Fixative for fresh stool samples Good preservation of trophozoites and cysts	Not recommended for concentration procedures Poor adhesive for mucoid or liquid samples Interferes with immunoassays
	Merthilate-iodine-formalin (MIF) solution	Both fixative and stain Long shelf life	Morphology preservation is inferior compared to PVA and Schaudinn's fluid
Non-mercury preservatives	Formalin solution (5%, 10%)	Good fixative Easy to prepare Compatible with immunoassays	Trophozoites are not preserved Alters morphology in permanent stained smears
	Sodium acetate-acetic acid-formalin (SAF) fixative	Long shelf life Compatible with immunoassays	Poor adhesive; requires albumin-coated slides
	Modified PVA (uses copper or zinc base in place of mercury)	Allows permanent stain and concentration techniques	Inconsistent staining characteristics Copper-based modification poorly preserves trophozoite morphology Interferes with several immunoassays

with the duodenal content. The procedure is used for detecting *Giardia* trophozoites in duodenal specimens.

4. **Sputum:** Expectorated or induced sputum is collected in a sterile screw-capped container. Thick and tenacious samples may require sodium hydroxide treatment. Examination of sputum is recommended for detection of *Cryptosporidium* spp., eggs of *Paragonimus westermani* and rarely larvae of *Ascaris*, *Strongyloides*, etc.
5. **Perianal swab:** Perianal swabs are collected by NIH swab or cellophane tape preparations for diagnosis of enterobiasis in children. In this procedure, cellophane tape is applied with NIH swab or a tongue depressor to expose the outer adhesive surface and is firmly pressed against the perianal folds to collect the nocturnally deposited ova; smear is prepared on the slide and examined microscopically for demonstration of eggs of *Enterobius vermicularis*.
6. **Urogenital samples:** Vaginal and urethral discharges, urine sediments and prostatic massage fluids are collected. Multiple samples increase the diagnostic yield. Urine is examined for demonstration of microfilariae of *Wuchereria bancrofti*, trophozoites of *Giardia lamblia*, vaginal and urethral discharge for trophozoites of *Trichomonas vaginalis*, etc.
7. **Aspirated materials**: These include pus from liver abscess to demonstrate trophozoites of *Entamoeba histolytica*, aspirations from hydatid cyst to demonstrate scolices and hooklets of hydatid cyst, body fluids, bronchoalveolar lavage for larvae of some nematodes, etc.
8. **CSF samples**: Collected by lumbar puncture to demonstrate neural parasites such as

Naegleria species causing meningoencephalitis.

9. **Biopsy materials:** Tissue samples for parasitological examination require impression smears by lightly pressing against sterile slides forming a thin smear and squash preparation apart from standard histopathologic procedures.
10. **Intact/part of worm:** Intact worms or part of worm like tapeworm proglottides should be submitted in physiological saline solution.

Microscopy

Microscopy is important for proper parasitological diagnosis. Morphological identification of parasitic forms must be observed under a light microscope, stereoscopic microscope or simple dissecting microscope. It is preferable to have a calibrated microscope with an ocular micrometre. Alternatively, polystyrene beads of standardized diameter can be used. While the main aim is to look for the parasites, mimickers or artefacts should be considered.

Stool Microscopy

1. **Microscopy of direct smear**: Direct smear is made by suspending 2 g of stool in a few drops of saline and iodine followed by examination under dry power objectives (Fig. 1). The examination is performed with low power (10×) for screening and then with a higher power (40×) in a systematic fashion.
   (a) **Saline wet mount:** Used to demonstrate motile protozoan trophozoites and helminthic ova and larvae (Fig. 2). Trophozoites appear as pale and transparent but refractile objects. Tapping and application of heat enhance motility.
   (b) **Iodine wet mount:** Lugol's iodine, D'Antoni's iodine or Dobell and O'Connor's iodine solution is used. It is primarily used to demonstrate the protozoal cysts which appear with refractile nuclei, yellow cytoplasm and brownish glycogen material. Trophozoites get killed in this preparation.
   Apart from the parasitic elements, microscopy also reveals Charcot-Leyden crystals (indicate disintegrating eosinophils) and other artefacts (Table 2, Fig. 3).
   Recently, Parija et al. recommended lactophenol cotton blue preparation of stool to demonstrate intestinal parasites. Cotton blue stains the protozoal cysts and helminth ova deep blue. Internal structures of the coccidian oocysts can be seen.
   Egg count is valuable in *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms for predicting the worm burden. Stoll's dilutional egg count is commonly used.
2. **Microscopy of stool smear after concentration:** To maximize the yield, the stool sample is concentrated by sedimentation or floatation techniques (Table 3).
   (a) In the sedimentation technique, all the helminth ova, larvae and protozoal cysts can be concentrated in the sediment. The formol-ether sedimentation technique is commonly performed where formalin fixation renders the preparation non-infectious to the operator. A half teaspoon of stool is taken in a 15 ml container with 5–10% formalin and is allowed to stand for 30 min. The faecal suspension is filtered through two layers of gauze in a funnel into a centrifuge tube. Saline is added, and it is centrifuged two times each at 500 × *g* for 10 min, followed by discarding the supernatant in between and replacing with saline. Final sediment is suspended in formalin with the addition of ether or ether substitutes. Then the tube is closed and shaken vigorously for 30 s. Then it is subjected to final centrifugation. Four layers are formed—the topmost layer containing the ether, the faecal debris layer, the

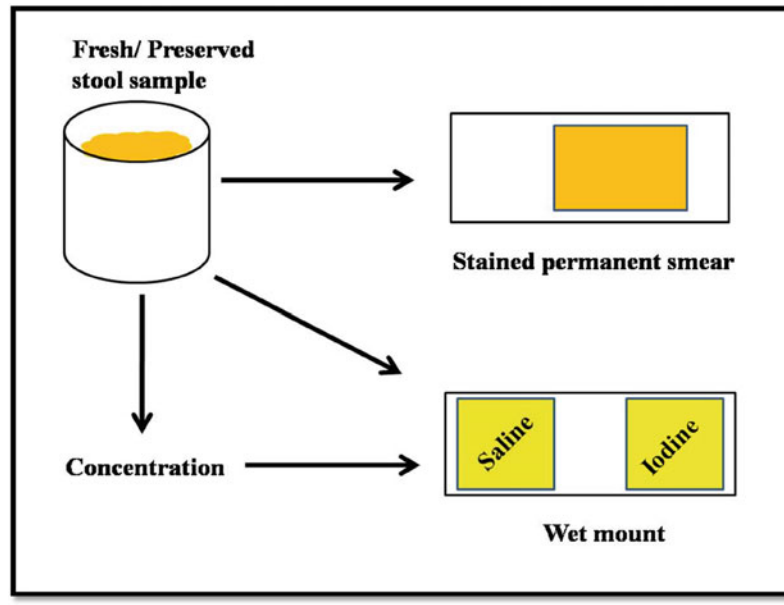

Fig. 1 Initial processing of stool sample

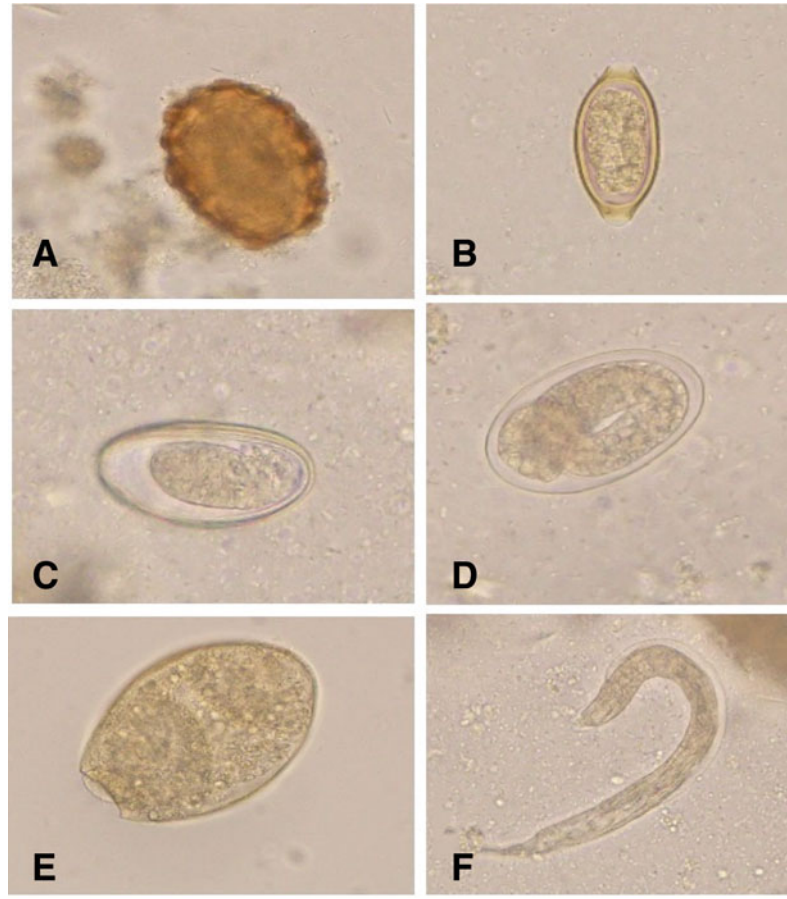

Fig. 2 Various helminth ova and larvae observed in stool sample. (**a**) Fertilized *Ascaris lumbricoides* ova, (**b**) ova of *Trichuris trichiura*, (**c**) *Enterobius vermicularis* ova, (**d**) ova of *Ancylostoma duodenale*, (**e**) *Fasciolopsis buski* ova and (**f**) *Strongyloides stercoralis* larva (Image courtesy of Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India)

Table 2 Various artefacts in different samples

Sample	Artefacts	Confused with	Usual differentiating characters of the artefact
Stool	Yeast cells	Coccidian oocysts	Yeast cells are oval with budding, thick-walled without the internal structures
	Pollen grains, plant seed	Helminth ova	Thick-walled, less uniform and may contain groove, spine, etc.
	Plant root hairs	Nematode larvae	Clear and refractile without iodine stain and internal structures
	Pus cells	*Entamoeba* spp. cyst	Pus cells have less dense cytoplasm with irregular cell outline. Usually smaller in size
	Macrophages	*Entamoeba histolytica/ Entamoeba dispar* trophozoite	Macrophages have larger and irregular nuclei with coarse cytoplasm with or without ingested red blood corpuscles
	Starch granules	Protozoan cysts	Refractile rounded structure without internal structure and stained with iodine
	Pineapple crystals	Charcot-Leyden crystals	Difficult to distinguish. Simultaneous examination required
Blood	Platelets, RBC inclusions, stain precipitate	Malaria parasite *Babesia* spp.	Platelets stain uniform colour without the internal structures
	Cotton/dust fibres	Microfilariae	Contain no internal nuclei
Urine	Non-pathogenic flagellates from stool contamination	*Trichomonas vaginalis*	Different motility patterns in the wet film
Respiratory sample	Ciliated epithelial cells	Flagellate protozoa	No internal structure

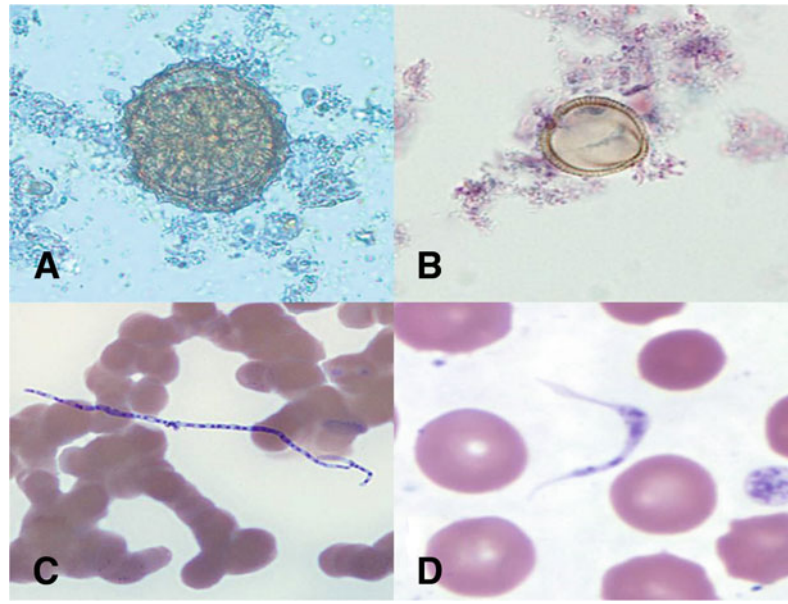

Fig. 3 Various artefacts noted in microscopy: (**a**) Pollen grain in a concentrated wet mount of stool mimicking *Ascaris* ova; (**b**) pollen grain in a trichrome-stained stool specimen mimicking *Taenia* ova but without hooklets; (**c**) fungal spore of *Helicosporium* spp., an airborne contaminant mimicking microfilaria; (**d**) platelet in a thin blood smear resembling *Trypanosoma* spp. (Image courtesy of DPDx, Centers for Disease Control and Prevention; https://www.cdc.gov/dpdx)

formalin layer and the bottom-most layer containing the parasitic contents. The sediment is examined as a wet mount after carefully decanting the upper layers. Protozoan cysts may be identified to the species level (e.g. *Iodamoeba butschlii*);

Table 3 Advantages and disadvantages of different stool concentration methods

Concentration methods	Advantages	Disadvantages
Sedimentation	Less technical error More sensitive Morphology is preserved Less infectious	Contain more faecal debris
Floatation	Less faecal debris Simple to perform without the need for a centrifuge	Operculated trematode ova, unfertilized *Ascaris* ova, *Taenia* ova and *Strongyloides* larvae do not float High specific gravity fluids alter the morphology

H. nana ova may be identified. *Isospora belli* oocysts are identified, but other coccidian oocysts require modified acid-fast staining for identification.

(b) In floatation techniques, a liquid with a high specific gravity (e.g. saturated salt solution, zinc sulphate) is used to float the lighter helminthic ova and protozoal cysts. A 15 ml flat-bottomed container is used to prepare the stool solution; any coarse faecal matter that floats up is discarded, and a $3'' \times 2''$ glass slide is placed in a fashion such that the centre of it touches the surface of the liquid. After 30 min, the glass slide is removed carefully, and the bottom side is examined microscopically. Protozoan cysts may be identified to the species level (*Giardia lamblia* cysts); hookworm ova may be identified.

3. **Microscopy of the permanent stained smear**: These are useful for detailed morphological examination even at later times and can be sent for expert opinions. Trichrome stain and iron-haematoxylin stains are the commonly used stains. These procedures are performed on both the fresh and preserved samples. The internal structures of the cysts and trophozoites are stained to permit better identification. Helminthic ova and larvae may not be easily identified due to stain retention.
Microscopy of permanent stained preparations of stool has played a crucial role in the diagnosis of cryptosporidiosis. Staining methods like Giemsa and Jenner's stain were used initially for the identification of the oocysts but later replaced by acid-fast Ziehl-Neelsen (ZN) and its modified version (modified ZN) that has become the most widely used method for detection of *Cryptosporidium* oocysts. Acid-fast oocysts stain red with a blue background (Fig. 4). Safranin-methylene blue fluorogenic stain and auramine-phenol methods are the other recommended stains. Smears stained with auramine-phenol or ZN stain have an advantage that the stained oocyst can be scraped off from the slide for subsequent DNA extraction for speciation. The detection limit for unconcentrated stool sample by microscopy has been reported to be 1×10^4 to 5×10^4, while concentration increases the sensitivity by tenfold.

Blood Microscopy

1. **Microscopy of the direct wet mount of blood**: The trypanosomes and microfilariae are identified by their size, shape and motility in moderate to heavy infections by this method.
2. **Microscopy of stained blood smears.**
   (a) **Thin blood smear:** Leishman's stain, Wright stain, Giemsa stain, Field's stain, etc. are used. These methods are useful for specific identification and speciation of *Plasmodium* spp. *Babesia* spp. may be misdiagnosed as that of *Plasmodium falciparum*, although the absence of malarial pigment and gametocyte forms along with the tetrad/Maltese cross forms are suggestive of *Babesia* spp.
   (b) **Thick blood smear:** Methanol fixation is omitted to permit lysis of the red blood cells and dehaemoglobinization. It is much more sensitive for detection of

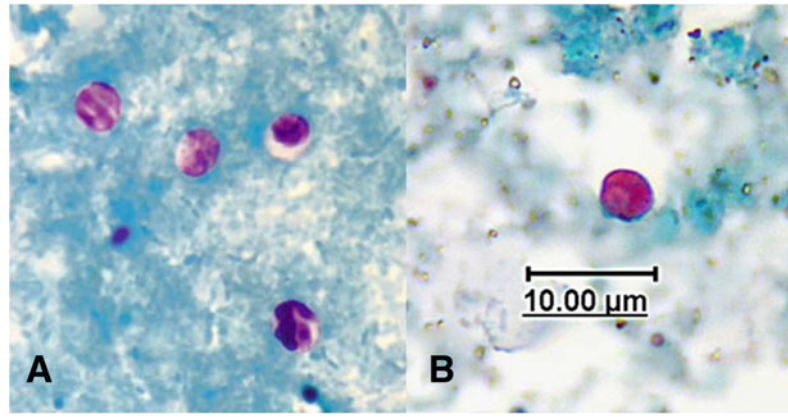

Fig. 4 (**a**) *Cryptosporidium* spp. oocysts stained with modified acid-fast stain; (**b**) fungal element in an acid-fast stained stool specimen should not be confused with *Cryptosporidium*. (Image courtesy of DPDx, Centers for Disease Control and Prevention; https://www.cdc.gov/dpdx)

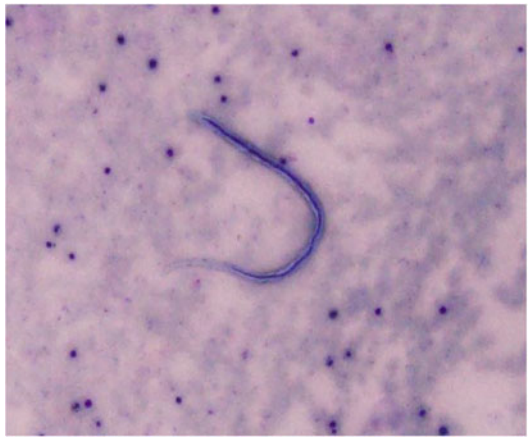

Fig. 5 Microfilaria of *Wuchereria bancrofti* in a thick blood smear, stained with Giemsa. (Image courtesy; Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India)

malarial parasites than the thin smear, but speciation is often difficult. Microfilariae can also be demonstrated (Fig. 5).

3. **Microscopy of concentrated blood smears.**
   (a) **Microhaematocrit centrifugation:** Finger-pricked capillary blood is collected in a microhaematocrit tube and centrifuged. The interface of plasma and red cells is examined for the presence of malaria parasites and trypanosomes under 100× objective.
   (b) **Triple centrifugation:** Citrated venous blood is centrifuged serially twice. Sediment is examined for trypanosomes.
   (c) **Buffy coat preparation:** This is examined for demonstration of amastigotes of *Leishmania donovani*.
4. **Fluorescence microscopy of blood smears**: Acridine orange-stained thin blood smear is examined with an epifluorescence microscope for detecting malaria parasites. It is more sensitive than Giemsa stain even at low parasitaemia.

Urogenital Specimens Microscopy

Low-power microscopy with a dull illumination reveals jerky motility of the *T. vaginalis* trophozoites; undulating membrane can be seen with higher magnification. Urinary sediment examination also helps in diagnosing urinary schistosomiasis by observing the characteristic terminal-spined ova of *Schistosoma haematobium*. Chylous urine may reveal microfilariae of *W. bancrofti*.

Duodenal Fluid Microscopy

Motile larvae of *Strongyloides* are easily seen, whereas falling-leaf-like motility of *Giardia* trophozoites may be observed by microscopy of duodenal fluid. Sedimented mucus-containing areas are examined for the presence of trophozoites of *Giardia*.

Sputum Microscopy

Sputum microscopy, following Giemsa stain or silver stains, is useful for demonstration of the migrating larvae of *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Ancylostoma duodenale* and *Necator americanus*; hooklets of *Echinococcus granulosus*; and clusters of *P. westermani* ova. Trophozoites of *E. histolytica* may be found in pulmonary complication of amoebic liver abscess. The method is also useful for demonstration of *Trichomonas tenax*, *Cryptosporidium* spp. and Microsporidia spp. Trichrome stain differentiates *E. histolytica* from *E. gingivalis*.

Perianal Swab Microscopy

Perianal swab microscopy is useful for detection of the non-bile-stained planoconvex eggs of *Enterobius vermicularis* ova and also the eggs of *Taenia* spp. and *Schistosoma mansoni*.

Aspirated Materials Microscopy

Giemsa stain for *Toxoplasma gondii*, trichrome stain for amoebae, modified ZN/Kinyoun stain for *Cryptosporidium* and modified trichrome stain for microsporidia are the most frequently used methods. Stained bone marrow aspirates are examined for demonstration of *Plasmodium* spp. and amastigotes of *Leishmania* and *Trypanosoma cruzi*. Bronchoalveolar lavage or washing materials are centrifuged and microscopically examined for *T. gondii* and *Cryptosporidium* oocysts. Aspirated liver abscess collected from the margin of the abscess may reveal trophozoites of *E. histolytica* trapped in the viscous anchovy sauce-like pus. Cyst material for hydatid disease shows protoscolices, hooklets and calcareous corpuscles in various stages of degeneration. Addition of 10% potassium hydroxide solution enhances visualization of the diagnostic hooklets.

CSF Microscopy

Microscopy of CSF is important to demonstrate trophozoites of *Acanthamoeba* spp., *Naegleria* spp. and *Balamuthia* spp. Also trypomastigotes of *Trypanosoma brucei*, *Trypanosoma rhodesiense* and *Trypanosoma gambiense* may be seen. In cases of eosinophilic meningitis, the larvae of *Angiostrongylus* can be found.

Biopsy Materials Microscopy

Microscopy of tissue sections and tease mounts are important for demonstration of:

1. LD bodies in the splenic aspirate, lymph nodes, bone marrow and liver
2. *Onchocerca volvulus* microfilariae in skin snips
3. Demonstration of larvae of *Trichinella spiralis*, *Taenia solium* and *Multiceps multiceps* in muscle biopsy samples
4. Diagnosis of neurocysticercosis by observing the scolex and typical tegumental anatomy in sections of brain tissue
5. *S. haematobium* ova in urinary bladder mucosal biopsy
6. Free-living amoebae in brain biopsy

Examination of Intact/Part of Worm

Adult worms of *A. lumbricoides* should be differentiated from those of earthworm and identified with the help of a dissecting microscope by observing the external anatomy. Observance of the genital girdle and genital opening helps in gender determination. Submitted tapeworm proglottids are examined for primary lateral branches of the uterus; fewer than 13 branches indicate the segment as that of *T. solium*. Microscopy is required in case of a thick segment after staining with aceto-alum carmine. Surgically extracted tiny worms like hookworm need microscopical identification.

Table 4 Culture methods for protozoa

Protozoa	Culture method	Utility
Entamoeba histolytica	Stool culture in Robinson's media and NIH polyxenic culture media	Diagnosis of chronic and asymptomatic cases and for zymodeme pattern determination
Acanthamoeba spp.	Non-nutrient agar with *Escherichia coli* overlay	Diagnosis of amoebic keratitis
Naegleria fowleri	Non-nutrient agar with *Escherichia coli* overlay Tissue culture	Recovery of free-living amoebae
Leishmania spp.	Novy-MacNeal-Nicolle medium (NNN medium), *Drosophila* medium	Demonstration of the motile promastigotes
Trichomonas vaginalis	Diamond's TYI medium	The most sensitive and gold standard for diagnosis
Plasmodium spp.	Tissue culture in RPMI 1640 medium	Drug resistance analysis, antigen preparation, vaccine development

Culture of Specimens

Culture methods are employed for isolation and refined identification of the cultivable parasites. Culture for protozoa is carried out for certain protozoa to confirm the diagnosis with inconclusive microscopy or serology (Table 4). Culture for helminths is mainly done for hatching out the larva and for distinguishing eggs of parasites which have similar morphology (Table 5). Culture methods, however, are not routinely employed in diagnosis.

Schistosome Egg Viability Test

Schistosome eggs in stool and urine are checked for viability by hatching in dechlorinated water. Presence of living miracidia indicates an active infection.

Immunodiagnosis

Immunodiagnosis of parasitic diseases includes intradermal skin tests, antibody-based serological tests and antigen-based serological tests.

Intradermal Skin Tests

Intradermal skin tests in parasitic diseases are based on hypersensitivity reactions. Immediate hypersensitivity reactions are observed in helminthic infections, e.g. filariasis, echinococcosis, schistosomiasis and ascariasis. Delayed-type reactions are utilized for protozoan infections like leishmaniasis, toxoplasmosis and amoebiasis. These tests are infrequently employed not only due to the lack of specificity but also due to difficulties in procurement and standardization of the crude antigen.

The Montenegro skin test (MST) was used earlier in the diagnosis of cutaneous leishmaniasis. This test is highly specific with good sensitivity and is also very simple to use. The major drawback of this test is the requirement of MST antigen preparation which further impacts the test sensitivity. Also this test cannot differentiate between current and previous infections.

Antibody-Based Serological Tests

Antibody production, whether IgG, IgM or IgE, in parasitic diseases has been utilized as a surrogate marker for diagnostic purposes.

Complement fixation test (CFT) was one of the earliest tests in parasitology. The test is used but rarely for paragonimiasis, leishmaniasis and Chagas disease. Immuno-electrophoresis (IEP) is used in amoebiasis, cysticercosis, trichinellosis and hydatid disease, with high specificity. Indirect haemagglutination (IHA) and indirect immunofluorescent antibody (IFA) tests are frequently used for diagnosis of amoebiasis, echinococcosis,

Table 5 Culture methods for helminths

Helminth	Culture method	Utility
Hookworm	Harada–Mori filter paper method of stool culture: stool sample is incubated over a filter paper strip inside a test tube containing water	Demonstration of the filariform larvae, differentiation of *Ancylostoma duodenale* and *Necator americanus* from the stool sample
Strongyloides spp.	Harada–Mori filter paper method Baermann funnel method Agar plate method	Being most sensitive, employed in suspected cases with negative microscopy

filariasis, cysticercosis, strongyloidiasis, etc. with variable sensitivity and specificity.

Other antibody-based immunoassays that are increasingly used currently in the diagnosis of zoonotic parasitic diseases include the following:

1. **Enzyme-linked immunosorbent assay (ELISA):** ELISA is the most frequently used test in the diagnosis of a wide range of protozoa and helminths. Newer formats include:
   (a) **FAST-ELISA:** Falcon assay screening test ELISA uses synthetic and recombinant peptides to detect antibodies against a known antigen. FAST-ELISA has been used in malaria, fasciolosis, schistosomiasis and taeniasis. Major drawbacks are cross reaction and selective immunogenicity of the epitope selected.
   (b) **Dot-ELISA:** Plastic plate of the regular ELISA is replaced by a better binding matrix of nitrocellulose membrane in Dot-ELISA resulting in improved sensitivity and specificity from a lower sample volume. The principle is similar to immunoblotting. The dotted membrane is incubated with an antigen-specific antibody followed by the addition of enzyme-conjugated anti-antibody and precipitable chromogenic substance. Formation of a coloured dot is read visually to interpret. It can be used as a field assay and is easy to perform and fast. Dot-ELISA is increasingly being used in diagnosis of amoebiasis, babesiosis, fasciolosis, leishmaniasis (cutaneous and visceral), cysticercosis, echinococcosis, malaria, toxoplasmosis, trypanosomiasis, toxocariasis, trichinellosis, fascioliasis, etc. Studies have showed better sensitivity and specificity of Dot-ELISA in CSF samples from African sleeping sickness by detection of antineurofilament and anti-galactocerebroside antibodies compared to the conventional ELISA kits.
   (c) **Luciferase immunoprecipitation system (LIPS):** It is a modified ELISA-based assay to detect specific antibodies by the production of light. The antigen of interest is fused with enzyme reporter Renilla luciferase and expressed in mammalian cells for undergoing posttranslational modifications. The crude protein extract is incubated with the test serum and protein A/G beads. The Renilla luciferase-antigen fusion gets immobilized on the beads. Addition of a coelenterazine substrate facilitates light production which indirectly gives the measurement of the antibodies. LIPS is sensitive and takes 2.5 h. LIPS has been applied for diagnosis of infection caused by *S. stercoralis* and *Loa loa*. Quick LIPS (QLIPS) is a modified rapid version of LIPS assay taking only 15 min. This method is recommended as the most specific method for detection of *Onchocerca volvulus*-specific antibodies in serum samples.
2. **Electroimmunotransfer blot (EITB):** This is the most widely used serological assay for cysticercosis. Seven glycoproteins derived from *T. solium* are separated and blotted on a nitrocellulose strip. Serum or CSF samples are

incubated with the strips and washed to remove the unbound antibodies. Binding is visualized by an enzymatic colour change in band pattern. Then it is compared with a control strip with proper alignment. Presence of bands indicates a positive result for specific antibodies.

3. **Radioimmunoassay (RIA):** Radioimmunoassays use radiolabelled molecules in a stepwise formation of the antigen–antibody complex. A known quantity of antigen is made radioactive by labelling with radioactive isotopes and mixed with a known amount of antibody. Addition of patient's serum alters the ratio and is interpreted accordingly observing the resultant radioactivity by a gamma counter. Radioimmunosorbent test (RIST) and radioallergosorbent test (RAST) are used in echinococcosis for detection of IgE antibodies.

Simple Serological Tests

A wide number of serological tests are being evaluated in diagnosis of many parasitic diseases in resource-poor settings, in less equipped laboratories and in the field. These are:

- Agglutination tests for amoebiasis, trypanosomiasis and echinococcosis.
- Direct agglutination test for visceral leishmaniasis.
- Carbon immunoassay for toxoplasmosis and amoebic liver abscess: The test is based on binding of the carbon particles present in Indian ink by *Toxoplasma*-specific or *Entamoeba*-specific antibodies combined with trophozoites.
- The (card agglutination trypanosomiasis test): CATT implies antibody-mediated agglutination of fixed trypanosomes which has specific surface glycoproteins and has a good sensitivity in detecting antibodies specific to *T. b. gambiense* in blood in human African trypanosomiasis (HAT). However, CATT is usually not used for *Trypanosoma brucei rhodesiense* diagnosis as these surface glycoproteins are not present in *T. b. rhodesiense.* Since the 1980s this test has been progressively used for screening the population at risk in western and central Africa where the gambiense form of the disease is prevalent. Since the 2010s, the tools for screening of gambiense HAT have been complemented by the development of rapid individual serological tests that are better adapted to passive screening at health facilities.
- Staphylococci adherence test for Chagas disease and amoebic liver abscess is based upon the affinity of staphylococcal protein A for IgG antibodies. Epimastigotes of *T. cruzi* or trophozoites of *E. histolytica* are fixed to the glass slide and incubated with test sera and staphylococcal suspension. Giemsa staining reveals the surface of these parasites covered by cocci in positive cases.

The limitations of antibody-based immunoassays are as follows:

1. They often cannot differentiate between recent and past infection.
2. They cannot assess the degree of parasitic infection.
3. They tend to be non-specific due to cross reaction.
4. They are unreliable in patients with immune suppression, malignancy, HIV/AIDS and congenital parasitic infections.
5. They show certain technical difficulties:
   (a) Availability of antigen is challenging. Sources include a culture of certain developmental stages, parasites maintained in animals or parasites obtained from infected veterinary or human cases which are difficult to procure.
   (b) Inadequate standardization procedures.
   (c) Higher cost, particularly with fluorescent techniques and radioimmunoassay.

Antigen-Based Serological Tests

Detection of antigen in various samples is generally indicative of recent infection. Low yield of microscopic identification can be substantially fortified with relevant antigen testing. Besides, antigen-based tests are frequently used as an

important prognostic marker, since antigen disappears from the serum after parasitological cure of the disease.

ELISA, immunochromatographic tests, counter-immunoelectrophoresis (CIEP), enzyme-linked immunosorbent assay (ELISA) and bacterial coagglutination are the most frequently used tests to detect antigen in serum, urine, saliva and other body fluids in a wide range of parasitic infections.

Antigen testing using serum samples has been applied widely in the diagnosis of amoebiasis, toxoplasmosis, malaria, leishmaniasis, cysticercosis, echinococcosis, lymphatic filariasis, schistosomiasis, fascioliasis, etc. Immunochromatographic tests are also known as rapid antigen detection tests (RDTs) where soluble protein antigens are captured by specific antibodies embedded on a nitrocellulose strip. A drop of blood sample is applied to the strip and is eluted by addition of buffer solution. Antigen–antibody complex formation is visualized as a coloured line. Technological improvements have rendered RDTs stable at temperatures up to 40 °C, user-friendly and fast (around 15 min). RDTs are useful in the identification of *P. falciparum* and *Plasmodium vivax*. Other *Plasmodium* species including the zoonotic *Plasmodium knowlesi* cannot be diagnosed with these kits. Also, false-negative results may occur in low parasitaemia.

Parasite antigens that have been excreted in the urine, saliva, etc. are detected for diagnosis of leishmaniasis, amoebiasis, cysticercosis, hydatid disease, schistosomiasis, lymphatic filariasis, etc. The advantage of urine or saliva as a specimen is that it can be collected by non-invasive methods and can be collected frequently without causing any inconvenience to the patient.

Stool antigen testing, also known as *coproantigen* detection, has been used for detection of specific antigen in stool samples for diagnosis of intestinal amoebiasis, giardiasis, cryptosporidiosis, etc. The coproantigen-detection ELISA is a highly sensitive test used for diagnosis of intestinal taeniasis in humans. The ELISA using polyclonal antibody to the adult *Taenia* stage is employed to detect *Taenia* antigen in the supernatant of stool. The test is not only useful for diagnosis of cases, but is also useful to detect *Taenia solium* carriers.

Antigen-based immunoassays have certain limitations, which are as follows:

1. Circulating antigens often combine with antibodies to form immune complexes; this hinders antigen detection and may lead to false-negative tests.
2. Intermittent rupture of parasitized cells, as observed in toxoplasmosis, may produce a false-negative result.

Molecular Diagnosis

Relative non-specificity of antibody tests and limitations of microscopy can be overcome with nucleic acid amplification methods.

Polymerase Chain Reaction (PCR)

PCR is a molecular technique of in vitro synthesis of a specific nucleic acid sequence. PCR has been employed for diagnosis of giardiasis, amoebiasis, malaria, leishmaniasis, toxoplasmosis, cryptosporidiosis, etc. PCR can distinguish between microscopically indistinguishable *E. histolytica*, *E. dispar* and *E. moshkovskii*. PCR is helpful in the detection of drug resistance in malaria parasites (Fig. 6).

Besides the conventional PCR, real-time PCR, loop-mediated isothermal amplification (LAMP) and Luminex-based assays have emerged as new approaches for the parasitic diagnosis. All of these tests offer greater sensitivity and specificity than other diagnostic tests, as well as permitting diagnosis from a very low concentration of parasites including asymptomatic and window periods. Multiplexing facilitates simultaneous detection of numerous pathogens and clinching the pinpoint diagnosis early.

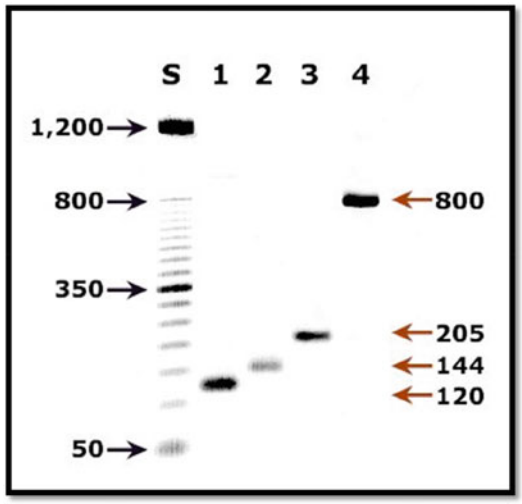

Fig. 6 Agarose gel (2%) analysis of a PCR diagnostic test for species-specific detection of *Plasmodium* DNA. **Lane S:** Molecular base pair standard (50-bp ladder). Black arrows show the size of standard bands. **Lane 1:** The red arrow shows the diagnostic band for *Plasmodium vivax* (size, 120 bp). **Lane 2:** The red arrow shows the diagnostic band for *Plasmodium malariae* (size, 144 bp). **Lane 3:** The red arrow shows the diagnostic band for *Plasmodium falciparum* (size, 205 bp). **Lane 4:** The red arrow shows the diagnostic band for *Plasmodium ovale* (size, 800 bp). (Image courtesy of DPDx, Centers for Disease Control and Prevention; https://www.cdc.gov/dpdx)

Real-Time PCR

This technique avoids the procedures and problems of post-PCR gel electrophoresis. Various fluorescent chemistries like SYBR green, TaqMan probes, fluorescence resonance energy transfer (FRET) and scorpion primers allow measuring the original template's concentration in terms of threshold values (Ct value). It allows high-throughput analysis of different sequences in a single-closed tube reaction. This has been used to diagnose *Plasmodium* spp. infection including *P. knowlesi* and also to detect pathogenic *E. histolytica*. Real-time PCR has also been used for the detection of *T. cruzi* infection following heart transplants; this allows immediate treatment much before the reactivation of Chagas disease.

Loop-Mediated Isothermal Amplification (LAMP)

Unlike a conventional PCR, LAMP is carried out in a constant temperature (around 65 °C), thus negating the need for a thermal cycler. Usually, six different primers are used to recognize eight distinct regions on a target gene. Thus amplification will occur only if all the primers can bind to form a product. Production of pyrophosphates in large quantities results in the formation of a white precipitate visible to the naked eye. LAMP has been used to detect *Entamoeba*, *Trypanosoma*, *Plasmodium*, *Babesia*, *Cryptosporidium*, *Theileria* and *Taenia*. Vector mosquitoes harbouring *Plasmodium* and *Dirofilaria immitis* have been identified with this highly specific technology. A sophisticated LAMP test, named RIME LAMP, has been developed to diagnose *T. b. rhodesiense* and *T. b. gambiense* from blood and CSF samples.

Luminex Technology

It is a bead-based flow-cytometric assay. Microsphere beads are covalently bound to antigens, antibodies or oligonucleotides to serve as probes in the assay. Up to 100 such microspheres emit unique fluorescent signals upon excitation by laser facilitating identification of the targets. Luminex technology has been utilized in the identification of *Cryptosporidium* and malaria.

DNA Probe

DNA probe is a labelled oligonucleotide sequence prepared as a complement to a unique part of the parasitic genome. A clinical sample containing the parasite would facilitate binding of the DNA probe upon addition and result in hybridization and subsequent detection. DNA probes are used in the diagnosis of infections caused by *Trypanosoma*, *W. bancrofti*, *Onchocerca volvulus*, *P. falciparum*, etc.

Proteomics

Recent interest in protein analysis stems from the fact that proteins are involved in the structure, signals and molecular machinery of parasites. The proteomic strategy identifies a protein in two ways—classic top-down strategy and bottom-up strategy. The top-down strategy uses two-dimensional gel electrophoresis to detect proteins. The bottom-up strategy displays peptides after shattering proteins in a biological fluid in a spectrum. The resultant spectrum is compared against a predetermined database to identify the organism. Surface-enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS) is a new advancement allowing sample binding to different chemically active protein hip surfaces providing automated high-throughput analysis of protein spectrum based on mass-to-charge ratios. SELDI-TOF MS has been utilized in trypanosomiasis, fascioliasis and cysticercosis.

For quite some time, antibody detection had been the mainstay for diagnosis of toxoplasmosis. Although its importance in testing during pregnancy has not diminished, *T. gondii* DNA detection through PCR, in body fluids (bronchoalveolar lavage fluid, cerebrospinal fluid, vitreous and aqueous fluids, blood and brain tissues), is becoming the preferred method for diagnosis of cerebral, ocular, congenital and disseminated toxoplasmosis where antibody detection is not always conclusive. It is also successfully used for early detection of intrauterine *T. gondii* infection. For PCR amplification, B1 gene, 18S rDNA gene, 529 bp repeat element, GRA1 SAG1 and SAG2 are the target genes. Real-time PCR by targeting amplification of the B1 gene is the most recommended diagnostic technique for congenital toxoplasmosis in comparison to nested and conventional PCR. LAMP assay has also been developed, which targets the *T. gondii* oocyst wall protein (OWP) genes, 529-bp repetitive element, SAG1, B1, SAG2, GRA1 and 18S rRNA for the medical and veterinary samples and also water samples.

Miscellaneous Tests

Animal Inoculation

In certain suspected infections, animal inoculation is performed to diagnose and maintain the parasite for further studies. Examples include hamsters for isolation of *Leishmania* spp., rats for *Trypanosoma* spp. and mice for *T. gondii*. Generally, the intraperitoneal route is used.

Xenodiagnosis

It is a tedious method of diagnostic infection of a vector by exposing it to a infectious sample and subsequent demonstration to confirm the diagnosis. This is an excellent method to diagnose chronic Chagas disease. A suspected patient is exposed to 30–40 nymphs of reduviid bug for 3 consecutive days. The bugs are maintained in the laboratory with periodic examination of their faecal matter over 3 months to demonstrate developmental stages. Xenodiagnosis had been used for trichinellosis in the past. The muscle tissue sample is fed to uninfected rats, and after the appropriate time, the diaphragmatic muscles are checked for the *T. spiralis* larvae.

Case Study

A mother brings her 6-year-old son to you with complaints of abdominal pain and sleep disturbances. You notice that the child is mildly restless and frequently bites his nails. On enquiry, the mother tells you that her son is experiencing perianal itching with marked frequency in the early morning while going to school. You suspect intestinal worm infestation and advise a stool examination for ova, parasite and cyst. On the next visit, the report is negative. However, the child is still having the same problems. You order Scotch tape preparation of the perianal area and clinch the diagnosis.

Questions:

1. What are the clues that hint towards parasitic infection?
2. What could be the reasons for the negative stool report?
3. What could be the Scotch tape preparation finding?
4. What could be the reason for early morning perianal itching?

Research Questions

1. What approaches should be taken to develop reliable, rapid, point-of-care tests for parasitic diseases? Except for malaria, filariasis, leishmaniasis and trypanosomiasis, no such test exists. Antibody- or antigen-based rapid tests for parasites like amoebiasis, toxoplasmosis, cryptosporidiosis and the vast majority of helminthic infections need to be developed.
2. How do we develop a complete standardized database for a specific and reliable gene target for parasites which will help in reproducibility of results and in development of more commercial PCR-based products?

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Chemotherapy of Parasitic Infections

Kolukuluru Rajendran Subash
and Shanmuganathan Padmavathi

Learning Objectives

1. To have a working knowledge of important antiparasitic agents.
2. To know the indications of the antiparasitic agents and the limitations.

Introduction

The drug development and discovery for parasitic diseases has gained more attention in the recent past after the appreciation of the novel works by William C. Campbell, Satoshi Ōmura and Youyou Tu on parasite-fighting therapies for discovering effective and novel antiparasitic therapies. C. Campbell and Satoshi Ōmura discovered avermectin, the derivatives of which were proven to be effective against river blindness and lymphatic filariasis. Similarly, artemisinin, a novel antimalarial agent, discovered by Youyou Tu, significantly reduced the mortality and morbidity due to malaria. These two discoveries have revolutionized the treatment of these debilitating diseases and paved newer pathways to the antiparasitic drug discovery process. These have caused a huge impact globally, particularly in the developing countries, where the only way of combating these diseases is by effective chemotherapy. Recently, in 2019, triclabendazole has been recommended for the treatment of fascioliasis, which shows that there is an increased focus on the treatment of parasitic diseases. This chapter broadly reviews the antiparasitic agents used to treat both protozoal and other infections.

Goals of Chemotherapy

A multidimensional approach is needed for effective control of parasitic diseases. Mass drug administration through long-term community health programmes and increased awareness of parasitic disease are important in containment of parasitic diseases globally. Successful management of parasitic disease by antiparasitic drug therapy in rare diseases is also equally important. A coordinated approach between nursing care for appropriate monitoring and clinical pharmacists to monitor and avoid dosing, administration error and potential drug–drug interactions in chemotherapy of parasitic infection are crucial. The severe adverse drug reactions observed with many cases during antiparasitic therapy have led

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to many compliance issues. Hence the role of direct observed therapy by healthcare workers benefits the outcomes of community health outreach programmes. Finally, there is a continuous need for research and development for newer drug molecules, potentially safe to combat resistance and treat parasitic infections, especially neglected tropical parasitic diseases.

Antiparasitic Agents

The key principles of use of antiparasitic agents include selecting an appropriate drug for appropriate indication, right dosage according to the individual conditions (age, comorbid conditions, drug-to-drug interactions) and right duration of treatment.

The antiparasitic drugs are broadly classified into antiprotozoals and anthelmintics (Fig. 1). The drugs effective against protozoal infections such as amoebiasis, leishmaniasis, toxoplasmosis, trypanosomal infections, trichomoniasis, malaria, etc. are antiprotozoals, whereas the drugs effective against cestodes, trematodes, nematodes and ectoparasites are categorized under anthelmintics.

Limited efficacy and potency of antiparasitic agents, high toxicity of the drug prevention, and their use in mass administration and development of resistance are the frequently encountered challenges with antiparasitic agents.

Chemotherapy of Protozoal Infections

Chemotherapy of Gastrointestinal Protozoa

Antiamoebic agents include metronidazole, tinidazole, secnidazole, ornidazole, satranidazole, paromomycin and iodoquinol.

Metronidazole, secnidazole, ornidazole, satranidazole and tinidazole are 5-nitroimidazole derivatives. Metronidazole (dose, 500–750 mg PO tid for 7–10 days) has activity against various protozoal infections including giardiasis and trichomoniasis. It acts by producing reactive toxic intermediates within the parasite, thus making it effective as both luminal and extraluminal amoebicide. Metronidazole metabolizes into acid metabolites and hydroxymetabolites. The latter act on the parasitic deoxyribonucleic acid (DNA) and cause DNA disruption, leading to inhibition of protein synthesis. Other nitroimidazoles have similar actions to that of metronidazole with high cure rate, long half-life and better toxicity profile for protozoal infections.

Paromomycin is an aminoglycoside antibiotic that acts by inhibiting parasite 30S ribosome resulting in inhibition of protein synthesis. Iodoquinol is a halogenated hydroxyquinoline which acts as a chelating agent. The compound

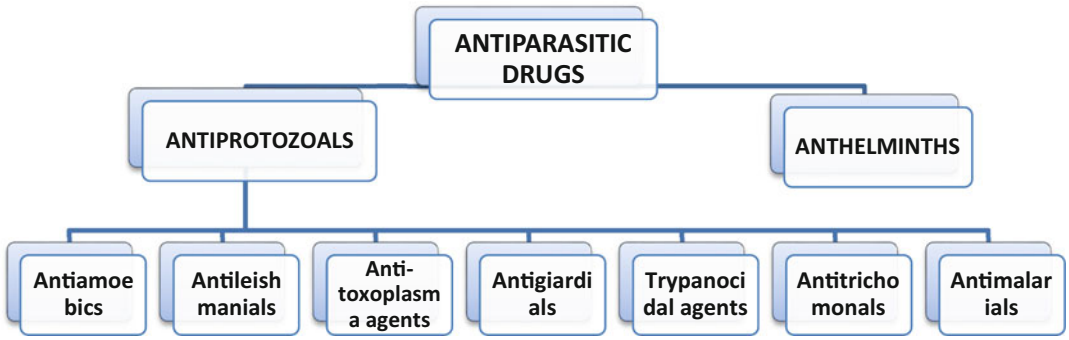

Fig. 1 Classification of antiparasitic agents

within the parasite reduces ferrous ions, thereby increasing protein-bound serum iodine and finally interfering with protozoal metabolism.

The clinical setting determines the choice of drugs used in amoebiasis. Asymptomatic intestinal infection caused by *Entamoeba histolytica* in adults is managed by treatment with luminal agents such as diloxanide furoate, iodoquinol or paromomycin. In mild to moderate and severe intestinal infection, metronidazole or tinidazole along with luminal agent is prescribed. The alternative treatment includes the use of luminal agent along with tetracycline or erythromycin. Metronidazole or tinidazole along with a luminal agent is used for the treatment of extra-intestinal amoebic infections. Iodoquinol is used for treatment of *Dientamoeba fragilis* infection.

Metronidazole and tinidazole are effective against giardiasis caused by *Giardia lamblia*. Paromomycin is recommended for treatment of giardiasis in pregnancy. Nitazoxanide and furazolidone are used in treatment of giardiasis resistant to metronidazole and tinidazole. *Balantidium coli* infection is treated best with tetracycline and alternatively with metronidazole.

Nitazoxanide is effective for treatment of cryptosporidiosis. Co-trimoxazole (trimethoprim 160 mg – plus sulfamethoxazole 800 mg) twice daily for 10 days is effective for treatment of *Isospora belli* and *Cyclospora cayetanensis* infections. Albendazole is the first drug of choice for the treatment of microsporidiosis. Paromomycin is the alternative drug.

Chemotherapy of Genital Protozoa

Anti-trichomonal agents include metronidazole and tinidazole. Metronidazole and tinidazole are the drugs of choice for treatment of infections caused by *Trichomonas vaginalis*. Metronidazole given orally in a single dose of 2 g or 250 mg three times daily for 7 days is effective. Tinidazole given in a single oral dose of 2 g is very effective for treatment of trichomonas infections resistant to metronidazole.

Chemotherapy of Blood and Tissue Protozoa

Sodium stibogluconate and meglumine antimoniate are pentavalent antimonial compounds. They act by decreasing viability of *Leishmania* spp. by inhibiting their glycolysis and citric acid cycle by preventing the conversion of ADP and GDP to ATP and GTP. An antifungal agent such as amphotericin B liposomal preparation acts by binding to an ergosterol precursor of the parasite and disrupting the parasite's membrane. Miltefosine, a derivative of alkylphosphocholine, acts by preventing synthesis of parasite cell surface molecules or by interfering in lipid metabolism of the parasite resulting in disruption of parasite cell signal transduction.

Sodium stibogluconate is prescribed at a dose of 20 mg Sb/kg/day IV or IM for a duration of 28 days to treat visceral leishmaniasis and 20 days for treatment of cutaneous leishmaniasis. Miltefosine given for 28 days, paromomycin for 21 days at a dose of 15 mg/kg/day IM or amphotericin, preferably liposomal preparations, is also effective. Pentamidine at a dose of 2–3 mg/kg IV or IM daily for 15–30 days in visceral leishmaniasis and meglumine antimoniate are the alternate drugs used effectively for intralesional application in leishmaniasis.

Both nifurtimox (8–10 mg/kg/day PO in three to four divided doses for 90 days) and benznidazole (5–7 mg/kg/day PO in two divided doses for 60 days) are the drugs of choice against Chagas disease caused by *Trypanosoma cruzi.* Both these compounds, on activation by parasite mitochondrial nitroreductase, result in the formation of intracellular nitro radical anions. These anions subsequently form a covalent attachment with parasite macromolecules resulting in cellular damage and death of the parasite.

Pentamidine isethionate 4 mg/kg/day IM or IV for 7 days and suramin sodium 100 mg IV followed by 1 g IV on days 1, 3, 5, 14 and 21 are effective against hemolymphatic stage, while melarsoprol 2.2 mg/kg/day IV for 10 days and eflornithine 400 mg/kg/day IV in four doses

for 14 days are effective against CNS stage of *Trypanosoma brucei* causing sleeping sickness.

Melarsoprol is a pro-drug metabolized to an active metabolite melarsen oxide. The mechanism of action of this drug is still unknown. It is suggested that melarsen oxide-trypanothione acts as an inhibitor of trypanothione reductase resulting in the formation of adducts and reduce trypanothione levels of the parasite. The reduction of trypanothione reductase may have a lethal effect on parasitic cells.

Anti-toxoplasma agents include pyrimethamine, sulfadiazine, clindamycin and spiramycin. Pyrimethamine and clindamycin along with folinic acid are recommended for treatment of acute, congenital and immunocompromised toxoplasmosis. Alternatively, pyrimethamine and sulfadiazine along with folinic acid are used. In case of pregnancy, spiramycin, 1 g three times per day until delivery, is recommended. Pyrimethamine prevents DNA and protein synthesis in the parasite by inhibiting dihydrofolate synthase. Sulfadiazine inhibits dihydropteroate synthase which is essential for folic acid synthesis in the parasite; sulfadiazine together with pyrimethamine is used for its synergistic action against toxoplasmosis.

Antimalarial Agents

Among the five *Plasmodium* species known to cause human infections, *Plasmodium falciparum* causes severe disease and death in humans, whereas *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* cause less severe disease. *Plasmodium knowlesi* is primarily a parasitic infection of monkeys and has recently been recognized to cause illness and severe disease among humans in Asia.

The species, geographic distribution and severity of the patient's infection determine the choice of drug to treat malaria. Chloroquine is the drug of choice to treat uncomplicated malaria caused by *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. Amodiaquine in combination with artesunate, atovaquone with proguanil and artemether with lumefantrine are used for the treatment of drug-resistant *P. falciparum* infections. Oral quinine is indicated in pregnancy. Primaquine, mefloquine, atovaquone-proguanil and doxycycline are used for chemoprophylaxis in malaria.

Mechanism of Action of Antimalarial Agents

Chloroquine is a 4-aminoquinoline that prevents haeme detoxification and biosynthesis of nucleic acid in the parasite. Amodiaquine and 4-aminoquinolone act by inhibiting haeme polymerase activity, thereby preventing detoxification of haeme. The free haeme accumulated is toxic to the parasite and makes it a better alternative in chloroquine-resistant strains. Quinine and mefloquine also inhibit haeme detoxification inside the food vacuole of the parasite. Lumefantrine, an aryl amino-alcohol group, is highly lipophilic and has a similar mechanism of action as quinolones, but it is prescribed only as a fixed combination since it is not recommended for monotherapy.

Primaquine acts by producing free radical-induced damage to the parasite by inducing production of intracellular toxic oxidative changes. Tafenoquine has a long plasma t½ of 16–19 days compared to 6–8 h of primaquine and thus reduces treatment to 3 days compared to 14 days with primaquine. Doxycycline is a broad-spectrum antibiotic that acts against the malarial parasite by disturbing the normal functions of malarial apicoplasts. Atovaquone inhibits the parasite cytochrome electron transport system, and proguanil inhibits dihydrofolate reductase, and thus both act synergistically by inhibiting folic acid synthesis in the malarial parasite.

Artemisinin (*qinghaosu*) is the active component of Chinese herbal medicine known for its antipyretic effect for over 2000 years. The drug is a sesquiterpene lactone endoperoxide, the exact mechanism of action of which is not known. Nevertheless, it is suggested that the iron-catalysed cleavage of the artemisinin endoperoxide bridge in the parasite food vacuole leads to formation of free radicals. These free radicals induce damage and lysis of the parasite or act by inhibiting plasmodial sarcoplasmic-endoplasmic

Table 1 Antimalarial drugs

Drug	Dosage schedule	Adverse side effects
Chloroquine	1 g (600 mg base) PO, then 500 mg (300 mg base) 6 h later, then 500 mg (300 mg base) at 24 and 48 h	Retinopathy, methemoglobinemia, pruritus, muscle weakness
Quinine	650 mg PO q8h × 3 or 7 days	Cinchonism
Mefloquine	750 mg PO followed 12 h later by 500 mg	Seizure, QT prolongation, neuropsychiatric symptoms
Primaquine	30 mg base/d PO × 14 days	Haemolytic anaemia
Sulfadoxine-pyrimethamine	500 mg/25 mg tab as single dose	Megaloblastic anaemia, Stevens-Johnson syndrome, toxic epidermal necrolysis
Atovaquone-proguanil	1g/400 g (adult Tablets Strength) PO once/day for 3 days	Gastrointestinal symptoms, headache
Doxycycline	100 mg PO bid × days	Gastrointestinal symptoms, photosensitivity, tooth discolouration in children
Artemether-lumefantrine	20 mg/120 mg of six doses over 3 days (4 tabs/dose at 0, 8, 24, 36, 48 and 60 h)	Haemolytic anaemia, bradycardia
Artesunate	2.4 mg/kg/dose IV for 3 days at 0, 12, 24, 48 and 72 h	Haemolytic anaemia, bradycardia

calcium ATPase labelled 'Pf ATP6' in the parasite. The newer drug pyronaridine, a Mannich base acridine, has also been studied as an antimalarial for many years. It has a similar mechanism of action to chloroquine and is now available in combination with artesunate.

Quinine is very effective in complicated falciparum malaria. Quinine in combination with clindamycin and atovaquone with azithromycin are used effectively for treatment of *Babesia microti* infection. The antimalarial drugs dosage and common adverse effects are summarized in Table 1.

Chemotherapy of Helminthic Infections

The therapeutic goals of anti-helminthic drugs include elimination of parasites, prevention of transmission and control of infections. The anthelminthic agents act against parasites by interfering with their neuromuscular functions, microtubular structure, calcium permeability or energy metabolism, thereby causing death of the parasite. Poor efficacy of therapy against certain parasites and frequent re-infection in endemic areas which require mass treatment campaigns are a few of the challenges faced during chemotherapy of helminthic infections.

Chemotherapy of Cestodes and Trematodes

Praziquantel acts by increasing the influx of calcium from endogenous stores of both cestodes and trematodes, leading to an intense muscular contraction of the parasite followed by its expulsion. Niclosamide acts by blocking ATP synthesis leading to death and expulsion of the parasite from the body.

Metrifonate is an organophosphorus compound that acts by inactivating acetylcholinesterases of the parasite. This leads to depolarizing neuromuscular blockade followed by expulsion of the parasite. Oxamniquine acts by intercalation of the parasite DNA, leading to blockade of nucleic acid and protein synthesis causing death of the parasite. Triclabendazole is a benzimidazole that acts by inhibiting parasite microtubule formation and protein synthesis. Bithionol blocks ATP synthesis and inhibits parasite energy derived from anaerobic energy metabolism leading to death.

Chemotherapy of Nematodes

The broad-spectrum benzimidazole group of drugs such as thiabendazole, mebendazole, albendazole and triclabendazole have lethal effects on the cytoskeletal structure of the

parasite. The cytoskeletal structure of nematodes includes microfilaments, microtubules and beta-tubulins. They act by inhibiting microtubule synthesis. Benzimidazole binds to beta-tubulins and prevents their assembly leading to inhibition of microtubule formation, followed by inhibition of glucose uptake leading to depletion of parasite glucose stores resulting in reduced ATP formation and death.

Piperazine activates the GABA-gated chloride channel in the nematode leading to flaccid paralysis and also produces a depressed acetylcholine response followed by expulsion of live parasites. Pyrantel pamoate inhibits the parasite's acetylcholine esterase and acts as an agonist at the cholinergic receptor, which leads to depolarizing neuromuscular blockade, thereby causing parasite paralysis. This leads to attachment failure within host intestinal lumen followed by expulsion from the host. Diethylcarbamazine acts by altering the membrane surface characteristics of microfilariae, thereby exposing them to phagocytosis, thus reducing the number of circulating parasites in the blood circulation. Ivermectin is a nematode-specific glutamate-gated agonist activating chloride channels in the parasite pharyngeal muscles leading to hyperpolarization and paralysis.

Chemotherapy of Ectoparasites

Permethrin, ivermectin, hexachlorocyclohexane, crotamiton, sulphur, malathion and benzyl alcohol are used for treatment of infections caused by ectoparasites such as lice and scabies.

Permethrin is toxic to *Pediculus humanus*, *Pthirus pubis* and *Sarcoptes scabiei*. Pyrethroids act on the neuromuscular system causing neurological paralysis by altering sodium and potassium channels on nerve membrane. Ivermectin is approved for head lice treatment as lotion and applied to the hair and scalp, but has limited use. Lindane is a gamma isomer of hexachlorocyclohexane, effective as a shampoo against *Pediculosis capitis* or *Pediculosis pubis*. It acts by affecting the nervous system by penetrating the chitinous layer, thereby killing lice and mites. Combining lindane with benzyl benzoate prevents development of resistance and improves cure rate.

Crotamiton (10%) cream or lotion is a scabicide and pediculicide with antipruritic properties. Because of lower efficacy and repeated application, it is the second choice as a scabicide and pediculicide. Malathion and dicophane are insecticides, poorly absorbed through the skin but able to penetrate the exoskeleton and act as an arthropod neurotoxin, but are rarely used. Sulphur, which is non-irritating to the skin, is the oldest scabicide used. On coming in contact with the skin, sulphur is reduced to hydrogen sulphide and gets oxidized to sulphur dioxide and pentathionic acid, which is lethal to arthropods. However, the compound has an unpleasant odour with staining; thus patient compliance is poor.

Drug Resistance

The emergence of drug resistance in parasites to the available drugs is a major challenge. Chloroquine-resistant *P. falciparum*, metronidazole-resistant *Giardia*, sulfonamide-resistant *Toxoplasma gondii* and diloxanide-resistant *E. histolytica* are a few examples of emerging drug resistance in parasites of public health importance.

Several molecular mechanisms are suggested to play an important role in the development of drug resistance among parasites (Table 2). The efflux process through efflux transporters such as P glycoprotein is one major mechanism suggested for development of resistance in parasites. Evidence supports that this kind of resistance can be partially reversed with verapamil. Other mechanisms include alteration in the affinity of binding or the structure of the target receptor (levamisole target acetylcholine nicotinic receptor). Emergence of drug resistance in parasites can be reduced or prevented using a combination of drugs with different mechanisms of action such

Table 2 Mechanism of action and mechanisms of resistance of commonly used antiparasitic agents

Drug	Mechanism of action	Mechanism of resistance
Chloroquine	Haemozoin formation from the haeme is inhibited, and this free haeme leads to parasite death by lyses of its membranes	Due to altered transport properties, there will be a decreased accumulation of the drugs inside the parasite
Artemisinins	Mechanism is unknown. Ideas are controversial, and they are proposed as (1) artemisinin-derived free radicals induce damage and lysis of the parasite or (2) act by inhibiting plasmodial sarcoplasmic-endoplasmic calcium ATPase labelled as 'Pf ATP6'	Mechanism is unknown
Metronidazole	Acts against the parasite by producing reactive toxic intermediates within the parasite. It metabolizes into acid metabolites and hydroxymetabolites of which it later acts on the parasitic deoxyribonucleic acid (DNA) and causes DNA disruption, leading to inhibition of protein synthesis	Decreases level of enzymes necessary for the activation of nitro group
Miltefosine	Prevents synthesis of parasite cell surface molecules or by interfering lipid metabolism of the parasite resulting in disruption of parasite cell signal transduction	Increased drug efflux
Albendazole	Binds to beta-tubulins and prevents their polymerization, followed by inhibition of glucose uptake leading to depletion of parasite glucose stores resulting in reduced ATP formation and death	Alteration in the high-affinity binding to β-tubulin of the parasites
Praziquantel	Increases the influx of calcium from endogenous stores of both cestodes and nematodes, leading to intense muscular paralysis of the parasites	Increased drug efflux

Table 3 Chemotherapy of parasites

• High-dose albendazole used longer than 3 months (as for hydatid disease) may cause hepatotoxicity
• Ivermectin should be avoided in children below 5 years old and has recently been approved for topical treatment of inflammatory lesions of rosacea
• Fastest-acting drugs against malaria are artemisinins
• Miltefosine can be administered orally for kala-azar
• Albendazole is the drug of choice for all nematode infestations including cutaneous larva migrans, visceral larva migrans and neurocysticercosis **except** *Enterobius* (mebendazole), *Wuchereria bancrofti* and *Brugia malayi* (DEC), *Onchocerca* and *Strongyloides* (ivermectin) and *Dracunculus* (metronidazole)
• The drug of choice for all trematode and cestode infections is praziquantel except *Fasciola hepatica* (triclabendazole) and hydatid disease (albendazole)

as artemisinin-based combination therapies (ACTs) in malaria and also by preventing the misuse of drugs.

Antiparasitic drugs (Table 3) are highly insoluble, and hence to increase their clinical effectiveness, they are given in large doses. To overcome this, scientists have developed a new way to deliver these drugs more efficiently by using nanotechnology. They have developed a novel nano-capsule formulation of triclabendazole (drug used for fascioliasis) to enhance its efficacy and reduce its toxic effects. Abametapir-A is an example of such a new drug being recently recommended for the treatment of *Pediculosis capitis* infections.

Case Study

A 19-year-old boy was admitted to casualty with severe abdominal pain, fever and bloody diarrhoea. On examination vitals were stable, and the patient was mildly dehydrated. The stool sample was sent for examination and it was positive for *E. histolytica*. The patient was admitted for 1 day and discharged with advice to take metronidazole 750 mg PO tid for 7 days. On the fourth day of treatment, the patient returned with dizziness, throbbing headache, chest and abdominal discomfort but no diarrhoea. History of alcohol consumption was noted.

1. Rationalize the cause for the symptoms presented on the fourth day of treatment.
2. Suggest a suitable plan of management for the above case.
3. What are the alternative drugs which can be used in this condition?

Research Questions

1. How to improve the discovery of antiparasitic drugs which are limited in number and sometimes ineffective because of resistance?
2. How is the incomplete knowledge of the mechanism of action of many antiparasitic agents leading to poor understanding of their toxicity and resistance pattern?
3. How is the lack of availability of effective vaccines playing a challenging role in controlling parasitic infections?

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Prevention and Control of Parasitic Zoonoses

Sanjoy Kumar Sadhukhan

Learning Objectives

1. To understand the difference between prevention and control as applicable in epidemiology.
2. To describe salient preventive and control measures which are commonly used for parasitic infections.

Introduction

Prevention of parasitic diseases deals with their interception. Control measures are used to check the possibilities of dissemination of the infection. By controlling the infection, the aim is to minimize and sustain low level of parasitic infections prevalent in the general population. The control methods intend to eliminate the disease at the level of its reservoir and source.

The basic measures for prevention and control of parasitic zoonotic infections are similar to those of any infectious disease. These include reduction/elimination of source/reservoir for parasites, breaking/interfering the chain of transmission and reduction/elimination of the susceptibility of host(s) at risk of infection. The prevention and control of parasitic infections is a challenging task as the containment of these infections essentially requires a change in human behaviour, political/administrative support and implementation of proper control measures for parasitic diseases. The matter is complicated further by the fact that a number of *zoonoses* such as taeniasis, hydatid disease, toxocariasis, etc. affect rural populations residing closer to domestic animals. In addition, *Cryptosporidium*, *Toxoplasma* and other parasites cause opportunistic infections in immunocompromised hosts with HIV/AIDS or following immunosuppressive therapy. The current concept of the 'One Health' approach involving all lives on earth with inter-sectoral coordination and international cooperation is therefore essential for prevention and control of parasitic zoonoses.

Preventive and Control Measures

The prevention and control measures for parasitic diseases can be broadly categorized under the following headings (overlapping exists between measures).

Prevention and Control of Zoonotic Infections in Humans

Protection of the susceptible host, controlling the reservoir and interrupting the transmission of the

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parasitic infections are important components of the prevention and control of parasitic infections among humans.

Protection of the susceptible host: This can be achieved by immunoprophylaxis, chemoprophylaxis or personal prophylaxis.

1. **Immunoprophylaxis**: It is carried out by active or passive immunization. Active immunization by the vaccines being developed and evaluated in parasitic infections aims:
   (a) To interrupt the chain of transmission at any specific stage in the life cycle of the parasite.
   (b) To minimize the morbidity and mortality due to disease by producing a vaccine.
   There are multiple reasons for the non-availability of vaccines in parasitic infections. These include (1) the complex nature of parasitic antigens, which makes them difficult to characterize, (2) the difficulty in the identification of the protective antigen for use in vaccines with available techniques and (3) intricate mechanisms of most parasites to evade the host immune system.
   Despite these challenges, significant progress has been made in the field of development of vaccines for malaria and amoebiasis. RTS,S/AS01 (RTS,S) is the first and, to date, the only vaccine which has demonstrated significant reduction of falciparum malaria and life-threatening severe malaria in young African children. Moreover, three nations—Ghana, Kenya and Malawi—introduced the vaccine in selected areas of moderate and high malaria transmission in 2019.
2. **Chemoprophylaxis:** Chemoprophylaxis, carried out either at the individual level or at the community level, has been used successfully for many parasitic infections. For example, reduction of the reservoir of infection for paragonimiasis is achieved by means of mass treatment of the population with praziquantel or bithionol. Annual mass drug administration of diethylcarbamazine has achieved significant chemotherapeutic control of *Wuchereria bancrofti* infection in a community. Chemoprophylaxis has also been recommended in malaria. It is recommended for travellers from non-endemic areas and as a short-term measure for soldiers, police and labour forces serving in highly endemic areas (Table 1).
3. **Personal prophylaxis**: Human behaviour is crucial for prevention and control of parasitic zoonoses. Avoidance of raw or undercooked food and food preparations prevents transmission of parasitic diseases. For example, adequate cooking of fish kills the infective plerocercoid larva of *Diphyllobothrium latum*, thereby preventing transmission of diphyllobothriasis to humans. Thorough cooking of meat kills the cysticerci in the infected beef or pork, a useful strategy to prevent *Taenia saginata* and *Taenia solium* infections, respectively. Paragonimiasis is prevented by avoiding eating raw or partially cooked crab or crayfish. Avoiding eating raw or undercooked pork and regular inspection of meat prevent transmission of trichinellosis to humans.
   Health education to discourage ingestion of fresh and raw aquatic plants prevents the risk of transmission of *Fasciolopsis buski* infection to humans. Health education, especially on behaviour change, is necessary for implementation of the preventive and control strategies for low- and middle-income countries where resource crunch is a real issue. Health education with improved nutrition supplemented with dietary iron prevents anaemia due to hookworms. Treatment of persons suffering from ascariasis and deworming of school children for intestinal helminthic infections not only improves personal health but also prevents pollution of soil by eggs and larvae of soil-transmitted helminths. Avoidance of the practice of open-air defecation especially near water reservoirs and washing hands after playing with and feeding dogs are the best practices that prevent many parasitic infections, including intestinal protozoal and helminthic infections and other nematode infections.

Table 1 Chemoprophylaxis of malaria

Drug	Dosage
Chloroquine	300 mg (base) = 3 tablets of 100 mg or 2 tablets of 150 mg once a week, on the same day each week or 100 mg (base) = 1 tablet of 100 mg daily for 6 days per week
Proguanil	200 mg = 2 tablets once a day (in combination with chloroquine)
Mefloquine	250 mg = 1 tablet once a week, on the same day each week
Doxycycline	100 mg = l capsule once a day

Since many parasitic infections are area, country or continent specific, following 'travellers' guides' while visiting any such place would prevent such infections.

Controlling Infection in the Reservoir Hosts

1. **Early diagnosis:** Early diagnosis followed by early treatment reduces mortality and morbidity due to diseases to a great extent. Hence adequate laboratory support is crucial. Laboratories should be well equipped to perform not only simple microscopy, serological and other simple tests like card agglutination tests but also newer advanced diagnostic tests. However, the facilities to perform tests such as Western blot (WB), enzyme immunoassay (EIA), luciferase immunoprecipitation system, polymerase chain reaction etc., although important in select specific infections, they are not widely available in many laboratories of most low- and middle-income countries.
2. **Surveillance:** The primary measure for controlling parasitic zoonoses in humans is proper surveillance, both passive (usual) and active. *Passive surveillance* includes proper analysis of routinely available data regarding parasitic zoonoses from the health system. Persons having symptoms suggestive of zoonoses, high-risk professions (e.g. animal breeders, shepherds, butchers, restaurant workers, etc.), travellers coming from or having a history of travel in endemic regions and persons having immunosuppressive disease or taking immunosuppressive drugs need special attention and care. Inclusion of important zoonoses in national disease surveillance programmes (e.g. Integrated Disease Surveillance Project (IDSP) in India) based on the analysis of data-based indicators of the infection is useful. Any early warning signal (e.g. clustering of cases over time, place and person) would immediately alert the system to impending outbreak of such zoonoses.
   Active surveillance, e.g. arranging a special survey with or without using a field-level diagnostic kit or establishing sentinel surveillance for detection of such zoonoses, is also essential.
3. **Treatment:** Treatment of parasitic zoonoses in humans depends on the type of infection (s). Chemotherapy against many of the parasitic infections is effective. Treatment by combined therapy, with more than one drugs, is mostly effective. It is usually effective against the 'active' form of parasites, such as trophozoites and not cysts e.g. *Entamoeba histolytica* and other protozoal infections. Barring a few, most of drugs currently available are relatively economical, and occurrence of drug-resistant parasites is relatively lower unlike that of bacterial infections. Treatment or prophylaxis for zoonoses in immunocompromised persons (e.g. cryptosporidiosis, toxoplasmosis, etc. in patients with HIV/AIDS) by specific chemotherapy along with concomitant antiretroviral therapy is important. Surgical removal of large-sized hydatid cysts with or without pre- and post-intervention medicines is an example of surgical intervention in certain parasitic diseases.

Interruption of Transmission Cycle

Interruption of the transmission cycle to prevent transmission of parasitic infections includes the following measures.

Sanitation Measures Including Good Personal Hygiene

Sanitation measures include consumption of adequately cooked food, especially pork, beef and fish, and drinking of water that has been boiled, filtered or chlorinated. However, the concentration of chlorine used for water disinfection is not sufficient to kill certain parasites such as the cysts of *Entamoeba*, *Giardia*, etc. Iodination with tetracycline hydroperiodide or filtration using 0.22 μm filtration membrane is recommended for such cases. Regulation of slaughter houses with proper meat surveillance and hygienic cattle and pig rearing is also important. Thorough handwashing with soap and water is essential, especially for food handlers. It should also be practised in other situations like after using the toilet; after changing a child's diapers or cleaning a child who has used the toilet; before, during and after preparing food; before eating food; before and after caring for a sick person; before and after treating a cut or wound; after touching an animal or animal waste; after outdoor activities, etc. Sanitary disposal of faeces along with preventing open defecation, use of sanitary latrines, adequate sewage treatment, etc. are of paramount importance. Improved personal hygiene with the objective of keeping oneself clean (frequent bathing, wearing clean clothes, trimming nails, using clean bedding and checking for parasites, etc.) is equally important to prevent parasitic zoonoses.

Pet and Stray Animals Management

An effective registration system, drastic reduction (elimination), periodic surveillance with stool examination and deworming of infected dogs (by single dose of praziquantel at a dosage of 5 mg/kg of body weight) are some of the important steps to reduce the intensity of parasitic infection in pet animals such as dogs or rats. Avoiding contact with cat faeces containing oocyst, especially for high-risk individuals like immunodeficient patients and pregnant women, is an important measure for prevention of acquired and congenital toxoplasmosis. Contraceptive vaccination is an effective measure to control stray animals such as dog populations.

Reducing Vector Population and Vector Bites

Reducing vector populations and bites essentially depends on environmental measures, chemical measures and personal protective measures.

Environmental measures include general cleanliness, closing rodent burrows, locating cattle sheds and poultry away from homes, avoiding water collections and repairing cracks and crevices in walls, etc., preventing replication of insect vectors. Source reduction measures such as flooding and flushing of breeding places and biological measures by using *Gambusia* fish, etc. are various methods that are being used to control mosquito vectors of malaria, filariasis, etc. (Table 2).

Chemical measures include spraying the breeding site with oil and insecticides such as DDT, pyrethrum and temephos to kill mosquitoes and other vectors. Similarly, the use of suitable insecticides in homes and outhouses is useful to control sandfly vectors of leishmaniasis, mosquito vectors of malaria, filariasis, etc. Use of traps and bait impregnated with insecticides is useful in controlling tsetse fly populations transmitting sleeping sickness. Avoiding exposure to ticks by use of tick repellents helps in preventing transmission of babesiosis.

Personal protective measures to prevent being bitten by vectors are varied and many. These include the use of screens on doors and windows, insecticide-impregnated mosquito nets, insect repellents like DEET (diethyltoluamide), use of long pants and long-sleeved shirts which may be insecticide impregnated, and use of protective shoes while going to the forest. Avoidance of sleeping in open areas and on floors of mud houses prevents bites from insect vectors.

In effect, the interruption of the transmission cycle can be achieved in multiple ways. For example, Fig. 1 describes the integrated approach which is needed to control schistosomiasis, while

Table 2 Environmental management as per World Health Organization (WHO) guidelines

Environmental modification	Long-lasting or permanent transformation of land, water and vegetation to prevent, reduce or eliminate vector or intermediate host breeding habitats (water-related, vector-borne diseases) or environmental conditions which favour waterborne and water-washed disease transmission	Grading, filling, drainage, land levelling, housing, urban drainage
Environmental manipulation	Changes of environmental conditions to create temporary unfavourable breeding conditions for vector breeding or transmission	Water-level fluctuations, water velocity changes, flushings, weed clearing, salinity changes
Modification or manipulation of human habitation or behaviour	Any environmental manipulation of modification measures to reduce man–vector and/or man–pathogen contacts	Bed nets, personal protection, house screening, safe bathing and laundry places, latrines, wastewater treatment, water supply

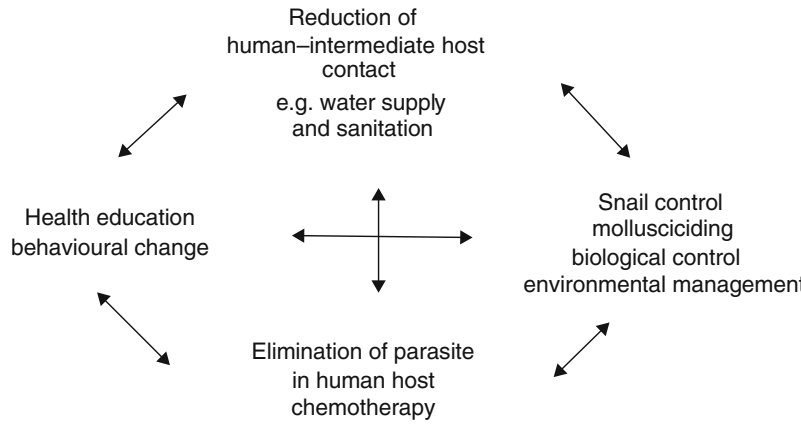

Fig. 1 Integrated approach to control schistosomiasis (Source: http://www.fao.org)

Fig. 2 shows the multiple ways by which malaria transmission can be reduced in the community.

Prevention and Control Among Animals

The basic principles for controlling parasitic infections in animals are essentially similar to those of human infections. Veterinary Public Health (VPH) action, defined as that part of public health action which is committed to the protection and improvement of human health through application of the capabilities, knowledge and professional resources of veterinary science, plays a key role.

The VPH actions are based on surveillance, animal control measures, control of livestock, supervision of the meat industry, slaughterhouses and the market, and control of vectors and vehicles.

Surveillance

Constant surveillance is an essential component of the control system and requires accurate, complete, timely and reliable information on specific diseases on a regular basis. Notification of important parasitic zoonoses is also a basic step in the overall surveillance system. Screening and testing of animals and humans is essential to assess the prevalence of zoonotic parasites in the community. Improved access to diagnostic tools and tests for detection of such parasites in humans, animals and the environment is an effective measure.

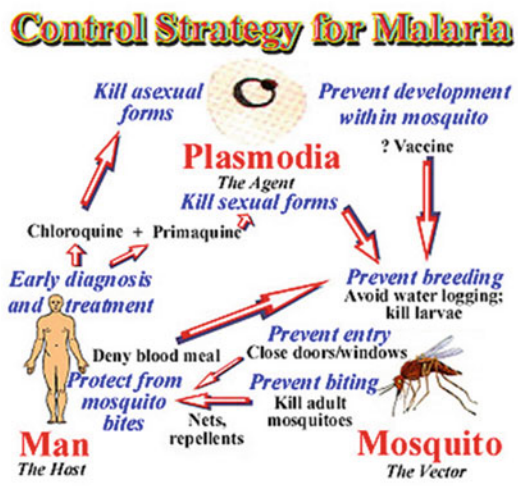

Fig. 2 Multiple control measures which are adopted for malaria (Source: https://www.malariasite.com/control-of-malaria/)

Slaughterhouse surveys are important in controlling echinococcosis, taeniasis, cysticercosis, trichinellosis and other zoonoses. Isolation and typing of the zoonotic agents are helpful in leishmaniasis, trypanosomiasis, etc. Epidemiological studies provide important knowledge of zoonoses and help to specify the suitable method (s) of control. They help to identify incidence of such zoonosis in humans and animals and determine the sources of infection associated with arthropods, animals, environment, crops, etc. Statistical data on dog and other animal populations helps in the control of parasitic zoonoses, particularly where dogs act as a main vector as in echinococcosis/hydatidosis.

Animal Control Measures

Animal control measures are an important goal of VPH action. The interventions include (1) ensuring proper feeding hygiene such as by discouraging raw meat and offal as feeds for both pet and stray animals; (2) quarantine of suspected animals, when transported for long distances for livestock import–export, pet trade, etc.; (3) testing and segregation/destruction of diseased and infected animals; and (4) immunization of exposed animals with effective vaccines.

Treatment of diseased and infected animals is an effective measure to control zoonotic parasitic infections such as preventing echinococcosis by deworming of animals and destruction of the excreta. Reducing parasitic load by deworming of pets, street animals and farm animals is an effective control measure. Control/elimination of 'stray' animals such as dogs to prevent echinococcosis and leishmaniasis; of cats to prevent toxoplasmosis; and of rodents to prevent leishmaniasis, toxoplasmosis, etc.

Control of Livestock, Meat Industry and Market

The control of livestock, the meat industry and the market is important to control parasitic zoonotic infections that are transmitted by the consumption of inadequate or undercooked meat and meat products. Preventive measures include (1) raising pathogen-free animals, (2) decontamination of feed, (3) regular inspection of slaughterhouses and markets with prohibition of slaughter of diseased animals, (4) ensuring adequate cooking of meat before serving and (5) health education of animal breeders, butchers, restaurant workers, cook, etc. regarding hygienic practices.

Overall, proper scientific reorganization of livestock, the meat industry and the market with suitable legislative and control measures to develop hygienic farming techniques is a key factor to reduce zoonoses in animals.

Vector Control Measures

Vector control measures prevent transmission of zoonotic pathogens from animals to humans. These measures include (1) proper feed hygiene to control toxoplasmosis, trichinellosis, etc.; (2) avoidance of feeding raw meat and offal to dogs to prevent echinococcosis and avoidance of feeding untreated refuge and meat products to cats to prevent toxoplasmosis; (3) arthropod control to reduce sandfly-transmitted leishmaniasis, tsetse fly-mediated trypanosomiasis, etc.; and

(4) biological control methods such as sterile male technique for mosquitoes to reduce mosquito populations.

Impact of Control Measures

Parasitic zoonoses are of diverse origin and also not uniformly distributed all over the world, and hence one single worldwide programme may not be applicable to prevent or control parasitic zoonoses. Specific targeted programmes for parasitic zoonoses in different parts of the world are few in number.

The control programme for cystic echinococcosis (hydatid cyst) in Uruguay in 2005 used dog surveillance by ELISA followed by anthelmintic treatment and dog control by castration and spraying. Human surveillance for cyst was done by ultrasonography. Over a 5-year follow-up, it showed significant reduction of dog positivity from nearly 10% to 2–3% and a corresponding reduction of human cyst from 6.5% to 2%. The trichinellosis prevention programme for Inuit communities in Nunavik, Canada (1992–1997), through meat screening, clinical and blood examination and use of anthelmintic (albendazole) with adequate community participation, was a success.

Programmes for control of congenital toxoplasmosis operate in many European countries with maternal screening for IgM and IgG antibodies. In a similar programme in Londrina, in the state of Parana, Brazil, there was significant reduction of affected pregnant women by 63% and affected children by 42%. Human African trypanosomiasis control programmes running in many African countries, including the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) with direct supervision and assistance from WHO, along with NGO and private sector support, showed a significant decline of 63% of the number of reported cases from 2000 to 2009. A Chagas disease control programme, based primarily on spraying indoor insecticides, in the Montalvania area of Brazil showed success by the total reduction in *Trypanosoma cruzi* infection from a high rate of 83.5%. Cross-sectional comparisons for the age groups 2–6 years and 7–14 years indicated a 100% reduction in *T. cruzi* incidence rates.

Conclusion

Prevention and control of parasitic infections remains a daunting and complex task and there is a need for convergence of multiple disciplines along with adequate administrative support. This has to be supplemented with adequate environmental and ecological changes to bring down the parasite population in the area and also to reduce the risk of transmission (Table 2). The provision of adequate financial resources at local and national level as well as through international funding would accelerate activities and efforts to control and prevent parasitic diseases.

Case Study

Insecticide-treated mosquito nets together with appropriate behaviour-change education is considered an effective preventive measure for visceral leishmaniasis (VL). Thus, during an epidemic of VL in eastern Sudan, 357,000 insecticide-treated mosquito nets were distributed to 155 affected villages. It was reported that an estimated 1060 VL cases were prevented between June 1999 and January 2001, giving a mean protective efficacy of 27%. Thus community distribution of insecticide-treated nets is a good measure for reducing the incidence of VL in a community. The village community should also be educated about the dangers of sleeping outdoors without using mosquito nets.

1. What are the hurdles in implementing prevention or control measures in resource-poor countries?
2. What are the international parasitic control programmes which are ongoing?
3. Name a few parasitic diseases which have been substantially controlled worldwide using public health measures.

Research Questions

1. How do we change the unfavourable human behaviour which is responsible for ongoing transmission of many of the parasitic zoonoses?
2. How do we develop and apply feasible, effective and efficient mechanisms to invoke political/administrative will among a large section of politicians/administrators in low- and middle-income countries?
3. How can we best improve and formulate proper control measures or national/international programmes for many endemic parasitic diseases?

Further Readings

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Part II

Zoonotic Protozoal Infections

Toxoplasmosis

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Learning Objectives

1. To understand the importance of the different modes and vehicles of transmission.
2. To know the importance of serological tests in the diagnosis of different forms of toxoplasmosis and their interpretation in pregnancy.
3. To review the preventive measures which are needed in pregnancy and in immunocompromised hosts.

Introduction

Toxoplasma gondii is an apicomplexan protozoan parasite and is responsible for the cosmopolitan zoonotic infection of toxoplasmosis. The members of the family Felidae like cats are the only known definitive hosts of *T. gondii*. The life cycle of *T. gondii* is completed within a wide variety of hosts, especially in all warm-blooded animals along with its two reproductive phases – sexual and asexual. The sexual reproductive phase occurs only in domestic cats or the wild Felidae family members, while the asexual reproductive phase of the parasite occurs in both intermediate (birds or mammals) and final or definite (domestic cats) hosts. *T. gondii* has three major genotypes – type I, type II and type III. All of these genotypes vary in pathogenicity in the hosts and their prevalence. It is generally asymptomatic in immunocompetent individuals, or it may manifest as flu-like symptoms and other non-specific clinical signs. Humans acquire *T. gondii* through ingestion of undercooked meat, drinking contaminated water, transplantation of a contaminated organ and contact with feline faeces. In humans, *T. gondii* is frequently associated with congenital infection and abortion. Infections of *T. gondii* are usually minor and self-limiting but severe in case of immunocompromised patients, including HIV-infected individuals, in whom it can cause encephalitis. The control of toxoplasmosis is dependent on accurate diagnosis which determines the therapeutic options. However, available options for toxoplasmosis chemotherapy are limited.

History

The word "toxoplasma" comprises two words, i.e. "toxon" and "plasmid", both originating from the Greek; the former word means "bow"

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and the latter means "form". Therefore, the original Greek meaning of the word "Toxoplasma" is a bow-shaped organism. *T. gondii* is a member of the Apicomplexa, which is an assorted group of several parasitic protozoans such as *Babesia*, *Cyclospora*, *Cryptosporidium*, *Isopora* and *Plasmodium*. The organism was first identified in Tunis in 1908, isolated from a common gundi (*Ctenodactylus gundi*). Thereafter, Splendore discovered the same parasite in Brazil, which was isolated from a rabbit. After thorough microscopic study of several tissues and experimental studies in 1909, Nicolle and Manceaux recommended the present term *T. gondii* after considerable microscopic analysis of several tissues and experimental studies.

Six clades of *T. gondii* have been featured by seeking knowledge of population genetic structure studies which indicates the origin of diverse isolates from rare ancestral lineages. It has been delineated that *T. gondii* appeared first in South American felids and then expanded through migratory birds and mostly through the transatlantic slave trade culture that involved migration of domestic cats, mice and rats. The first observance of human infections was made in the 1920s in a series of cases of congenital diseases characterised by choroidoretinitis, hydrocephalus and encephalitis. After the advent of the HIV pandemic in the 1980s, toxoplasma encephalitis due to reactivation of latent infection came to light.

Taxonomy

The genus *Toxoplasma* belongs to the subfamily, Toxoplasmatinae; the family Sarcocystidae; the order Eucoccidiorida; the subclass Coccidiasina; and the class Conoidasida in the phylum Apicomplexa.

Toxoplasma gondii (Nicolle &Manceaux, 1908) is the only species in the genus *Toxoplasma*.

Genomics and Proteomics

In 2003, the initial output of the *T. gondii* genome sequencing effort was accomplished. The Toxoplasma Genome Consortium undertook 10 X shotgun genome sequencing and annotation of the type II strain ME49 in partnership with the University of Pennsylvania and the Institute for Genomic Research (TIGR) that resulted in a draft version of the 80 Mb genome sequence. The type II ME49 strain was the first to be sequenced, which was followed by the other two strains, GT1 and VEG, as well as chromosomes Ia and Ib of the RH strain. The ME49 strain chromosome map was utilised as a template to build the chromosomes for the GT1 and VEG strains. The genomic sequences of the three strains are between 61 and 64 Mb in size in the most recent release of ToxoDB (version 5). A recent version of the genome annotation for the ME49 strain, as well as a brand new genome annotation for the GT1 and VEG strains, has been released. *T. gondii* has an estimated number of genes of 8102 for the ME49 strain, 8145 for the GT1 strain and 7945 for the VEG strain.

The rapid development and implementation of *T. gondii* proteome analysis has been aided by the sequencing and annotation of the parasite's genome. The methods of host cell invasion, the structure and composition of apical organelles, the organisation of the cytoskeleton and the "entire" proteome of tachyzoites have all been studied. The tachyzoite has been the subject of proteome research since it is *Toxoplasma*'s active, infectious stage. No significant data has been reported to date on the other life cycle stages. Most proteome investigations have employed type I strain RH tachyzoites because they have essentially little bradyzoite differentiation in vitro under typical growth conditions. The first large-scale proteomic study of *T. gondii* tachyzoites revealed over 1000 *Toxoplasma* proteins. Advances in mass spectrometry have enabled the use of high- and medium-throughput proteomics approaches to study various aspects of protein functions. These include analysis of subproteomes, analysis of post-translational modifications and identification of macromolecular complexes.

The Parasite Morphology

T. gondii exists in three forms: the trophozoite/tachyzoite, the bradyzoites and the sporozoites. All these three forms are necessary for infections. These stages undergo sexual (*gametogony*) or asexual (*schizogony*) reproduction, depending on the host. While the trophozoite and bradyzoite stages are represented by the schizogony, the sporozoite stage is formed by either gametogony or sporogony. All three forms can occur in domestic cats as well as in other felines which are the definitive hosts of these parasitic forms and provide sustenance to both the schizogony and gametogony, while on the other hand, out of the three forms, two forms, i.e. trophozoites and bradyzoites, also exist in other warm-blooded animals including birds and humans, which are the intermediate hosts for them.

Trophozoites/Tachyzoites

The term "Tachyzoite" (*tachos* = speed in Greek), previously called "trophozoite" (*trophicos* = feeding in Greek), was coined by Frenkel. It is the rapidly multiplying form which occurs intracellularly in the intermediate hosts and also extracellularly in the definitive host. Endodyozoites and endozoites were other terms used for tachyzoites. Various aggregated tachyzoites are called groups, clones or terminal colonies.

The tachyzoite is about 2 by 6 μm in size and appears as crescent-shaped, having a rounded and pointed posterior and anterior (conoidal) end (Fig. 1). Ultrastructurally, the tachyzoite comprises a number of cell organelles which include micronemes, mitochondrion, rhoptries, endoplasmic reticulum, Golgi complex and a multiple-membrane-bound plastid-like organelle (a Golgi adjunct or apicoplast) apart from a number of inclusion bodies. The nucleus is central in position with a prominent nucleolus.

Tachyzoites are the dissemination form. They have the ability to invade all cell types of the vertebrate and can divide in a parasitophorous vacuole. Tachyzoites enter the host cells either by phagocytosis or by penetration. Once inside the cell, the tachyzoite becomes ovoid in shape and comes to lie inside a parasitophorous vacuole. Both the multiplication and invasion rates vary and are mostly dependent on the *T. gondii* strain and the type of host cells.

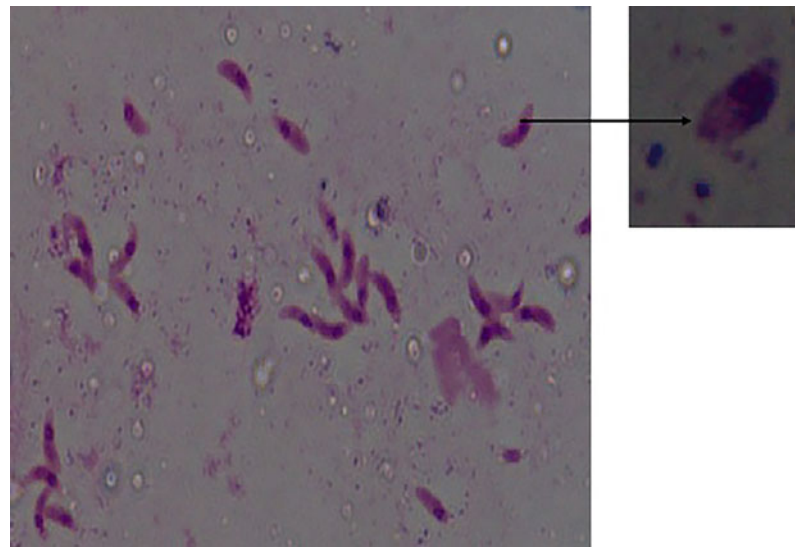

Fig. 1 Tachyzoites of *Toxoplasma gondii*, stained with Giemsa, smear was made from peritoneal fluid obtained from a laboratory-inoculated mouse. (Image courtesy: Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India)

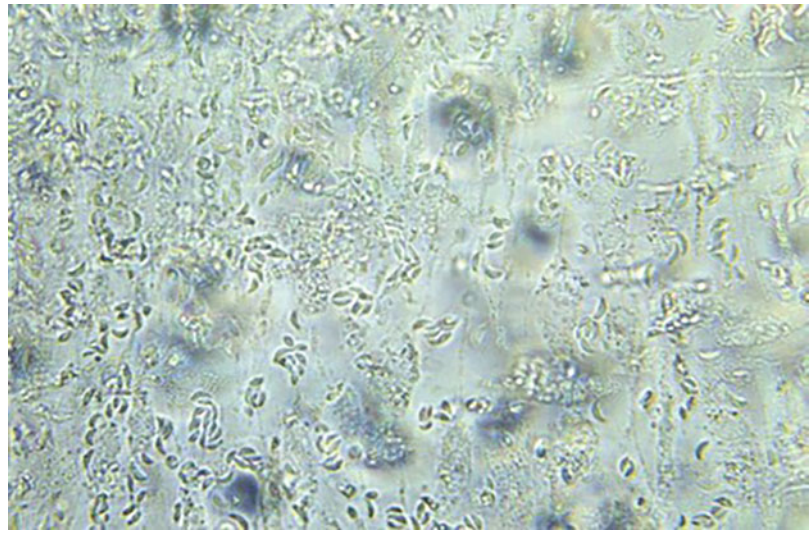

Fig. 2 Photomicrograph of a tissue sample showing a darkly stained, *Toxoplasma gondii* tissue cyst, which contained numbers of spherical-shaped bradyzoites (Courtesy: PHIL; CDC/ Dr Green)

Bradyzoite/Tissue Cyst

The term "bradyzoite" (*brady* = slow in Greek) was also coined by Frenkel to describe the organism that can divide slowly inside the tissue cyst and is also called cystozoite. Tissue cysts remain intracellular and expand, as the bradyzoites inside them multiply by *endodyogeny*. These tissue cysts have a thin (0.5 μm) and elastic wall that can accommodate hundreds of bradyzoites (Fig. 2). There is variation in the size of the tissue cysts, i.e. younger tissue cysts can be small at 5 μm in diameter and can only contain two bradyzoites, whereas the older ones may have hundreds of bradyzoites. Tissue cysts are found to be spheroidal in the brain and rarely as large as 70-μm in diameter, whereas intramuscular cysts are mainly elongated and can be 100-μm in size. Tissue cysts grow in visceral organs, such as the kidneys, lungs and liver, but are mainly found in the muscular and neural tissues, including the cardiac and skeletal muscles, brain and eyes. Intact tissue cysts are mostly harmless and can persist lifelong without inducing any inflammatory response inside the host.

Bradyzoites differ slightly from tachyzoites in structural appearance. The nucleus is located at the posterior end in bradyzoites, while it is centrally positioned in tachyzoites. The bradyzoites usually have electron-dense rhoptries (Fig. 3), whereas tachyzoites have labyrinthine ones. Bradyzoites are more resistant to proteolytic enzymes in comparison to tachyzoites, and this explains why cats have a longer prepatent period if fed with tachyzoites in comparison to ingestion of bradyzoites. After the definitive host ingests the tissue cysts, the cyst wall gets ruptured by the proteolytic enzymes present in the small intestine and stomach. Thereafter, the released bradyzoites invade intestinal epithelial cells and start generating numerous *T. gondii*.

Sporozoites

Sporozoites are found in mature oocysts. Oocysts have ovoid structures and are mainly 12 to 13 μm in size. After sporulation oocysts have two sporocysts, each accommodating four sporozoites. The wall of the oocyst has a multilayered structure which shields the parasite from any chemical and mechanical damages and enables it to persist in a moist environment for a longer period (>1 year). When definitive hosts like cats and other felines become infected by the ingestion of either oocysts or tissue cysts, the parasites begin further development in the host's intestinal epithelial cells, where both the schizogony and gametogony take place. Therefore, the

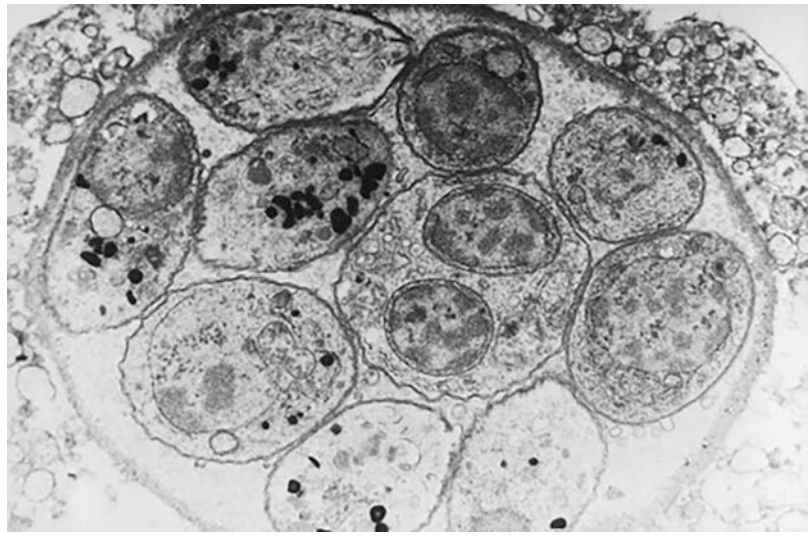

Fig. 3 This transmission electron microscopic (TEM) image reveals some of the ultrastructural details displayed by a *Toxoplasma gondii* tissue cyst, within which bradyzoites could be seen developing (Courtesy: PHIL; CDC)

definitive hosts shed millions of oocysts each day in faeces, but the freshly passed oocysts are not infectious. They become infectious only after development in water or in the soil for a few days according to the availability of temperature and aeration.

Ultrastructurally, the sporozoite is the same as the tachyzoite, but with fewer rhoptries, micronemes and amylopectin granules. They are $2 \times 6–8$ μm in size with a subterminal nucleus.

All the forms of *T. gondii*, i.e. trophozoite, bradyzoite and sporozoite, are crescent-shaped, but ultrastructurally, they vary in the size of inclusion bodies and in certain organelles. Often all of these three forms have similar numbers of rhoptries but the appearance of these is different in each stage.

Cultivation of Parasites

There are various cell lines, such as transformed cell lines (HeLa, CHO, Vero, LM, MDBK, 3 T3, etc.), and culturing techniques which are being employed to maintain tachyzoites in vitro. Tachyzoites are obligate intracellular forms multiplying every 6–9 h depending on the strain. Once the host cell reaches a count of 64–128 parasites, the cells burst with the release of a fresh batch of tachyzoites which infect new healthy cells.

T. gondii strains do not grow uniformly in all of the cell lines. Human foreskin fibroblast cells (ATCC CRL-1634™) are best suited to maintain *T. gondii*. Dulbecco's Modified Eagle Medium (DMEM) and RPMI 1640 medium, with added growth factors like glutamine and foetal bovine serum along with antibiotic supplementation, have also been found to be alternative satisfactory media. Since low CO_2 and high pH can affect the parasite's growth, culture media should be incubated at pH 7.2 in an atmosphere of 5%. All work should be carried out in a biosafety level 2 laboratory as many *T. gondii* strains are extremely virulent and can easily penetrate any human tissue.

Laboratory Animals

Guinea pig was the first animal model established by Markham in the year 1937 for studying toxoplasmosis. Later, in 1951, Hogan produced the first animal model for ocular toxoplasmosis in rabbits using intracarotid injection, and Frenkel managed an intraperitoneal injection in a hamster in 1953, following the same lineage. Thereafter, nonhuman primates, cats, dogs and pigs have

been explored. Toxoplasmosis can be identified in an experimental model by looking for *T. gondii* cysts in biopsies using a particular colour reaction and immunohistochemistry or by the PCR method.

Among various laboratory animals, the most common are mice, rabbits, pigs and nonhuman primates, which are used for testing the efficacy of any drugs against *T. gondii* infection. Rats are partially resistant to infection by *T. gondii*. The type of laboratory animal has a significant impact on the infection's prognosis. Mice are the most regularly utilised animals in studying the efficacy of drugs. However, in the case of congenital toxoplasmosis, rats and sheep are found to be more relevant. Furthermore, the mouse strain, parasite strain (virulence and lethality versus non-lethality), route of infection (oral versus intraperitoneal) and size of parasite inoculum are the main factors that determine intensity of infection. In animal models, coinfection with various microbes has been explored to simulate a similar situation to immunocompromised hosts, which is a prevalent feature in immunocompromised individuals, particularly during AIDS. In an attempt to explain the pathogenicity of *T. gondii* in hosts with virus-induced immunodeficiencies, experimental models of dual infections were created. Mice infected with *T. gondii* and the retrovirus LP-BM5, which causes murine acquired immunodeficiency syndrome (MAIDS) in mice, and cats infected with *T. gondii* and the feline immunodeficiency virus (FIV) are more vulnerable to primary acquired toxoplasmosis; however, reactivation of chronic infection is not always detected. *T. gondii* was found to be related to other opportunistic pathogens in various experimental models of concurrent infections. In immunocompromised rats, infection with *Pneumocystis carinii* and *T. gondii* was obtained, and this model was utilised to test the efficiency of combined prophylaxis against both diseases.

Further, use of genetically immunodeficient animal models clearly illustrates the role of immunity as a major adjunctive factor in the management of acute infection. These models, on the other hand, are more difficult to create and standardise, but they are meant to closely mimic the characteristics of clinical diseases and to help researchers better comprehend the complicated interactions between infections and host defence.

Life Cycle of *Toxoplasma gondii*

Hosts

Definitive Host

Cats and other felines.

Intermediate Host

Humans and other mammals like sheep, goat, pig, cattle and mice.

Infective Stages

1. Oocysts from ingestion of food or water or vegetables contaminated with cat faeces
2. Tissue cysts containing bradyzoites in undercooked meat (goat, sheep, pork, etc.) from herbivores that have ingested cat faeces

Transmission of Infection

The life cycle of *T. gondii* is completed within a wide variety of hosts, especially in all warm-blooded animals along with its two reproductive phases – sexual and asexual (Fig. 4). While the sexual reproductive phase occurs only in domestic cats or the wild Felidae family members, the asexual reproductive phase of the parasite occurs in both intermediate (birds or mammals) and final or definitive (domestic cats) hosts. During different periods of its life cycle, individual parasites convert into various cellular stages, which include the tachyzoites, bradyzoites (found in tissue cysts) and sporozoites (found in oocysts).

Asexual Cycle

When the intermediate host ingests the tissue cyst or oocyst, the parasites first invade the intestinal epithelial cells. Inside these cells, the parasites differentiate into the rapidly dividing tachyzoites. *T. gondii* have two phases of asexual development. During the acute stage, the first phase,

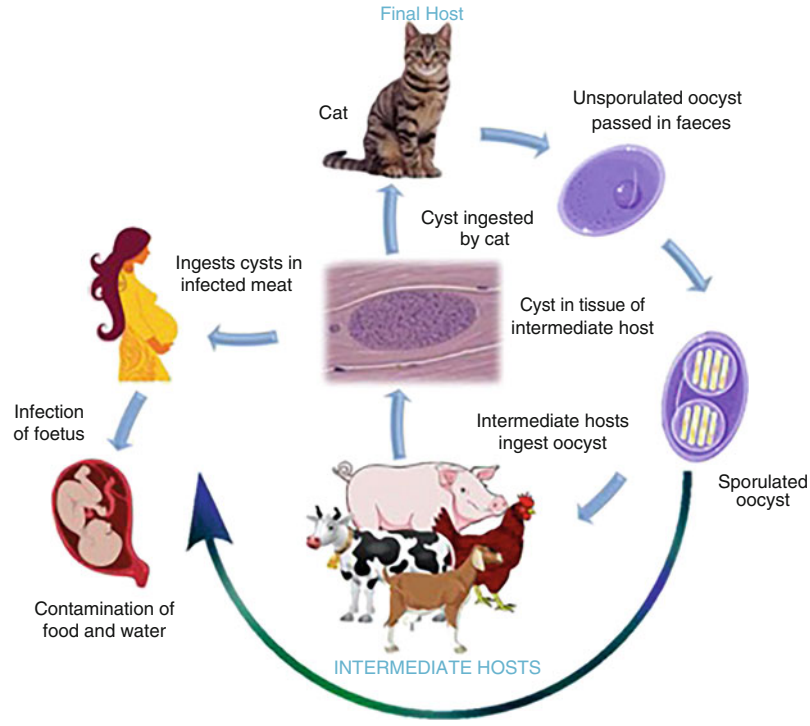

Fig. 4 Life Cycle of *Toxoplasma gondii*

tachyzoites replicate quickly in several discrete varieties of host cells. Inside host cells, the tachyzoites continue to multiply inside the parasitophorous vacuoles formed during entry into the cell. Ultimately the host cell ruptures, releasing the tachyzoites, which can disseminate to any organ of the body including the brain.

During the chronic stages of infection, pressure from the host's immune system causes tachyzoites of the last-generation stage to convert to bradyzoites to form tissue cysts. Tissue cysts in tissues such as brain and muscle tissue form approximately 7–10 days after initial infection. The tissue cysts are principally formed in the brain, eye and the striated muscles and can persist for a long time. Cysts usually range in size between 5 and 50 μm in diameter.

Inside the tissue cyst, gradual multiplication of bradyzoites (or cystozoites) by endodyogeny takes place. They are immediately infectious. Sometimes, in certain intermediate hosts, they may persist lifelong. Tissue cysts undergo lysis with release of the bradyzoites, which transform into tachyzoites that again infect healthy cells and form the tissue cyst.

Sexual Cycle

When a definitive host like a cat consumes a tissue cyst, the bradyzoites convert into merozoites inside intestinal epithelial cells. The merozoites start multiplying asexually by endodyogeny followed by repeated cycles of endopolygeny. During the final stages of this asexual cycle, gamogony and resulting oocyst formation occur. The unsporulated oocysts are then released by the intestinal epithelial cells and pass out with the faeces of the animal.

Pathogenesis and Pathology

In most cases human acquire toxoplasmosis mainly by ingesting tissue cysts which are present in infected meat or oocysts present in food which is contaminated with cat faeces. After ingestion, bradyzoites from tissue cysts or sporozoites from oocysts are released and enter intestinal epithelial cells and start multiplying. The tachyzoites so formed spread to the regional lymph nodes from where they may be carried to various organs through the lymphatics or blood. Necrosis of the

lymph nodes and affected organs is the hallmark of infection. The vulnerable organs include the adrenals, eye and heart. There is no toxin production by *T. gondii*, and necrosis occurs because of intracellular multiplication of tachyzoites.

In AIDS patients, there is reactivation of latent infection leading to opportunistic infection. The encephalitis, a main lesion of toxoplasmosis, is distinguished by necrosis in the tissue of these patients, which mainly leads to multiple abscesses.

Immunology

Both innate and adaptive immune responses play significant roles in toxoplasma infection. This comprises systematic and well-coordinated cellular interactions between the parasite, enterocytes, monocytes, dendritic cells (DC), macrophages, NK cells and neutrophils. The human cellular response to *T. gondii* infection is highly dependent on cell type and the infecting strain of *T. gondii.* In healthy humans and animals, *T. gondii* infection is asymptomatic because the host's innate and adaptive immunity suppresses the parasite's initial multiplication and eliminates the majority of the parasites. When a *T. gondii* tachyzoite infects monocytes, it triggers innate immune responses such as the generation of proinflammatory cytokines, which triggers adaptive immunological responses mediated by T and B cells. Activation of adaptive immunity also induces cell-autonomous immune responses in infected cells, causing *T. gondii* stage change into a bradyzoite (a form that develops slowly but evades host immunological responses) that eventually leads to chronic infection.

T. gondii trigger innate immunity, the initial line of defence for the host, which responds quickly and recognises pathogens via pattern recognition receptors (PRRs), such as TLRs, NOD-like receptors and C-type lectins. Ligand detection by PRRs triggers the production of proinflammatory cytokines such as TNF-, interleukin-1 beta (IL-1), IL-6 and IL-12 and plays a key part in the subsequent cascade of events. *T. gondii* activates innate immunity, and *T. gondii* produces a robust CD4 T cell response resulting in IFNγ production in acute as well as chronic stages of infection. CD8+ T cells may act as effector cells during *T. gondii* infection, while the CD4+ T cells provide the necessary help in maintenance of these cells. Depletion of both CD4+ and CD8+ T cell populations has been shown to result in the reactivation of latent toxoplasmosis and, as a result, infection in animals vulnerable to toxoplasma encephalitis. Additionally, antibodies generated during toxoplasma infection can destroy the parasites. Parasite-specific IgM, IgA, IgE and IgG2 antibodies can be detected in patients with toxoplasmosis and serve as important tools in distinguishing recent from past or chronic infections.

Infection in Humans

Toxoplasmosis symptoms vary based on parasite characteristics such as strain virulence and inoculum size, as well as host immune status and genetic background. The three genotypes of *T. gondii* differ in virulence and epidemiological pattern of occurrence.

T. gondii infects a huge percentage of the world's population but rarely leads to a clinically significant disease. Asymptomatic infection with *T. gondii* is seen most often with development of latent infection and formation of tissue cysts. Sometimes mild symptoms may appear as lymphadenopathy, which is the most remarkable clinical feature. Severe manifestations may occur such as encephalitis, sepsis or myocarditis but these are found to be rare in immunocompetent humans. However, some individuals are at high risk for fatal or life-threatening toxoplasmosis. These individuals include foetuses, newborns and immunologically impaired patients where *T. gondii* can lead to dangerous complications like encephalitis, chorioretinitis, congenital infection and neonatal mortality and postnatally acquired toxoplasmosis in immunocompetent humans.

Ocular toxoplasmosis can be a result of infection acquired either postnatally or during the prenatal period. The symptoms such as retinitis and retinochoroiditis manifest later in life.

Congenital toxoplasmosis is the most deadly form of toxoplasmosis and is caused by *T. gondii* transplacental contamination of the foetus during pregnancy. The severity of the disease is mostly determined by the gestational age at the time of transmission. Infection of the foetus during the first trimester of pregnancy can result in serious damage to the foetus, but later trimesters have less severe foetal disease. Early-stage infections can cause anaemia, chorioretinitis, jaundice, seizure and hydrocephalus in the foetus. Sensorineural deafness, microcephaly, mental retardation, visual deficiency and slow development are late consequences of congenital toxoplasmosis.

In immunocompromised individuals, an earlier acquired latent infection of *T. gondii* gets reactivated, which commonly manifests as encephalitis. Toxoplasma encephalitis and disseminated toxoplasmosis are commonly seen in patients with Hodgkin's disease, those on immunosuppressive therapy or bone marrow or other organ transplant patients. *T. gondii* is an important opportunistic pathogen in AIDS patients causing severe encephalitis and death in over 30% of these patients.

Infection in Animals

Infected domestic cats remain asymptomatic with no clinical disease. Nevertheless, clinical signs may appear with the intensity of infection that include fever, anorexia, ocular inflammation, lethargy, abdominal discomfort, pneumonia and central nervous system distress. Kittens are more vulnerable to clinical infection, and feral domestic cats are at a higher risk of infection as compared to indoor cats.

Domestic dogs can be infected with *T. gondii*, but clinical infection occurs less commonly than subclinical disease. However, clinical signs of the disease involve respiratory, neuromuscular or gastrointestinal systems and sometimes prove fatal. Stray dogs are supposed to be at higher risk, and mostly become infected by eating uncooked infected meat.

Toxoplasmosis is common in sheep, goats, pigs and chickens as intermediate hosts; however, horses and cattle are found to be resistant to the disease. In sheep, congenital infection causes stillbirth and preterm lamb loss. Infected lambs usually survive with normal growth, but because of its consumption, it represents a public health issue. Toxoplasmosis in adult goats is more intense than in sheep, and congenital infection leads to death of kids pre- or post-birth. Pigs may get infected with *T. gondii*, by ingestion of oocysts, congenitally by tachyzoite transplacental transmission and through intake of meat having *T. gondii* bradyzoite tissue cysts. Adult pigs barely show any clinical signs, but the meat of these infected pigs is the major source of human infection. Toxoplasmosis in young pigs proves fatal and they often die without participating in the human food chain. *T. gondii* infection in animals occurs mainly by environmental exposure to the oocysts, and roaming of outdoor domestic cats is found to be a risk factor for infection in various farm animals.

Epidemiology and Public Health

Toxoplasmosis is a significant public health problem worldwide. About one-third of the world population are estimated to be exposed to this parasite. Toxoplasmosis is usually more prevalent in moist, warm and low-altitude regions. This fact is associated with longer viability of *T. gondii* speculated oocysts in warm and humid areas. About 8–22% of the US population are infected, and a similar percentage of infected population is also estimated for the United Kingdom. In Central America, South America and continental Europe, estimates of infection range from 30 to 90%. Europe, in particular, has been found to have a wide range of prevalence, ranging from 10% in Iceland to 63% in Poland.

Toxoplasmosis can be classified as a One Health disease as it significantly affects the health of various creatures (humans, domestic animals, wildlife) and ecosystems and is viewed as a serious threat for all those who depend on animal resources. *T. gondii* infection in food-producing animals has become an important public health issue, as a source for human toxoplasmosis by

transmission of the parasite via pork and wild boar meat and meat products. Infected cats are a major contributor to environmental contamination. The presence of cats in the environment has been linked to greater *T. gondii* seropositivity in pigs (19%) and wild boars (23%), respectively, around the world. These infections have serious consequences that affect mortality and standard of life.

T. gondii have three archetypal clonal lineages. Diverse aberrant genotypes have been seen in the Americas and China numbering 189, and most of these come under genotypes 1 through 5. There is no dominant genotype reported in the Southern Hemisphere; however, a few genotypes are found in the Northern Hemisphere, particularly genotypes 1 (type II clonal), 2 (type III) and 3 (type II variant), which include most isolates, and these are mainly found in Europe. In North America, genotypes 2 to 5 (4 and 5, jointly known as type 12 and prevalent in wildlife) are common. In Africa, genotypes 2 and 3 predominate, whereas genotypes 9 and 10 are very common in China. Several genotypes are linked with intense virulence in humans and wildlife. Clonal lineages 1–4 are most abundant, with highly similar multilocus genotypes, a high degree of linkage disequilibrium and infrequent recombination. Type II strains, which are avirulent in mice, have been identified as the cause of more than 70% of human cases of toxoplasmosis in the United States and Europe as shown primarily in France. Type I, recombinant and atypical strains, has been associated with a higher frequency of ocular toxoplasmosis and severe toxoplasmosis in immunocompetent patients.

Diagnosis

The diagnostic methods mainly employed are serologic tests, molecular methods (PCR, RT-PCR), histological demonstration and bioassay. Other less preferred methods that help in detection are a toxoplasmin skin test, antigenaemia and antigen analysis in body fluids and antigen-specific lymphocyte alteration.

Microscopy

Tachyzoites detection in any histological sections from biopsies specifies an acute infection. Chronic toxoplasmosis can be confirmed through the detection of bradyzoites containing tissue cysts in histological samples. Stains such as haematoxylin and eosin as well as Wright stain are usually used for encysted tachyzoites and bradyzoites demonstration (Figs. 5 and 6). Immunoperoxidase staining has been found to be sensitive and specific, uses antisera to *T. gondii* and has been used successfully to detect *T. gondii* in the brain of AIDS patients.

Animal Inoculation

It is assessed as the gold standard for detection of *T. gondii* infection. Secretions, excretions, body fluids, lymph nodes and muscle and brain tissues are possible specimens, and mice and cats are the common animals which can be used. IFN-γ knockout mice are preferred, due to high sensitivity, or normal mice may be immunosuppressed by administrating dexamethasone. The tachyzoites are found in peritoneal cavity of mice after 6–10 days of inoculation.

Serodiagnosis

Serologic tests serve as the primary choice for diagnosis. Chronic infection is diagnosed by the serological detection of antibodies produced against parasite-specific antigens. For this, IgG and IgM ELISA assays in combination format are mostly used.

IgM antibodies are mainly traceable around 1 week post-infection and remain for several months or years. This makes IgM antibodies detection insufficient while confirming acute infection. Moreover, IgA antibodies are produced before IgM and remain for only several months, so they are considered to be a satisfactory and initial marker of acute infection. Also, IgG antibodies provide information about the

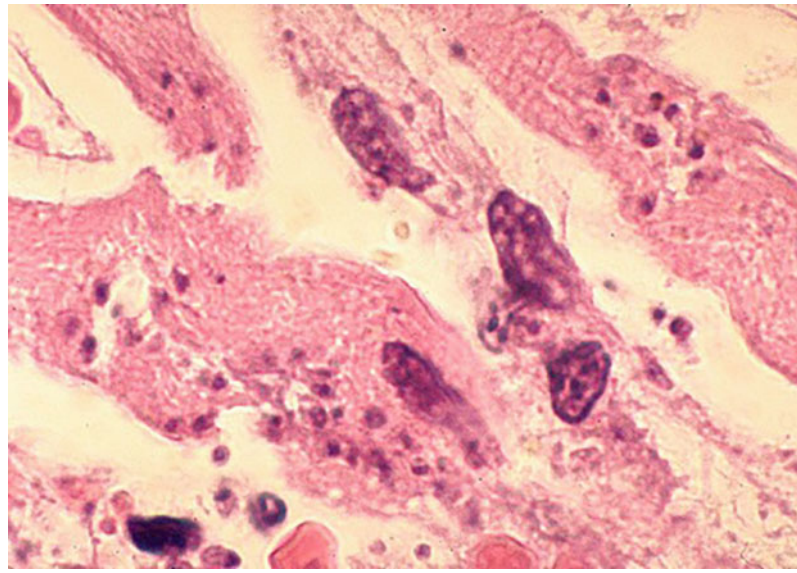

Fig. 5 This photomicrograph reveals some of the histopathology found in this cardiac tissue sample in a case of cardiac toxoplasmosis. The biopsy specimen was harvested from a patient with a fatal case of AIDS. Within the myocytes numerous *Toxoplasma gondii* tachyzoite can be seen. (Courtesy PHIL, CDC/ Dr. Edwin P. Ewing, Jr.)

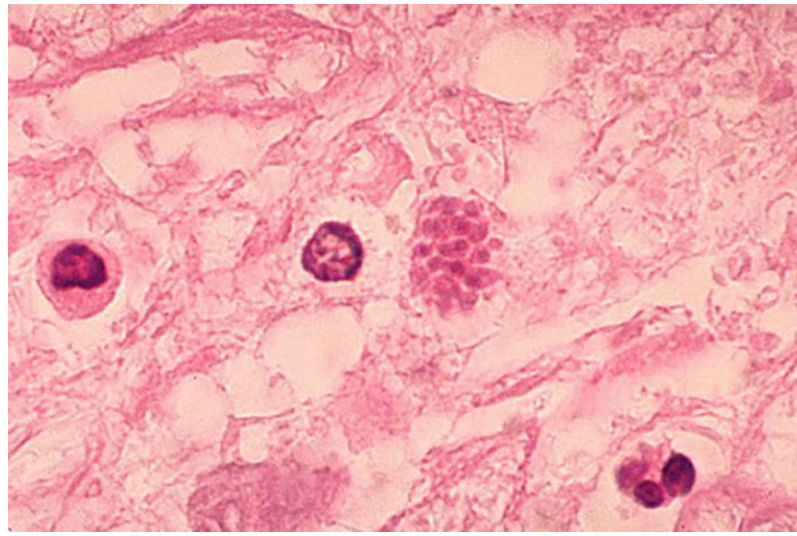

Fig. 6 This photomicrograph reveals some of the histopathology found in this brain tissue sample in a case of neuro-toxoplasmosis. The biopsy specimen was harvested from a patient with a fatal case of AIDS. A pseudocyst, containing numerous, *Toxoplasma gondii* tachyzoites is visible (Courtesy: PHIL, CDC/ Dr. Edwin P. Ewing, Jr.)

occurrence of infection, without revealing the timing of infection.

Although IgM indicates an acute infection, it may persist for a prolonged period following infection. Therefore, an IgG avidity test is usually done to distinguish between previous and current infections, because IgG affinity increases over time as it is obtained from the antigen-driven B-cell selection process. For confirmatory diagnosis, samples should be analysed in a reference laboratory where serological panel testing can be done comprising avidity testing; ELISA for IgA, IgM and IgE; the dye test used for IgG antibodies measurement and the differential agglutination test (Table 1). Other serological tests that can be employed are the latex agglutination test, the indirect haemagglutination test and indirect fluorescent antibody tests.

Molecular Diagnosis

T. gondii DNA detection through PCR in body fluids (bronchoalveolar lavage fluid,

Table 1 Serological tests used in toxoplasmosis

SN	Serological test	Principle	Remarks
1.	*Sabin-Feldman dye test (reference test)* IgG detection	Based on the inhibition of staining of live tachyzoites by antibody. Live tachyzoites from mice are incubated with patient's serum, and alkaline methylene blue solution is added. Antibodies will kill the tachyzoites and will not take up the dye and will appear colourless and thin or distorted	If less than 50% of the tachyzoites take up the stain, the test is considered positive Test is potentially hazardous and requires a high degree of technical expertise
2.	*Differential agglutination test:* IgG detection	Formalin-treated antigen (HS) and methanol-treated antigen (AC) used with a single sample. AC antigen is specific for membrane	To rule out recent infection. AC strong antibody response to AC in early infection, wanes after 6–12 months HS/AC$\geq$ 4: Infection has occurred more than 6 months earlier
3.	*Avidity test*: IgG detection	With increasing humoral response, there is increasing avidity of IgG. In early stage of infection, weak avidity antibodies are produced and in late stage those of strong avidity. Two parallel ELISA are run with untreated serum and one with serum treated with urea/guanidine/thiocyanate which dissociates Ag-Ab complex of weak avidity	High avidity index indicates infection in remote past Not always true since increase in avidity may be slow
4.	*IgM/IgA detection*	Uses cytosol antigens enriched with membrane antigen (P30, SAG 1) to enhance sensitivity	A titre of 1:256 in double sandwich IgM ELISA is considered diagnostic for recent acute infection IgA can be detected in serum or in aqueous or vitreous samples in case of ocular infections But IgM antibodies can persist for months to more than 1 year A negative or low titre of IgM does not exclude a positive diagnosis for cerebral toxoplasmosis since antibody production is suppressed in HIV infection
5.	*Western blot test:* IgG	Two samples tested in parallel: blood/CSF; blood/aqueous humour; maternal/neonatal blood	Additional bands in the second sample denote organ/neonatal infection

cerebrospinal fluid (CSF), vitreous and aqueous fluids, blood and brain tissues) has been employed for diagnosis of cerebral, ocular, congenital and disseminated toxoplasmosis. It is also successfully used for early detection of intrauterine *T. gondii* infection. For PCR amplification, B1 gene, 18S rDNA gene, 529-bp repeat element, GRA1, SAG1 and SAG2 are the target genes. Real-time PCR by targeting amplification of the B1 gene is the most recommended diagnosis technique for congenital toxoplasmosis in comparison to nested and conventional PCR. The LAMP assay has also been developed which targets the *T. gondii* oocyst wall protein (OWP) genes, 529-bp repetitive element, SAG1, B1, SAG2, GRA1 and 18S rRNA for testing medical and veterinary samples and water samples (Table 2).

A summary of all the diagnostic tests is provided in Table 3.

Treatment

Patients who are immunocompromised or who are immunocompetent but have severe or prolonged symptoms are frequently treated with pyrimethamine, sulfadiazine and folinic acid. Treatment lasts between 2 and 4 months, depending on the severity of clinical indications and symptoms. Trimethoprim/sulfamethoxazole,

Table 2 DNA target regions for detection of *Toxoplasma gondii* in various molecular methods

Molecular method for detection	DNA target regions
Conventional PCR	B1 gene, 529-bp repeat element, 18S rDNA gene, SAG1, SAG2 and GRA1
RT-PCR	B1 gene, 529-bp repeat element, 18S rDNA gene, SAG1
LAMP	B1, 529-bp repetitive element, SAG1, SAG2, GRA1, oocyst wall protein genes

Table 3 Laboratory diagnosis of toxoplasmosis

Diagnostic method	Target	Remarks
Microscopy of biopsy specimen by using haematoxylin-eosin or immunoperoxidase staining	Tachyzoites or tissue cysts	Invasive procedure
Animal inoculation in cats and mice	Tachyzoites are found in peritoneal cavity	Gold standard but not done routinely
Immunodiagnostics: Sabin-Feldman dye test, ELISA, avidity test, differential agglutination test (DAT)	IgM/IgG/IgA	Standard mode of diagnosis Dye test is the reference serological test Avidity test and DAT can be done to differentiate present from past infections
Molecular diagnosis: PCR, real-time PCR	B1 gene, 18S rDNA gene, 529-bp repeat element, GRA1, SAG1 and SAG2 genes	Highly useful for diagnosis of cerebral, ocular and disseminated toxoplasmosis. It is also used for early detection of intrauterine *Toxoplasma gondii* infection

on the other hand, is the same as pyrimethamine/sulfadiazine. Maintenance therapy is usually started after resolution of the acute phase and mainly consists of the same regimen as in the acute phase but at half dose. This regimen is followed for the rest of the patient's life or until the immunosuppression has resolved.

Prevention and Control

The prevention of toxoplasmosis can be done primarily by imparting health education related to this pathogen and disease and various precautionary measures to avoid personal exposure to the parasite. The infection can be prevented using precautionary measures such as the following: cooking meat to 66 °C throughout before eating; washing hands with detergent and water after touching meat; feeding cats cooked and dry/canned food instead of raw meat; keeping cats indoors and changing litter boxes daily and cleaning them with boiling water; flushing cat faeces down the toilet or burning it; and usage of gloves while gardening.

Currently no vaccine is available to protect humans and animals from congenital infections except for a live attenuated vaccine, Toxovax® (Intervet Schering Plough, Boxmeer, The Netherlands), available in New Zealand and Europe to prevent abortions in sheep.

Proper counselling and health education about risk factors can lower the incidence and probability of getting the infection, which is adapted by many countries to reduce incidence of congenital toxoplasmosis. Countries like Germany and Italy have reported surveillance of congenital toxoplasmosis. Health education may include instructing women about possible environmental exposure and ways to avoid it during pregnancy and receiving appropriate treatment without delay in case of acute infection.

Case Study

A 7-day-old newborn infant underwent a screening test and presented with good health with no symptoms. The mother of this newborn participated in a newborn toxoplasmosis survey conducted at the university hospital which

involved the detection of toxoplasmosis through filter paper screening and accordingly the survey and requirement of fresh blood collection after a few months were explained. The newborn during its first screening using filter paper tested negative for toxoplasmosis and was selected as negative control group. Six months later the second blood collection of both mother and baby was done.

The mother presented negative anti-*T. gondii* IgM and IgG results, while the 6-month-old infant tested positive for anti-*T. gondii* IgA and IgM and had low-avidity IgG and positive PCR assay, which was again confirmed through mouse bioassay and repeated PCR assay. To determine how such a young infant became infected, his mother was interviewed regarding epidemiological aspects. During this interview, she reported that she had given her 2-month-old baby a piece of undercooked beef to suck on. There was no house pets (cats or dogs) and the child mostly fed on breast milk and filtered water. Later on, the nursing infant presented with fever and swollen lymph nodes, which were confirmed as signs and symptoms of acquired toxoplasmosis. For treatment, sulfadiazine (100 mg/kg/day, every 12 h), pyrimethamine (1 mg/kg/day, once daily) and folinic acid (10 mg/day, every 3 days) were prescribed for 1 year with clinical follow-ups throughout his early childhood. The detection of acquired toxoplasmosis in a 6-month-old nursing infant is very rare. This report emphasises the importance of serological surveys for toxoplasmosis control in pregnant women and infants.

1. What is the significance of the above study?
2. How is the infant diagnosed about acquired toxoplasmosis?
3. Who is at risk for developing severe toxoplasmosis and what are the preventive measures?

Research Questions

1. How do we develop an experimental model which can exactly mimic the focal toxoplasma encephalitic lesions as found in immunocompromised humans?
2. Why is there variation in response to *T. gondii* infection by the different cell types in humans? What is the reason behind there being no unified defence strategy?
3. What are the challenges in the production of efficacious vaccines against toxoplasmosis?

Further Readings

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Leishmaniasis

Magda El-SayedAzab

Learning Objectives

1. To have a clear understanding of the classification, distribution and clinical manifestations of the large number of species of *Leishmania*.
2. To know the various diagnostic modalities which should be adopted in different forms of leishmaniasis.
3. To have a knowledge about the increasing importance of molecular diagnosis for precise species identification.

Introduction

Leishmaniasis is a tropical and subtropical disease endemic in most parts of the world including Asia, Africa, America and Mediterranean region. It has a wide range of clinical spectrum depending on the infecting species and many other factors. The clinical presentations can be broadly categorised into visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). Diagnosis of leishmaniasis is based on the presenting clinical condition and is confirmed mainly by a direct demonstration of stained amastigotes in biopsy aspirates or imprints from the affected tissues or in peripheral blood samples. Besides, in vitro culture to demonstrate isolated promastigotes and isolation of the parasite by experimental inoculation of mice, hamsters or guinea pigs is also done. Advanced methods like detection of parasite DNA in tissue samples by PCR are also in practice. Therapy of CL is by various local topical applications in addition to oral miltefosine, which is administered also in the mucocutaneous leishmaniasis (MCL) and VL cases. Intravenous liposomal amphotericin B (L-AmB) is recommended for treatment of the cases of VL. Despite various attempts, no vaccines or prophylactic chemoprophylaxis is currently available for inhabitants and travellers to endemic areas.

History

The prehistoric existence of *Leishmania* was recorded millions of years before in a fossil of sand fly, in addition to records of clinical cases resembling CL (2000 BC) and VL (1500 BC) in amplified *Leishmania donovani*. DNA samples from ancient Egyptian and Nubian mummies, besides demonstration of leishmanial DNA in Northern Sudan (800 BC), Peru (700 BC) and the Tigris-Euphrates basin (650 BC). Records of CL came later in the tenth, fifteenth and sixteenth centuries AD. In the eighteenth century, kala-azar

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was recorded from India by Russel in 1756 followed by a report on the probable involvement of the sand fly in transmission in the New World region by Cosme Bueno in 1764. In the nineteenth century, Villar in 1859 described the clinical cutaneous presentation of the Peruvian uta that was later similarly depicted as the Aleppo button.

The first description of *Leishmania* parasite (nineteenth–twentieth centuries), and its incrimination in the oriental sore and relation to Protozoa, was by Borovsky in 1898. The first demonstration of amastigote forms in smears from the spleen of a deceased Indian patient from *Dum-Dum fever* by Leishman in 1901 which was confirmed by Donovan in the same year led to recognition of the amastigotes as Leishman-Donovan bodies in kala-azar patients and was named *Leishmania donovani* by Ronald Ross in 1903. In the same year, Wright described *Leishmania tropica* and its conversion from amastigotes to promastigotes was described a year later by Leishman and Rogers in 1904. Also, in the same year, Rogers succeeded in culturing the promastigotes, and Laveran and Chatoin described the first case of VL in the Mediterranean region.

Taxonomy

The genus *Leishmania* Ross, 1903, belongs to family Trypanosomatidae Doflein, 1901; order Trypanosomatida Kent, 1880; class Kinetoplastidea Cavalier-Smith, 1981; and phylum Euglenozoa Cavalier-Smith, 1993, in the kingdom Protozoa Cavalier-Smith, 2002. The genus *Leishmania* was divided into two subgenera based on the restricted growth of promastigotes anteriorly in the sand fly alimentary tract (subgenus *Leishmania* Garcia 2001 distributed worldwide) or in the midgut and hindgut of the sand fly (subgenus *Viannia* Garcia 2001 limited to Central and South America). Subgenus *Sauroleishmania* (reptilian *Leishmania*) is another subgenus that includes species primarily pathogenic for reptiles. Subgenus *Leishmania* included four species pathogenic to humans such as *L. donovani*, *Leishmania major*, *L. tropica* and *Leishmania mexicana.* Subgenus *Viannia* included *Leishmania braziliensis* and *Leishmania guyanensis.*

Genomics and Proteomics

Sequenced genomes of *L. major*, *Leishmania infantum*, *L. donovani* and *Leishmania braziliensis* are composed of 8300 protein-coding and 900 RNA genes. These are randomly distributed throughout the genome, and an estimated 1000 *Leishmania*-specific genes are present. A few (about 200) species-specific differences exist in gene content between *L. major*, *L. infantum* and *L. braziliensis* genomes, of which around 8% occur as varying proportions in the three species, suggesting variable influences on disease pathology. Several protein-coding genes (about 65%) are without recognised function.

The Parasite Morphology

The basic morphological features of promastigotes and amastigotes are the same being composed of a plasma membrane overlying a system of microtubules, nucleus with a centrosome, mitochondrium, kinetoplast, Golgi apparatus, basal body (kinetosome), flagellar pocket and flagellum. Unlike other eukaryotes, the cytoskeleton of leishmanial pathogens is formed of a dense subpellicular microtubular spiral corset which defines their shape.

Promastigote

The flagellated motile form measures 8–15 μm (Fig. 1a). The flagellum arising from the kinetosome passes anteriorly through the flagellar pocket as an endogenous axoneme (5.4–5.9 μm) and then becomes free anteriorly. The periplast extends anteriorly to form the flagellar pocket which is supported by three to four flagellum-associated microtubules. The flagellum is covered by a sheath formed by the lining membrane of the flagellar pocket. The flagellar axoneme is formed

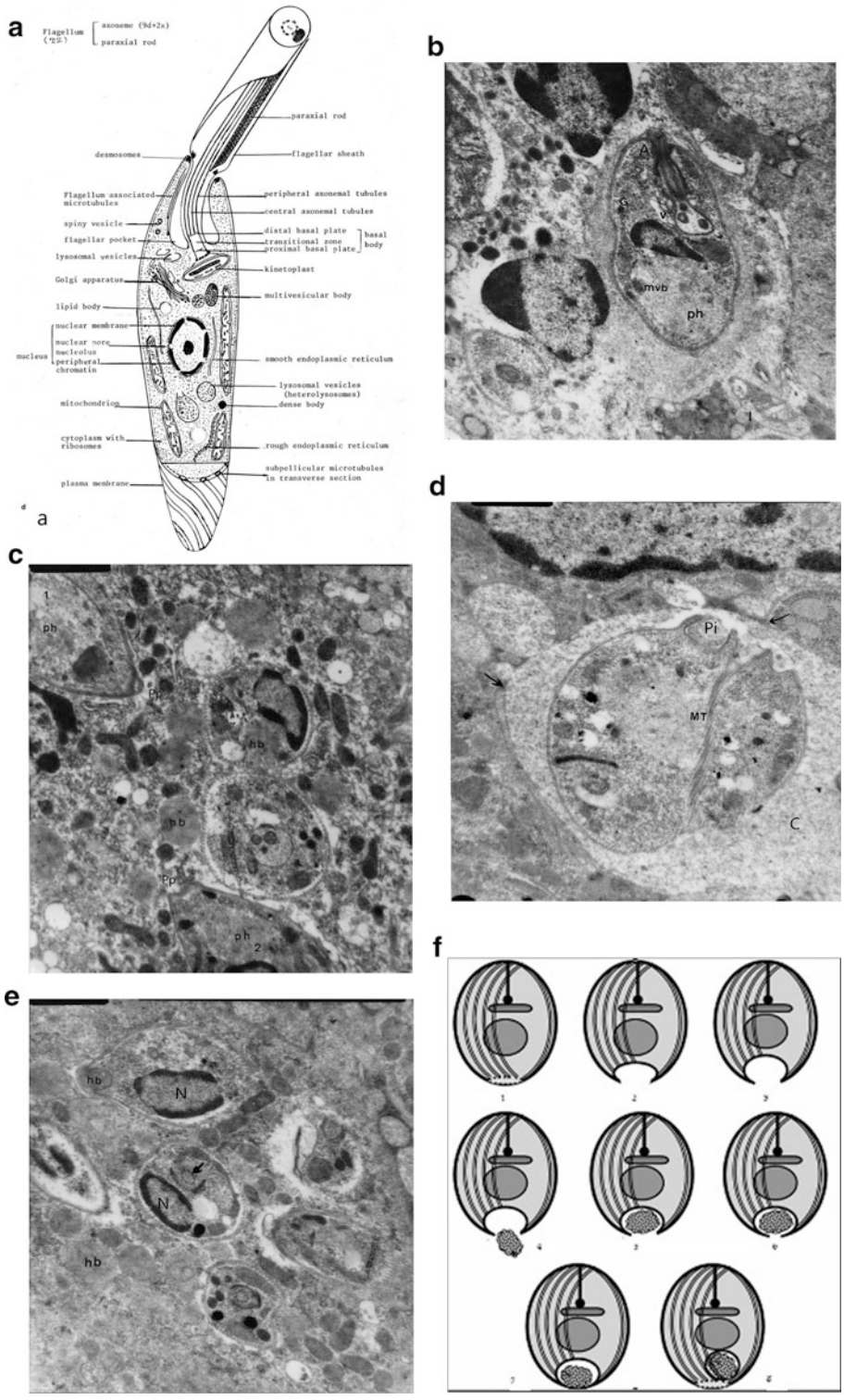

Fig. 1 (**a**) Ultrastructure diagrammatic adaptation of a *Leishmania* promastigote. **Source:** Thesis: Ultrastructure study of isolated *Leishmania* strains from Egypt. Mona A Abdel Mawala; Parasitology Dept., Faculty of Medicine, Ain Shams University, Cairo, Egypt. (**b**) Ultrastructure of a *Leishmania* amastigote. **A**, endocellular axoneme with

of nine pairs (doublets) of peripheral microtubules and two central microtubules (singlets), i.e. (9d+2s). At the base of the flagellar pocket, the peripheral axonemal microtubules are continuous with the proximal plate of the basal body, and the central ones originate from the distal plate. An intra-flagellar lattice like paraxial rod lies longitudinally alongside the axoneme. Feeding of nutritive material by promastigotes in the fluid milieu and excretion occur through the flagellar pocket by pinocytosis (cell drinking) and exocytosis, respectively. Binary fission of the promastigote starts posteriorly and proceeds anteriorly, and the anterior poles of the daughter parasites separate last.

Amastigote

The pellicular layer of the amastigote form is covered by the host's parasitophorous vacuole membrane (Fig. 1b). Anteriorly, the flagellar pocket contains a short endogenous axoneme. There is no free flagellum. The microtubular cytoskeleton forms a regular spiral network around the parasite. The posterior pole and flagellar pocket of amastigotes are devoid of the microtubular framework. The posterior bare area is covered by the plasma membrane (Fig. 1c). Evidently this is for the purpose of phagocytosis (cell eating), where the contraction of microtubules by built-in tubulin contractile elements results in suction of the plasma membrane in the microtubule-deficient area forming a deep invagination with two lips (Fig. 1d). With further contractions of the microtubules, the formed indentation or cup increases in depth up to 0.4 μm, engulfing host cell nutritive macromolecules into the parasite cytosol by endocytosis, which proves that amastigotes use the posterior pole bare area for feeding (Fig. 1e, f). This specific mechanism of engulfment of host material not only is a metabolic adaptation to the intracellular environment but also ensures protection from intracellular killing. Exocytosis of intraparasitic materials occurs through the flagellar pocket. Amastigotes are most active metabolically, and their nutrient uptake systems for glucose, amino acids, nucleosides and polyamines are optimal at acidic pH. The amastigotes multiply within two types of parasitophorous vacuoles in the macrophages. Type I is small and tight fitting over a single amastigote, as with *L. major* infection. Type II is large and occupied by more than one amastigote, as with *Leishmania amazonensis* infection.

Cultivation of Parasites

Active, motile promastigotes can be grown in in vitro culture of smear samples or biopsies. Culture media used are Nicolle-Novy-MacNeal biphasic blood agar medium; Schneider liquid medium; Grace insect liquid medium; and semi-synthetic, autoclavable, liquid medium. Cultures are incubated at 22–26 °C and examined microscopically twice weekly for the first 2 weeks and then once weekly for another 2 weeks before considering as negative.

Fig. 1 (continued) zonular desmosomes; **G**, Golgi apparatus; **V**, vesicle; **mvb**, multivesicular body; **ph**, phagosome. (**c**) Ultrastructure of a *Leishmania* amastigote. **ph**, phagosome (1 and 2); **Pp**, posterior pole (1 and 2); **hb**, homogeneous body (intracellular and in host cytosol). (**d**) Ultrastructure of a *Leishmania* amastigote. **MT**, microtubules; **Pi**, posterior invagination; **C**, host cell cytosol; **arrows**, parasitophorous vacuole wall. (**e**) Ultrastructure of a *Leishmania* amastigote. **hb**, homogeneous body (in posterior invagination and host cell cytosol); **N**, nucleus; **arrow**, axoneme. (**f**) Diagrammatic representation of sequence of endocytosis by *Leishmania* amastigote. **Electron microscopic figures adapted from:** Azab ME, Abdel Mawla MM. Ultrastructural analysis of posterior polar endocytic phagocytosis by *Leishmania* amastigotes. Read before the Egyptian Parasitologists United (EPU) Vth Conference: 'Parasitosis: A multidisciplinary Approach'. Ain Shams University Guest House, Cairo, Egypt; 24–25 March 2018

Laboratory Animals

Commonly used animals are mice or hamsters that are injected intradermally in footpad, ear or base of tail. Response in experimental animal depends on virulence of the strain. Lesions at the site of inoculation start to appear within 1–2 weeks. Visceral strains may also present with severe orchitis.

Visceral Leishmaniasis (VL)

Life Cycle of Leishmania Donovani

Hosts

Leishmania completes its life cycle in two hosts: vertebrate host consisting of man, dogs, rodents, etc. and invertebrate host female sand fly *Phlebotomus argentipes* (Fig. 2).

Infective Stage

Promastigotes in the alimentary canal of female sand fly are the infective forms.

Transmission of Infection

Bite of Sand Fly

The sand fly takes up the amastigote forms of the parasite from the vertebrate host during a blood meal. In the gut of the sand fly, a peritrophic matrix is formed to cover and separate the blood meal from epithelium of the midgut until the amastigotes differentiate into motile flagellated promastigotes in about 4 days. Procyclic flagellates (6.5–11.5 μm long; flagellum shorter than body length) are first formed, followed by differentiation into nectomonad flagellates (longer than 12 μm). These nectomonad flagellates escape the peritrophic matrix and attach by their flagella to microvilli of the gut prior to moving to the thoracic midgut and stomodeal valve where

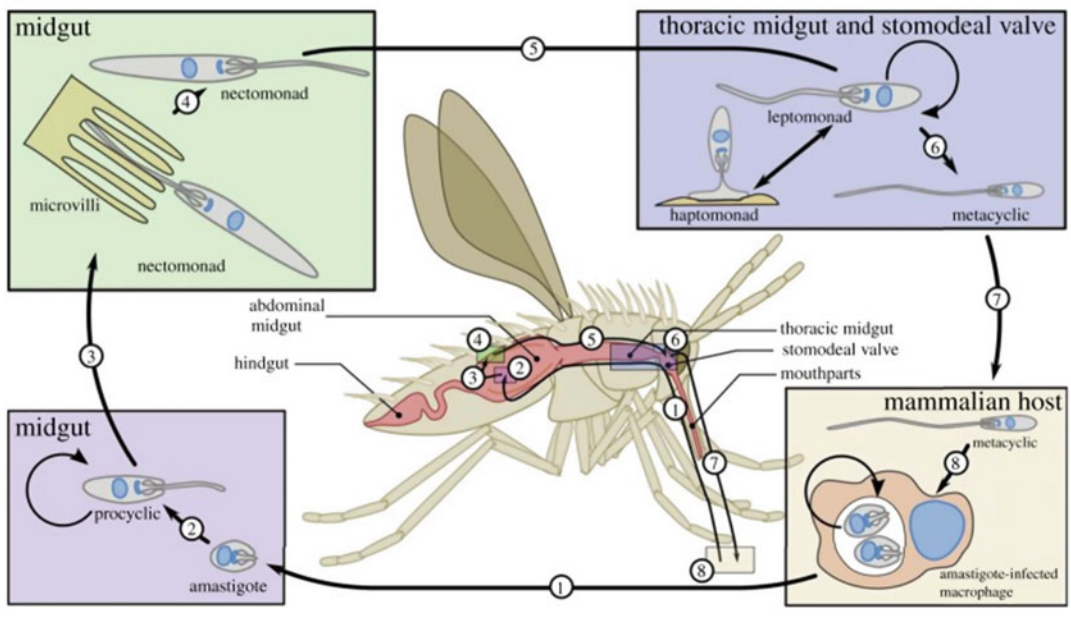

Fig. 2 Schematic presentation of *Leishmania* life cycle in the sand fly. (Source: By permission from Jack Sunter, Keith Gull. Shape, form, and function and *Leishmania* pathogenicity: from textbook descriptions to biological understanding. Open Biol. 2017 Sep; 7(9): 170165)

they become the leptomonad forms (6.5 and 11.5 μm long; flagellum longer than body length). Some leptomonads remain attached to the stomodeal valve as haptomonads by hemidesmosomal complexes on the enlarged tip of their flagella. Others differentiate into metacyclic infective forms (less than 8 μm long; flagellum longer than the body length) that migrate to the proboscis of the sand fly. This series of multiplications takes place by binary fission producing 10–1000 promastigote-infective stages per bite.

The promastigotes in the proboscis of the sand fly are introduced into the skin of the mammalian host. Inside the skin they face complement opsonisation in the preparation for phagocytosis by mammalian host skin macrophages. In the appropriate acidic milieu of the macrophage phagosome, the promastigotes transform into nonmotile amastigotes within 12–24 h, multiply by binary fission and eventually cause rupture of the host cells. Released amastigotes are re-phagocytosed by mononuclear cells of the reticuloendothelial system in the liver, spleen and lymph nodes throughout the body. The cycle is completed when the female sand fly ingests the amastigotes in a blood meal.

Pathogenesis and Pathology

Proteolytic activities by cysteine proteinases, metalloproteinases and serine proteinases are important targets because they are associated with tissue invasion, survival in macrophages and immunomodulation. Other parasite virulence factors that promote invasion and establishment of *Leishmania* pathogenesis in the mammalian host include lipophosphoglycan (LPG), glicoinositolphospholipids (GIPLs), proteophosphoglycan (PPG) and the 11 kDa kinetoplastid membrane protein (KMP-11). Apparently, these parasite factors modulate the relation between the parasite and its host immune cells.

LPGs are multifactorial surface virulence factors implicated in the preliminary phases of infection. *Leishmania* use the LPG coating to escape from complement lysis and immune evasion, by developmental modification of its constitution during stage transformation from metacyclic forms. Interaction of LPG with Toll-like receptor (TLR2) on host macrophages results in the induction of TNF-α, IL-12 and reactive oxygen species. The immune evasion is due to impairment of monocyte activation of B cells (NF-κB) resulting in decrease of IL-12 production and modulation of dendritic cells, thus inhibiting antigen presentation and promoting an early IL-4 response. GIPLs facilitate survival in macrophages by inhibiting nitric oxide synthase and protein kinase C. The PPG is a mucin-like surface glycoprotein expressed by amastigotes and found in parasite-free vesicles in macrophage cytosol. It contributes to activation of complement via mannose-binding protein attachment of *Leishmania* to host cells and maintenance of the parasitophorous vacuole. It also has an immunomodulatory effect on macrophage function inhibiting induction of TNF-α and collaborates with INF-γ to stimulate the production of nitric oxide by macrophages. The KMP-11 factor is a hydrophobic protein linked with LPG on surface membranes of promastigotes and in greater quantities in amastigotes. It is also expressed in the parasite's flagellar pocket and intracellular vesicles. The immunoregulatory properties include stimulation of T-cell proliferation, increase of IL-10 expression and arginase activity and reduction of nitric oxide production.

Protein folding is another strategy achieved by genes encoding heat shock proteins (HSPs) and includes cytosolic, mitochondrial, nuclear and endoplasmic reticulum proteins. Parasites exposed to stress conditions as pH extremes, increased temperature, oxygen and nutrient deprivation express these proteins that bind to the cellular proteins to sustain their folding. In addition, the different HSP systems are considered as important virulence factors because they stimulate innate immunity; induce production of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α and IL-12 by dendritic cells; and promote MHC-I and MHC-II pathways and adaptive immunity.

Spleen: The spleen becomes the largest reservoir of infected reticuloendothelial cells resulting in massive splenomegaly with extension of its

lower pole beyond the midline into the pelvis. Amastigote-containing macrophages are found in the red pulp composed of lymphocytes, macrophages and plasma cells in a network of splenic cords (cords of Billroth) and sinusoids (wide vessels) filled with blood. Hyperplastic white pulp lymphoid follicles consist of plasma cells, apoptotic lymphocytes and dendritic cells and the marginal zone between the two pulps is composed of B-cell lymphocytes. Subsequent atrophy of the splenic white pulp is associated with significant reduction in its size with disappearance of secondary lymphoid follicles, marginal zones and boundaries separating white and red pulps. Due to changed dissemination of cells, the normal immunological functions of the spleen are compromised.

***Lymph Nodes*:** Lymphadenopathy is localised in the CL and generalised in VL due to increased transfer of lymphocytes from the blood, promoted by activated dendritic cells via a Toll-like receptor (TLR9). Proliferations of plasma cells and histiocytes in the paracortex are associated with depletion of small lymphocytes in the paracortical areas. Concurrent populations of cells include histiocytes, plasma cells, multinucleated giant cell and mast cells with varying degrees of necrosis containing intracellular and extracellular amastigotes. Predominant plasma cell classes are those producing IgG and IgE immunoglobulins. Macrophages show a strong alpha-1-antitrypsin reaction. In kala-azar patients, the paracortical areas contain proliferations of histiocytes packed with amastigotes, occasional plasma cells and large lymphoid cells. The medullary cords are loaded with plasma cells and histiocytes.

Bone Marrow: In VL the bone marrow becomes hyperplastic. Normal haemopoietin tissue is replaced by parasitised macrophages resulting in ineffective haematopoiesis and peripheral cytopenias. Marrow tissue is replaced by free and intra-histiocytic amastigotes, increased lymphocytes, plasmocytes and eosinophils forming multifocal to diffuse granulomas. It is also associated with gelatinous transformation of marrow, megakaryocytic dysplasias and medullary aplasia, erythrocytic hypoplasia, leukaemic blasts, Reed-Sternberg-B-like cells, tart cells and foamy cells. As opposed to phagocytosis, in emperipolesis there is active penetration of red blood cells, neutrophil precursors and lymphocytes, into intact megakaryocytes. Hematopoietic stem cell disorders present as anaemia and/or leucopenia and thrombocytopenia.

Anaemia: Anaemia in VL is multifactorial due to alteration in RBCs' permeability; sequestration and destruction in enlarged spleen; immune mechanisms; reduction of plasma iron level due to abnormal iron retention by macrophages; dietary deficiency of iron, folate and Vit B12; increased sensitivity to complement; inhibition of erythrocyte enzymes; production of haemolysin by the parasites; and presence of cold agglutinins. The anaemia is normocytic normochromic (MCV 80–95 fl and MCH$\geq$27 pg) with a haemoglobin level of 7–10 g/dl and reduced haematocrit. Haemolysis poses as a major cause of anaemia.

Liver: Increased numbers of enlarged Kupffer cells and hyperplasia of macrophages filled by replicating amastigotes lead to liver enlargement. Hepatocytes may get infected. Associated changes include chronic mononuclear cell infiltration of portal tracts and lobules by lymphocytes and plasma cells, fibrin ring granulomas and diffuse fibrosis. Additional portal hypertension, severe hepatitis with cytolysis and cholestasis lead to hepatic failure with elevation of liver enzymes.

PKDL: Severe cases of VL are complicated by an asymptomatic skin rash in the form of macules, papules, nodules or plaques or as a mixture on the uncovered areas of the face, arms and upper chest. After some time, the whole body may be covered with variable intensity. Other cytopathological changes include discrete or confluent hypopigmented macules all over the body except for palms, soles, scalp and axillae and erythematous papules or nodules mainly on the face. Persisting amastigotes in the skin provoke an inflammatory response composed of mononuclear cells, a mixture of histiocytes, lymphocytes and occasional plasma cells. Histiocytes predominate in papulonodular lesions, mixed with many vacuolated macrophages indicating activation

and with epithelioid cells and plasma cells. In hypopigmented macular lesions, the lymphocytes predominate with some histiocytes and scarce plasma cells.

Immunology

The complement system plays an important role as the first line of defence against promastigotes in the blood. The classical and alternate complement pathways are both activated, but only the latter is concerned with eradication of C3-bound promastigotes and amastigotes in the presence of Mg^{2+} and not Ca^{2+} (Fig. 3). Neutrophils are the first host cells recruited within a few hours to the inoculation site of metacyclic promastigotes. Other activated cells include macrophages and dendritic cells. In response to antigen stimulation, activated Th-1 cells produce the cytokine IL-12, which in turn induces the production of IFN-γ and IL-2. The cytokine such as IFN-γ activates defence cells killing of amastigotes by nitric oxide synthase, neutrophil elastase, platelet-activating factor and neutrophil extracellular traps. Following the neutrophil initiative, recruited natural killer T cells, also involved in innate immunity, produce IFN-γ that promotes Th-1 response differentiation of $CD4^+$ T cells to restrict early parasite dissemination. Th-2 response leading to increased production of IL-10 and IL-4 occurs in patients developing active VL.

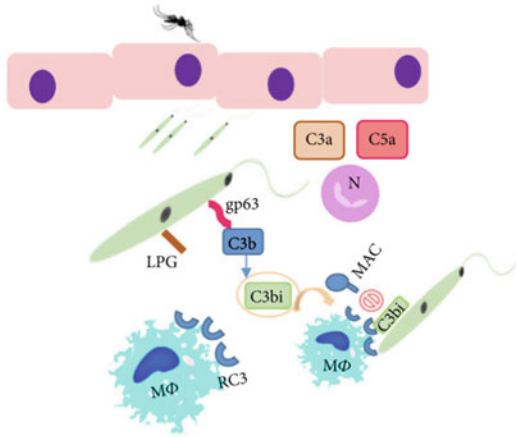

Fig. 3 Complement cascade post *Leishmania* inoculation. **Source:** (By permission**:** Gabriel A, Valerio-Bolas A, Palma-Marques J, Mourata-Gonçalves P, Ruas P, Dias-Guerreiro T, Santos-Gomes G. Cutaneous leishmaniasis: The complexity of host's effective immune response against a polymorphic parasitic disease. J Immunol Res 2019:2603730)

Infection in Humans

VL is the most severe form involving the liver, spleen and lymph nodes and may be fatal if untreated. The incubation period varies from 2 to 6 months. The condition may range from asymptomatic self-resolving to subacute and acute fulminant disease, or it may become chronic to manifest later in immunocompromised patients. Patients present with continuous or bouts of two-peaked high fever associated with rigors and chills. Fever lasts for weeks associated with night sweats, a palpable huge enlargement of the spleen, moderate enlargement of the liver, generalised lymphadenopathy and pancytopenia (Fig. 4f). Lymphadenopathy is more commonly seen with Mediterranean VL than Indian kala-azar. Complications include gastrointestinal dysenteric bleeding, peripheral oedema, acute renal failure and secondary bacterial infections. Generalised hyperpigmentation is a late feature of VL (Fig. 4g). PKDL occurs due to spread of the amastigotes that survived in the skin post VL treatment.

Infection in Animals

Wild canines and domestic dogs are the main reservoirs of zoonotic VL. Canine visceral leishmaniasis manifests variable signs and symptoms. These range from asymptomatic to development of mild symptoms or even death in severe cases. Infections in rodents are not overt as in the cases of canines, though rodents are also the reservoirs.

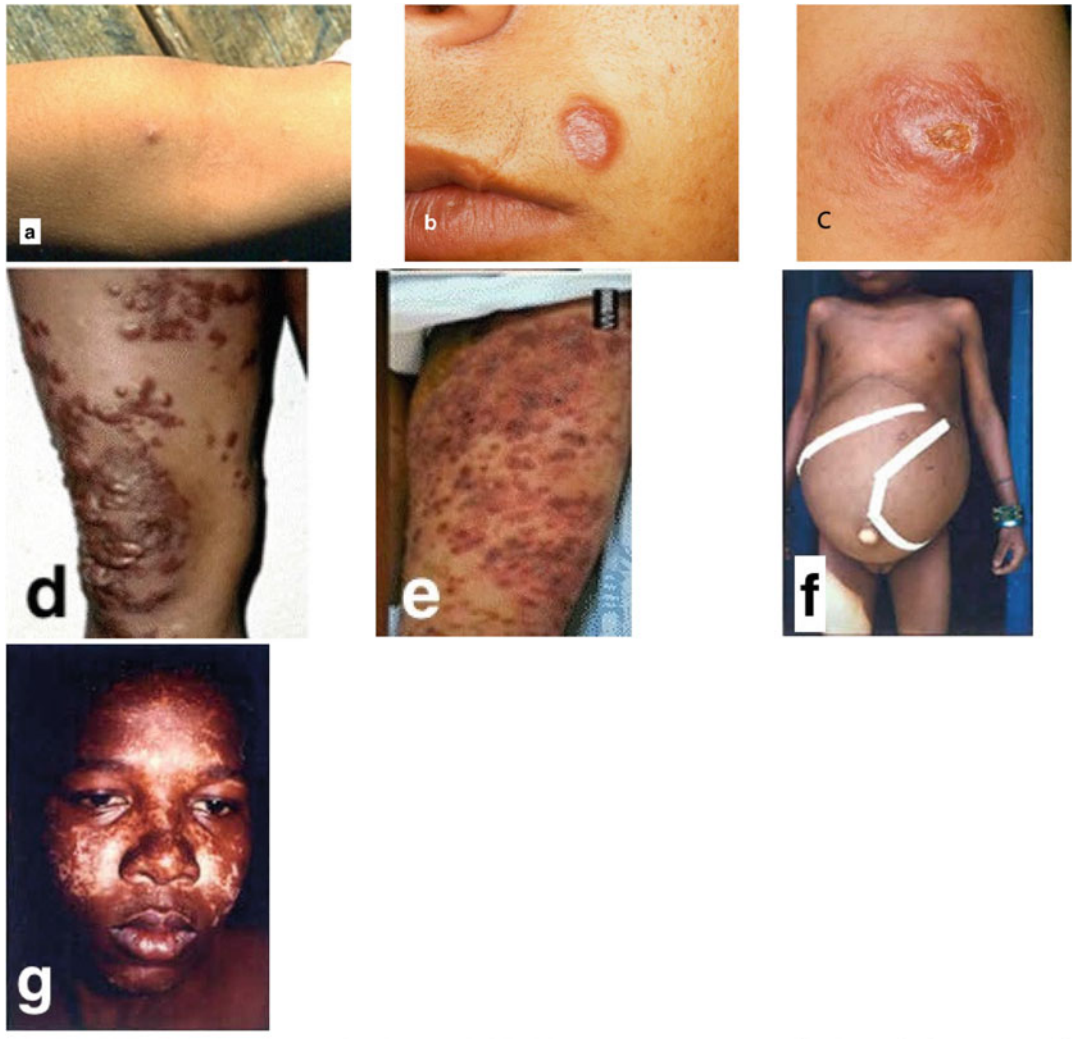

Fig. 4 (**a**) Papule of CL; (**b**) nodule of CL; (**c**) typical ulcer of CL; (**d**) diffuse CL case with various types of eruptions such as papules, nodules, infiltrated erythemas and brownish-coloured freckles on the lower extremities; (**e**) disseminated CL case with multiple and confluent lesions on a wide range of the lower extremities and partial ulcerations; (**f**) hepatosplenomegaly in VL; (**g**) skin colour changes in PKDL, Sudan (El Hassan). **Source for Figure 4a–c:** EMROPUB_2013_EN_1590.pdf. Permission granted for use from 'Manual for case management of cutaneous leishmaniasis in the WHO Eastern Mediterranean Region', https://applications.emro.who.int/dsaf/EMROPUB_2013_EN_1590.pdf?ua=1. **Source for Figure 4d, e:** Permission granted from Hashiguchi Y, Gomez EL, Kato H, Martini LR, Velez LN, and Uezato H. Diffuse and disseminated cutaneous leishmaniasis: clinical cases experienced in Ecuador and a brief review. Trop Med Health. 2016; 44: 2. **Source for Figure 4f, g:** Permission granted for use from 'Manual on visceral leishmaniasis control' WHO: https://www.who.int/leishmaniasis/surveillance/slides_manual/en/index1.html

Epidemiology and Public Health

Common reservoir hosts are domestic and feral dogs, rodents, foxes, jackals, wolves, raccoon dogs and hyraxes. An estimated 2.5 million dogs carry the infection in the Mediterranean basin. Common New World reservoir hosts include sloths, ant eaters, opossums and rodents. The reservoir of infection for Indian kala-azar is humans and is rodents for African kala-azar,

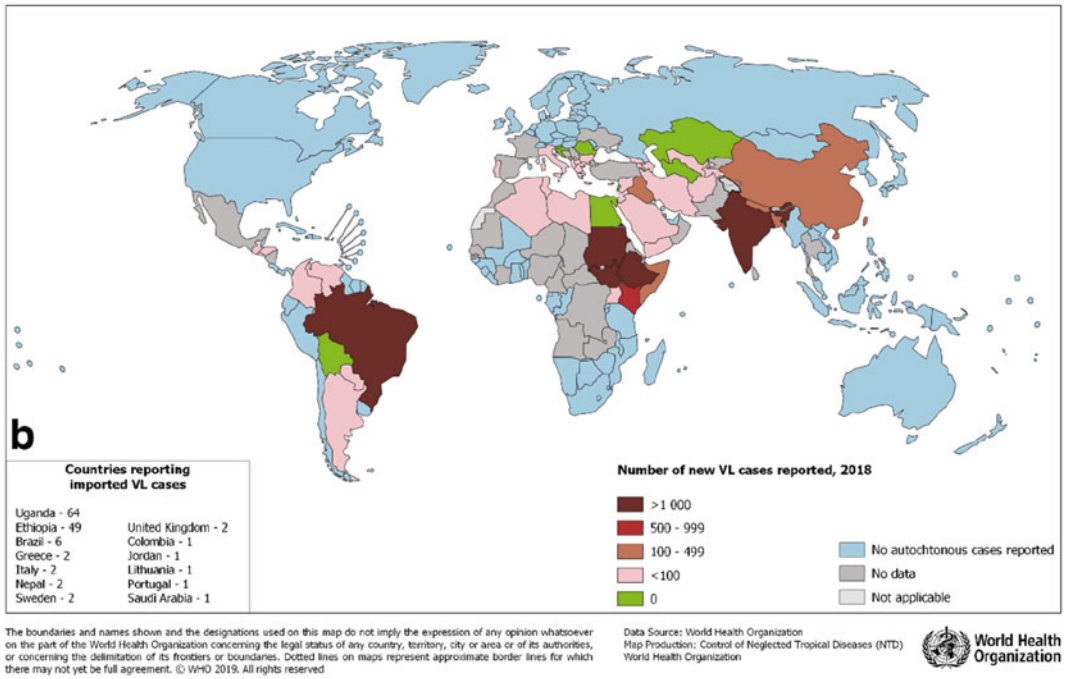

Fig. 5 Status of endemicity of visceral leishmaniasis worldwide, 2018 (Source: Permission granted by WHO; https://www.who.int/leishmaniasis/ burden/en/)

foxes in Brazil and Central Asia and canines for the Mediterranean and Chinese kala-azar. Other mammalian reservoirs for the *Leishmania* parasite include equines and monkeys. The phlebotomine sand fly vectors are tiny (1.5–3 mm) mosquito-like insects that live in wall cracks, animal burrows and dead leaves. They actively feed on blood of their hosts specifically at dawn and dusk.

The annual worldwide new case records of VL vary between 50,000 and 90,000, of which only 25–45% are reported to WHO. Most of those cases were from Brazil, Nepal, China, India, Iraq, Ethiopia, Kenya, Somalia and Sudan (Fig. 5). Leishmaniasis is apparently absent in New Zealand, Southern Pacific, Australia and Antarctica. The PKDL confined particularly to East Africa (mainly Sudan) and South Asia (India, Nepal and Bangladesh) manifests within months in the former areas and years later in the latter countries, after apparent successful treatment of kala-azar. In addition, susceptibility genes in band 22q12 have been found in an ethnic group in parts of Sudan that has a high prevalence rate of VL. In the afflicted areas, leishmaniasis affects mainly the poor sectors of the population suffering from malnutrition and compromised immune systems. The disease is caused by inadequate dwellings and migration of inhabitants to new infectious locations, in which ecological disruptions are provoked by deforestation, urbanisation, building of dams, irrigation schemes and wars. Uncommon modes of transmission include congenital transmission, contaminated needle sticks, blood transfusion and sexual intercourse.

Diagnosis

Microscopy

Microscopy is based on the demonstration of intracellular *Leishmania* amastigote (LD bodies) within macrophages by microscopy of smear samples from aspirates from the spleen, liver, bone marrow, lymph nodes, thick blood or buffy

coat of peripheral blood in VL and from skin in PKDL. Smears are stained by Giemsa, Leishman or Wright stains for the demonstration of amastigotes.

In Vitro Culture

Demonstration of active, motile promastigotes from in vitro culture of smear samples or biopsies is diagnostic of the *Leishmania* spp. Culture media used are Nicolle-Novy-MacNeal biphasic blood agar medium and Schneider liquid medium. Other media like Grace insect liquid medium and semisynthetic, autoclavable, foetal calf serum media are also used. Culture is used for identification of species and drug susceptibility testing.

Serodiagnosis

Complement fixation (using nonspecific antigen) was replaced by enzyme-linked immune assay (using monoclonal or polyclonal antibodies for detection of specific recombinant proteins) and immunofluorescent IgG and IgM antibody test (based on cytoplasmic or membrane fluorescence of intact promastigote as antigen) that are available for the diagnosis of VL. Direct agglutination test (using formalin-fixed promastigotes or freeze-dried promastigotes) is of 100% sensitivity and specificity in detecting *L. donovani* antibodies in blood or serum. Immunochromatographic test (using recombinant kinesin antigen, rk39) is 98% sensitive and 90% specific in detecting *L. infantum* antibodies (except in East Africa). The latter two are simple rapid techniques that do not need special equipment or expertise.

Serological detection of antibodies though sensitive may cross react in Chagas disease and leprosy resulting in false-positive results. Detection of antibodies does not differentiate between present and past infections and may be negative in immunocompromised patients.

Molecular Diagnosis

Detection of DNA can be done for the identification and differentiation of species, quantification of the load of infection and evaluation of treatment, and is achieved by various PCR assays targeting nuclear or kinetoplast minicircle DNA. Lymph node and bone marrow aspirates, tissues, blood or urine samples are the specimens examined by PCR. The use of buffy coat as a source of *Leishmania* DNA in PCR proved to be more sensitive than whole blood. The use of lymph node tissues for the detection of leishmanial DNA by PCR determines the post-treatment outcome of VL. Those who test negative neither relapse nor develop PKDL, while positively reacting patients may be prone to either relapse or the development of PKDL. Targeting conserved sequences in minicircles of kDNA of different *Leishmania* species by RFLP distinguishes between relapse and reinfection in treated VL patients.

A PCR-ELISA technique using a common primer from *L. infantum* strains proved to be more sensitive than other diagnostic techniques. It was able to detect even 0.1 promastigote or 1 fg of nucleic acid. A fluorescent DNA probe has been used to detect a conserved region of small subunit rRNA gene and a pair of flanking primers and was rapid and specific. The *miniexon* gene involved in trans-splicing nuclear mRNA, employed in a PCR-RFLP-based genotyping assay, detects *Leishmania* in various clinical samples, characterising the parasite at species level.

Treatment

For VL, pentavalent antimony (SbV) compound as sodium stibogluconate (SSG) and meglumine antimoniate (MA) has been the drug of choice despite toxicity and increasing resistance. Parenteral therapy with liposomal amphotericin (L-AMB; AmBisome) is now preferred due to growing resistance to antimonials. A single dose of L-AMB is curative but expensive. Oral therapy with miltefosine has been shown to be highly effective in India, especially in combination with L-AMB. Intramuscular paromomycin is a cheaper alternative having a good cure rate. In HIV cases, antiretroviral therapy should be added with the above drugs. PKDL is difficult to treat but symptoms improve with treatment by miltefosine.

Cutaneous Leishmaniasis (CL)

Causative Agents: Old World CL is caused by *Leishmania tropica* complex (*L. tropica*, *L. major*, *Leishmania aethiopica*). New World CL is caused by *L. braziliensis* complex and *L. mexicana* complex.

Life Cycle: It is similar to *L. donovani* except that the parasites in humans and animals remain in the skin particularly in the reticuloendothelial cells of skin.

Hosts

Primary Hosts

Humans and domestic animals such as dogs, rodents and gerbils.

Vectors

The vectors for Old World CL are sand flies of species *Phlebotomus sergenti*, *Phlebotomus papatasi*, *Phlebotomus causasiasus* and *Phlebotomus intermedius*. Sand fly of genus *Phlebotomus* and *Lutzomyia* transmit the agents of New World CL.

Pathogenesis and Pathology

Leishmania-induced popular skin tissue swelling progresses to ulceration with intense infiltration of inflammatory T and B lymphocytes, plasma cells and parasites. In chronic inflammation, mononuclear cellular infiltration predominates. Neutrophil-released TNF cytokine contributes to disease pathogenesis by inducing cellular adhesion, necrosis and cytotoxicity. $CD4^+$ T cells, CD8+ T cells and NK cells are the other cells that contribute to cytotoxic activity and inflammatory response. The NK cells through IFN-γ actively participate in parasite destruction and ulceration of lesions. Necrosis of ulcers occurs due to apoptosis of infected macrophages and of cells conveying parasite antigens. Excessive degradation of the extracellular matrix is induced by enzyme activation of pro-inflammatory cytokines.

Immunology

In CL, neutrophils present in the chronic nonhealing cutaneous lesions also confirm their role in mediating tissue injury. These polymorphonuclear cells play a significant role in early infection and in delaying progression of the disease. While adequate inflammatory response results in containment of the lesions, an exaggerated response often causes tissue damage. Therefore, both pro-inflammatory and anti-inflammatory regulatory cell populations are found in CL.

Infection in Humans

Clinically, CL begins as one or more variable-sized papules that progress to form painful nodules, on exposed areas of the skin (Fig. 4a). The nodules slough forming typical ulcers with raised indurated edges and flat bases, which are usually secondarily infected (Fig. 4b, c). Left untreated, slow spontaneous healing of the ulcer occurs as cell-mediated immunity develops, leaving a permanent fibrosed scar. Local draining lymph nodes become enlarged and painful. In diffuse cutaneous leishmaniasis (DCL), the entire body of the patient is covered by various sized slowly growing nodules that do not ulcerate (Fig. 4d, e). In mucocutaneous leishmaniasis (MCL), the ulcers start at the mucocutaneous junctions on the face and then metastasise through the mucous membranes. This results in severe disfiguring destruction of the nasal septum, lips and palate and may reach the pharynx and larynx.

Infection in Animals

Cutaneous infection in canines is a significant veterinary problem. The burden of the problem is quite severe especially as CL in animals is a

neglected disease. Infected dogs are the common victims. Clinical signs vary from dermatitis, loss of hair, ulcerations in skin, weight loss and ocular and nasal lesions typical for CL. The lesions often heal or otherwise become chronic leading to severe disfigurement.

Epidemiology and Public Health

The CL constitutes about 95% of cases in Middle East, Mediterranean basin, Central Asia and the Americas. A WHO report in 2018 shows the annual worldwide incidence of CL reaching 600,000 to one million of which 85% are newly acquired infections in nine countries (Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Iraq, Pakistan, Syria and Tunisia) (Fig. 6). The CL constitutes nearly 95% of cases in Middle East, Mediterranean basin, Central Asia and the Americas.

Leishmaniasis is present worldwide, mostly in tropics and subtropics and the Mediterranean basin. *Leishmania* spp. belonging to the subgenus *Leishmania* (one complex, five species) and subgenus *Viannia* (two complexes, one of them with two species) are the parasites of the New World. Subgenus *Leishmania* with three complexes containing eight species causes infection in the Old World (OW). The geographical distribution, reservoir host, vector and transmission caused by *Leishmania* spp. are shown in Tables 1 and 2.

Diagnosis

Microscopy

Specimens collected from the edges of the active lesions by punch biopsy and stained by Giemsa stain can be examined for the presence of amastigotes of *Leishmania*.

In Vitro Culture

Isolation of promastigotes from the specimens by in vitro culture is also useful for the detection and identification of *Leishmania* species causing cutaneous leishmaniasis.

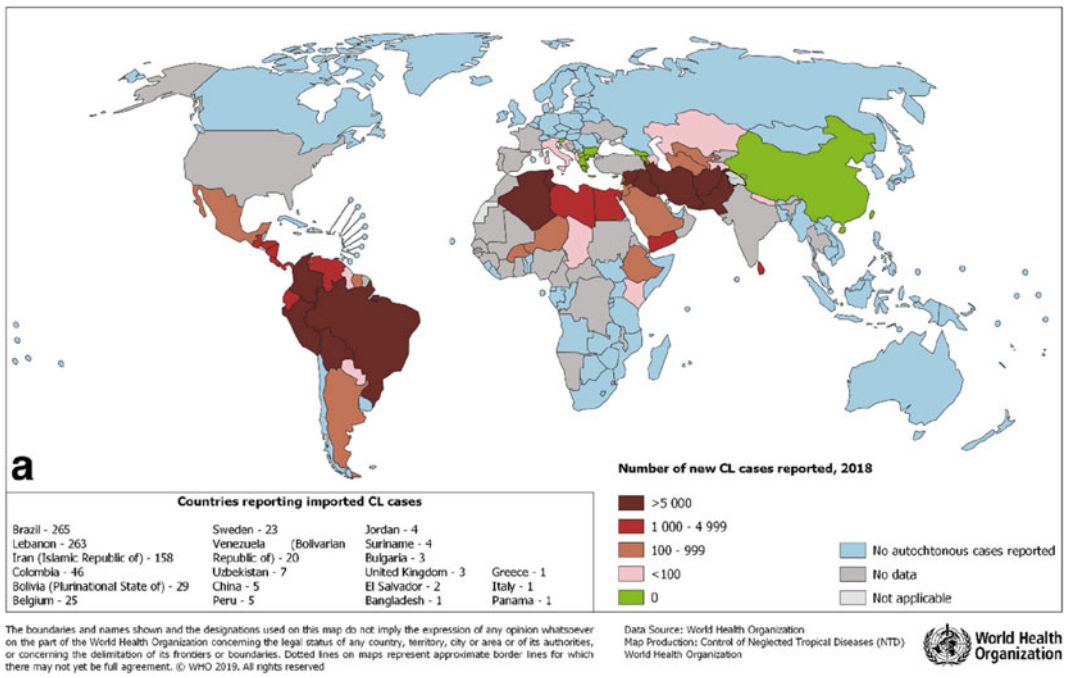

Fig. 6 Status of endemicity of CL worldwide, 2018 (Source: Permission granted by WHO; https://www.who.int/leishmaniasis/ burden/en/)

Table 1 Classification of old world *Leishmania*

Species	Reservoir host	Geographic distribution	Human disease
***Leishmania major* complex** *Leishmania major*	*Psammomys*	Middle East, Africa, Central and Western Asia, India, China	Cutaneous leishmaniasis
***Leishmania tropica* complex** *Leishmania tropica* *Leishmania killicki* (syn. *Leishmania tropica*) *Leishmania aethiopica*	Human	North Africa, Mediterranean region, Middle East, Western India	Cutaneous leishmaniasis, *Leishmania* recidivans
	Rodent spp., *Ctenodactylus gundi*	Northern Sahara, Northern Algeria, Tunisia, Libya	Cutaneous leishmaniasis
	Rodent spp., hyraxes	Ethiopia, Uganda, Kenya	Cutaneous leishmaniasis, disseminated cutaneous leishmaniasis
***Leishmania donovani* complex** *Leishmania donovani* (*archibaldi*)	Canines, hyraxes, equines, monkey	Middle East, Africa, China	Visceral leishmaniasis
	Human, rodents	Sudan, Ethiopia, Kenya, Uganda	Visceral leishmaniasis, post-kala-azar dermal leishmaniasis
	Humans	Indian subcontinent, China	
Leishmania donovani infantum (chagasi)	Canines	Mediterranean region, Middle East, Central Asia, China	Visceral leishmaniasis, cutaneous leishmaniasis

Serodiagnosis

Assay for specific cell-mediated immunity is done by Leishmanin skin test as a measure of delayed hypersensitivity. The antigen consisting of killed promastigotes is injected intradermally in the flexor surface of the forearm. A 5 mm or more induration appears after 72 h in competent individuals with active cell-mediated immunity in association with CL, and *L. recidivans*, and 6–8 weeks after recovery from VL; it is negative during active VL and in DCL associated with low cell-mediated immune responses.

Antibody-based diagnostic methods are not useful in the diagnosis of CL.

Table 2 Classification of new world *Leishmania*

Species	Reservoir host	Geographic distribution	Human disease
Subgenus: *Leishmania*			
***Leishmania mexicana* complex**			
Leishmania mexicana (*pifanoi*)	Canines	Northern and Central parts of South America	Cutaneous leishmaniasis Disseminated cutaneous leishmaniasis
Leishmania amazonensis (*garnhami*) *Leishmania aristides* (nov.sp.) *Leishmania venezuelensis* *Leishmania forattinii*	Forest rodents and sloths	Central and South America	
Subgenus: *Viannia*			
***Leishmania braziliensis complex*:** *Leishmania braziliensis* *Leishmania peruviana*	Forest rodents and sloths	Central and South America	Cutaneous leishmaniasis Disseminated cutaneous leishmaniasis Mucocutaneous leishmaniasis
Leishmania guyanensis complex *Leishmania guyanensis (shawi)* *Leishmania panamensis* *Leishmania lainsoni* *Leishmania naiffi* *Leishmania lindenbergi* *Leishmania utingensis* *Leishmania colombiensis*			

Molecular Diagnosis

Molecular methods that are used for VL are also used for the detection and identification of *Leishmania* spp. The specimens are collected from the specific cutaneous lesions and gene targets, and methods used are the same as for VL.

Treatment

Both local and systemic therapeutic treatments are available for CL.

Local Therapy

In some patients with CL, local therapy may be advocated which should be assessed depending on the nature, size and location of the disease. Local physical therapy in the form of a single session of thermotherapy at 40–42 °C or multiple cryotherapy sessions using liquid nitrogen is attempted. Intralesional infiltration of pentavalent antimony (SbV), undiluted sodium stibogluconate or meglumine antimoniate is also done. Other options include cryotherapy with intralesional SbV, photodynamic therapy with methylene blue/red light-emitting diode or topical applications of 15% paromomycin + 12% methyl benzethonium in paraffin/0.5% gentamicin.

Systemic Therapy

The traditional therapy for CL depends on pentavalent antimonial compound administered IV or IM for a duration of 20 days. Conventional amphotericin B deoxycholate is mainly used as a rescue therapy for CL. Lipid formulations of amphotericin B have a better safety profile compared to the conventional drug.

The oral agent miltefosine is FDA approved for the treatment of CL in adults and adolescents who are not pregnant or breastfeeding. However, the indications for therapy are limited to infections caused only by the species in the *Viannia* subgenus. Variable response has also been observed with miltefosine therapy in the New World CL cases. Similarly other oral agents like ketoconazole, itraconazole and fluconazole have been used with mixed results.

Mucocutaneous Leishmaniasis (MCL)

Mucocutaneous form of leishmaniasis is seen in a subset of populations with CL and is extremely rare in occurrence. Nevertheless, it is the most serious form of CL and is potentially life threatening. The condition is caused by *Leishmania* species of *Viannia* subgroup. The virulence of the parasite and host immunity decide the progression from CL to MCL.

Promastigotes in the alimentary canal of female sand fly are the infective forms.

The life cycle, pathogenesis, pathology and immunology of MCL are the same as those of CL.

Infection in Humans

The manifestations of MCL occur in majority of patients with scar from previous CL. The initial lesions typically present as erythema and ulceration of the nares. The condition gradually progresses to oedema and nasal septum perforation. The condition then progresses to gingival oedema, palatal ulcerations and periodontitis. Finally, the condition leads to gradual destruction of tissues resulting in disfigurement. Lymphadenopathy is also present which in due course shows ulceration and secondary infections.

Infection in Animals

Though rare, MCL has been reported in pet animals particularly in the dogs and the cats. Ulceration and involvement of the face are similar to that in man, often initiating from the nares and the nasal septum. Disfigurement of the face also occurs.

Epidemiology and Public Health

MCL is mostly limited to South and Central America. The MCL accounts for over 90% of cases in Bolivia, Brazil, Ethiopia and Peru.

Table 3 Laboratory diagnosis of leishmaniasis

	Diagnostic methods	Target	Remarks
1.	**Microscopy** Aspirates from the spleen, liver, bone marrow, lymph nodes, thick blood or buffy coat of peripheral blood in VL. The edges of the active lesions by punch biopsy in CL and MCL Giemsa staining used most commonly	Amastigote forms	Low sensitivity. Experienced microscopist needed
2.	**In vitro culture** Nicolle-Novy-MacNeal biphasic blood agar medium, Schneider liquid medium. Grace insect liquid medium	Motile promastigotes	Time-consuming and potentially hazardous
3.	**Immunodiagnostics** ELISA, immunofluorescent tests, immunochromatographic (ICG) tests	Antibodies against recombinant kinesin antigen (rk39) or other antigens	Cannot always distinguish between past and present infection ICG test can be used in field conditions
4.	**Molecular diagnosis** PCR, RFLP, PCR-ELISA	Nuclear or kinetoplast minicircle DNA	RFLP could be used to distinguish between relapse and reinfection in treated VL patients PCR-RFLP-based genotyping assay can be used for identification as well as for characterising the parasite at species level

Diagnosis

The major methods of diagnosis in MCL remain the same as that of CL. However, histopathology and imprint smears from the nasal mucosa reveal very few parasites unlike that in CL. Consequently, molecular diagnosis by detection of *Leishmania* DNA in the specimens is the most sensitive method for the diagnosis of MCL.

The diagnostic modes for all the forms of leishmaniasis have been summarised in Table 3.

Treatment

Systemic treatment is mandatory in MCL cases as the spread and localisation make local treatment impractical or ineffective. For Old World MCL, miltefosine or pentavalent antimonials for 28 days or liposomal amphotericin B is recommended, and treatment with above drugs has been reported to be effective. For New World MCL, the same drugs have been found to be useful with the addition of pentoxifylline with antimonials. Pentavalent antimonials are still the gold standard of treatment with an overall cure rate of 88%. Destructive mucosal lesions contain few parasites, while tumour necrosis factor (TNF) levels are high. Pentoxifylline downregulates TNF-α and inhibits leucocyte migration and adhesion. Combining antimonials with pentoxifylline has been found to be highly effective in refractory MCL, and in many instances the requirement for a second course with antimony compounds can be avoided.

Prevention and Control of Leishmaniasis

Personal precautions include avoidance of outside quests particularly at dawn and dusk, the optimal time for active feeding by sand flies, clothing covering the whole body and the spraying of repellents on exposed parts of the face and hands, as well as impregnation of bed sheets and nets by insecticides. Other preventive measures include the early diagnosis and treatment of patients to control spread of the disease, identification and control against reservoir host and elimination of vector outdoor habitats by the spraying of appropriate insecticides.

Vaccines for Leishmaniasis

Problems facing vaccine production are the existence of different infectious species of *Leishmania* and virulence factors between various species, as well as the existence of different reservoir hosts (*L. donovani* is anthroponotic, whereas *L. infantum* is zoonotic with canine species as the main reservoir). The guinea pig reservoir host of *L. enriettii*, in which T-cell responses to parasite antigens are established within 2 weeks and healing of cutaneous lesions occurs in about 10 weeks, presents an ideal model for CL. Another highly susceptible model is the BALB/C mice that suffer from large expanding and metastasising lesions, causing death. For VL the golden hamster mimics the infection in human. Being the main reservoir of *L. infantum* and *L. donovani chagasi*, the sequential responses to infection in mice are as in human.

First-generation live vaccinations were used in primitive attempts against CL in endemic areas for cosmetic reasons. Intentional inoculation of virulent amastigotes from infectious exudate was done on covered areas of babies' bodies to stimulate protective immunity, which usually becomes solid, and to avoid scars at the site of sand fly bite, especially if on the face. Second-generation vaccines relied on genetic modifications of *Leishmania* spp. Such vaccines used bacteria or virus transports to deliver genetically modified avirulent *Leishmania* parasites and epitopes of antigens in the form of synthetic peptides with adjuvants. A recent up-to-date advancement is the IDRI vaccine, alternatively known as LEISH–F3 + GLA-SE, introduced as a pure recombinant preparation for protection against VL. This vaccine incorporates two fused leishmanial parasite proteins (LEISH-F3) with the TLR-4 agonist adjuvant (GLA-SE). Preliminary administration of three 28-day apart injections elicited high ID93-specific antibody titres with increased IgG1 and IgG3 subclasses and Th-1-type cellular vaccination responses, indicating satisfactory protection.

Case Study

A 5-year-old boy presented to the Children's Hospital in Cairo with intermittent fever reaching up to 40 °C for 25 days that did not respond to usual antipyretics. The mother gave history of having spent their summer holiday 2 months previously in a resort in Agamy area on the West Mediterranean coast of Alexandria. On examination the spleen was enlarged about 10 cm below the coastal region, and the liver was likewise enlarged and tender on palpation. Some neck, axillary and inguinal lymph nodes were palpable. A complete blood picture revealed haemoglobin level of 6.5 g/dl, RBC count of 3.5 mill./cu mm, leucopenia of 2.5 Th./cu mm, neutropenia of segmented neutrophils 18% and thrombocytopenia of 95 Th./cu mm. ESR and C-reactive protein were raised to 60 mm and 125 mg/L, respectively. Liver function tests were raised. Preliminary examination of blood film and thick drop revealed apoptotic monocytes with scattered amastigotes of *Leishmania*. The condition was diagnosed as infantile VL and successfully treated with liposomal amphotericin (L-AMB) for 5 days, boosted on days 14 and 21.

1. Mention the serological tests which can be done to diagnose this condition.
2. What are the alternate drugs available for treatment?
3. How the disease mentioned here differs from similar disease in Indian subcontinent?

Research Questions

1. How to arrive at an updated and simplified taxonomic classification based on phylogenetic relationship of *Leishmania* species?
2. Is the loss of the free flagellum by intracellular amastigotes related to the acidic milieu of the macrophage phagosome?

3. How to fast-track the development of an effective vaccine based on genetic diversity and population structure of various *Leishmania* species?

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African Trypanosomiasis

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Learning Objectives

1. To understand the importance of innate immunity and factors associated with it in protection to infection.
2. To have an idea about the geographic distribution of different species and subspecies and their roles in disease causation.
3. To have a knowledge about the primary importance of serological tests in diagnosis.

Introduction

Trypanosomiasis is a disease that affects both humans and animals and has a detrimental impact on the socio-economy of numerous endemic countries. Trypanosomes are protozoan parasites mostly transmitted by blood-feeding vectors, which in many cases represent their primary obligate host. Two types of trypanosomes exist, stercorarian trypanosomes, released through insect faeces, and salivarian trypanosomes, transmitted through insect saliva. These two groups of trypanosomes are characterized by very distinct host-parasite interactions, and this chapter focuses on African trypanosomiasis only. From all the salivarian trypanosomes known to infect mammals, only three are to be considered zoonotic, all belonging to the subgenus *Trypanozoon*, and two of these might be even up for discussion when it comes to a zoonotic classification sensu stricto. The true zoonotic trypanosome is *Trypanosoma brucei rhodesiense*. This East-African trypanosome has an extended mammalian host reservoir that includes both game and domestic animals. The reservoir diversity is the main reason why eradication of HAT (human African trypanosomiasis) as such is considered unfeasible. *T. b. rhodesiense* causes an acute and most often deadly form of sleeping sickness. However, *T. b. rhodesiense* infections represent only a small fraction of all HAT cases reported, as

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Trypanosoma brucei gambiense parasite is responsible for an estimated 95–98% of all HAT cases. This infection is more chronic and has a much wider geographic distribution that covers West and Central Africa. *T. b. gambiense* infections are often considered to be anthroponotic. Indeed, it is now accepted that *T. b. gambiense* parasites represent a group of more diverse *Trypanozoon* organisms, of which the zoonotic infections might be much harder to control. Finally, *Trypanosoma evansi* is in general not considered to be a human parasite, despite the fact that several atypical human infections have been reported. These infections have only been reported outside Africa, but it is very well possible that due to a lack of surveillance, the number of aHT infections has been systematically under-reported.

History

T. evansi was the first salivarian pathogenic trypanosome to be discovered. The parasite was identified in the Indian subcontinent by Dr Griffith Evans in 1880, in horses and camels suffering from surra. David Bruce identified trypanosomes in the blood of infected cattle suffering from the African cattle wasting disease known as nagana between 1894 and 1910. While the name surra finds its origin in the Hindi word meaning 'rotten', nagana finds its origin in the Zulu language, meaning 'depressed' or 'low spirit', directly reflecting the clinical manifestation of this animal disease.

The first reports of HAT date back to the eighteenth century. However, it was only at the end of the nineteenth century, from 1896 until 1906, that the first properly recorded HAT epidemic occurred, coinciding with population displacements as a result of the colonial development of the Congo River basin. A second epidemic occurred during the 1920s. By the 1960s, transmission of HAT was nearly halted, helped by a combination of intense screening and treatment policies, as well as vector control. In the aftermath of decolonization, a loss of interest in the surveillance took place. In combination with the banishment of DDT insecticide, this led to a re-emergence of the disease in the 1970s. At the end of the twentieth century, the WHO estimated 300,000 people contracted the infection each year. After the renewed establishment of successful diagnosis and treatment programmes, this third epidemic now seems to be controlled. In 2019, less than 1000 *T. b. gambiense* infections were reported to the WHO, while the case report number for *T. b. rhodesiense* marginally surpassed 100.

Taxonomy

The taxonomical position of the genus *Trypanosoma* belongs to family Trypanosomatidae, order Trypanosomatida and class Kinetoplastida in the phylum Euglenozoa. *T. b. rhodesiense*, T. *b. gambiense* and *T. evansi* cause infections in humans.

Parasite Genomics and Proteomics

The genome of *T. brucei* has 11 megabase chromosomes (of 35 Mb total) as well as 5 intermediate (200–300 kb) and about 60–100 minichromosomes of sizes 30–150 kb. The genome contains 9068 predicted genes, including approximately 900 pseudogenes and approximately 1700 *T. brucei*-specific genes. Antigenic variation is one of the most interesting mechanisms that exhibited the trypanosomes to evade the host immune response. Upon injection by an infected tsetse fly, the metacyclic trypomastigotes reach the mammalian blood circulation, covered by unique surface glycoprotein called the metacyclic variant surface glycoprotein (mVSG). This protein acts as a defence layer against host antibodies and complement attacks and also helps in immune evasion. Large subtelomeric arrays contain 806 variant surface glycoprotein (VSG) genes. A single trypanosome has more than 1500 VSG genes, most of which are located in extensive silent arrays. Interestingly, most of these silent VSGs are pseudogenes, and ongoing studies are trying to understand how non-intact VSGs are recombined to produce genes encoding functional coats. Only 1 VSG is expressed at a

time from 1 of approximately 15 dedicated VSG expression site transcription units. Antigenic variation can be classified into two distinct types. VSG switching by recombination allows that VSGs are regularly being altered, while other genes associated with the expression site (ESAGs) remain unchanged. Alternatively, the active expression site is 'switched off', allowing mRNA elongation from a newly activated expression site. This changes the VSG, as well as the ESAGs. The latter is an advantage when the trypanosome needs to adapt to a new host, as will be outlined below in the case of adaptation to growth in human serum/blood.

Subspecies differentiation of *T. brucei* parasites is based on two specific 'resistance' genes that allow growth in human serum/blood. In *T. b. rhodesiense*, human serum resistance is linked to the presence of the SRA encoding gene (or serum resistance-associated gene). *T. b. gambiense* is mostly marked by the presence of the *TgsGP* gene. However, *T. b. gambiense* is not a homogeneous family of parasites and is currently being divided into two groups. The rather homogeneous Group 1 *T. b. gambiense* parasites show an invariable true NHS resistance phenotype, all having the *TgsGP* gene marker. Group 2 *T. b. gambiense* parasites are a much more heterogeneous, showing variable resistance, being much closer related to *T. b. rhodesiense* and *T. b. brucei* and representing the zoonotic side of gambiense HAT. There is no specific genetic marker for these parasites.

Although *T. evansi* was first discovered in India, it is generally accepted that the parasite is in fact a 'variant' of *T. brucei* that has lost the kinetoplast DNA (kDNA), which is essential for development in the gut of the tsetse fly. With respect to atypical HT, no genetic markers have been discovered so far that can explain how some *T. evansi* parasites have acquired a serum resistance mechanism. The notion that *T. evansi* mutations are far from understood has been made clear by the genetic analysis of a large group of *T. evansi* parasites found in a limited geographic location. Detailed microsatellite genotyping of parasites isolated in Kenya allowed grouping of *T. evansi* in at least four different clusters, with different evolutionary origins (Fig. 1). It is feasible that if similar studies would be done including *T. evansi* parasites of four different continents, an even more complex parasite ontology would be revealed.

Only a few studies have been done regarding the proteomic analysis of first- and second-stage HAT disease CSF protein profiles, showing that the number of differentially expressed proteins between the two stages is less than hundred. Two of these proteins, osteopontin and beta-2-microglobulin, were confirmed to be accurate markers of first and second stages of patients with sleeping sickness. The proteome of the insect stage and the human blood stage of the parasite have also been mapped. Comparing 4364 protein groups resulted in the identification of stage-specific proteins that can lead to a better

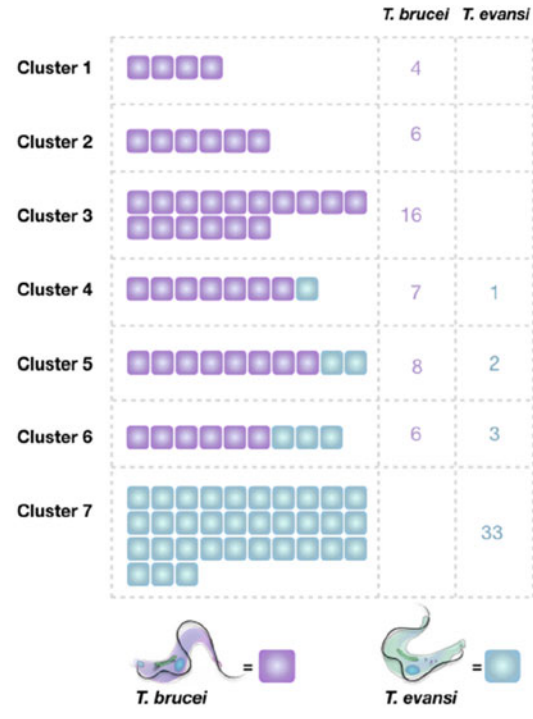

Fig. 1 Genetic clustering of a collection of Kenyan trypanosome field isolates. While some clusters contain distinct *Trypanosoma brucei* or *Trypamosoma evansi* parasites, other clusters contain a mix of the two with close genetic relation. Hence, different *T. evansi* parasites are considered to be derived from different *T. brucei* parasites

understanding of how parasites adapt to different hosts.

The Parasite Morphology

T. brucei is pleomorphic, with three main forms, all of which have a small kinetoplast and a conspicuous undulating membrane (Fig. 2).

Trypomastigotes or Long Slender Forms

These forms are 20–30 μm in length with a free flagellum, which may be up to one half of the length of the organism. The posterior end is pointed and the nucleus is central. The kinetoplast is situated in front of the posterior extremity. They are the proliferative stage of the parasite.

Metacyclic Trypomastigotes or Short Stumpy Forms

These forms may be 15–20 μm in length without a free flagellum. The kinetoplast is usually sub-terminal. In stained specimens, blue volutin granules are often present in the cytoplasm, often arranged in a line along the margin of the cell. They are the non-proliferative stage of the parasite.

Intermediate Forms

Intermediate forms of variable length, in-between two stages, are also found. In this form, a free flagellum is present. The nucleus is centrally placed. The posterior end is somewhat variable in shape, but is bluntly pointed. The kinetoplast is close to the posterior extremity. Volutin granules are occasionally present but neither as common nor as plentiful as in the short, stumpy forms.

The structural rigidity of the cell, in all these forms, is ensured by the microtubes that are aligned below the plasma membrane as longitudinal bundles. The only place where this structure is interrupted is at the level of the flagellar pocket, where a basal body anchors the single flagella and where all the endocytosis and exocytosis events take place. At the base of the flagellar pocket, the kinetoplast is located, which is made up of numerous circular DNA molecules. In the long slender form, the tip of the flagella is free and points towards the direction of motility of the trypanosome. The long slender form has a single simple mitochondrion extending anteriorly from the kinetoplast, and the cristae are short, few in number and tubular. The metacyclic stumpy form has larger mitochondrion extending anteriorly and posteriorly from the kinetoplast with numerous cristae and plate-like in appearance.

Cultivation of Parasites

In vitro cultivation methods are available for trypanosomes that allow a limited range of experiments to be executed, such as those that have been used in the context of the discovery of the quorum sensing regulation, i.e. parasite population density regulation. In short, most culture methods are based on the use of HMI-9 medium (Hirumi's modified Iscove's medium 9) that can be supplemented with 1.1% (w/v) methylcellulose, 15% (v/v) fatal calf serum and 5% (v/v) heat-inactivated human serum. Key to this medium choice is the presence of hypoxanthine (1 mM), bathocuproinedisulphonate (0.05 mM), β-mercaptoethanol (0.2 mM) and sodium pyruvate (1 mM). Procyclic cells can be grown in SDM-79 medium. In vitro, cell differentiation to the insect form (procyclic) parasites can be obtained by adding citrate and *cis*-aconitate (3 mM each) or 6 mM *cis*-aconitate and dropping culture temperature conditions from 37 °C to 27 °C. Cells need to be refreshed every 24–48 h.

Laboratory Animals

Most laboratory animal experiments have been performed in C57BL/6 mice (and many gene-deficient knockout variants) and BLAB/c mice. A smaller number of reports have documented results obtained in CBA/Ca mice, C3H/HeN

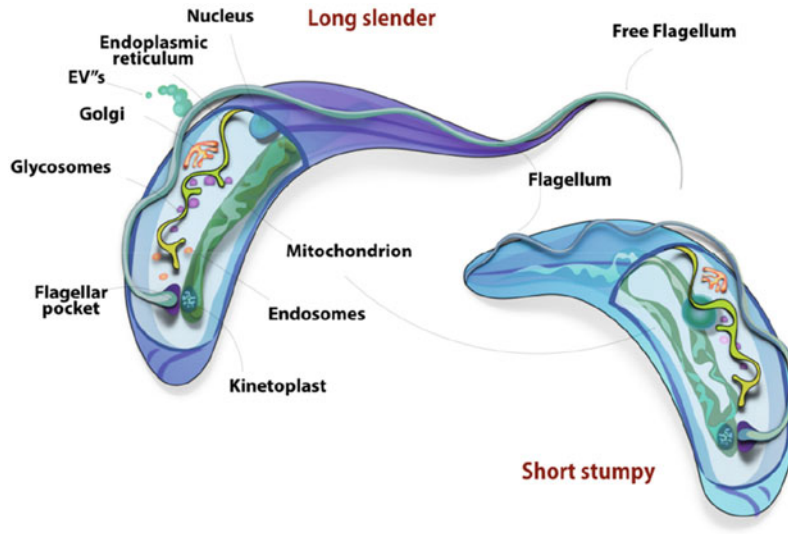

Fig. 2 *T. brucei rhodesiense* and *T. brucei gambiense* parasites are pleomorphic parasites, occurring in the blood of their mammalian host as both a long slender (LS) proliferating form and a short stumpy (SS) non-proliferating form. Both forms can be distinguished by their shape, the long free flagellum of the LS form and their motility, the latter being higher in the LS form. *Trypanosoma evansi* is only observed in the LS form in the blood of the mammalian host

(or J) mice or SWISS mice. F1 crosses have been mainly used in immunology hereditary determination experiments. Studies in AKR mice are of particular interest, as this stain has a natural C5 complement deficiency. The occurrence of 'regular' *T. brucei* parasitaemia control in this strain was the first indication that trypanosome growth in vivo can be regulated in large in a complement-independent manner. Experimental mouse infection is usually performed by intraperitoneal (IP) injection of around 5000 parasites (blood/PBS). This dose was determined based on the average trypanosome content of an infectious tsetse fly bite. If available, infections can be performed using infected tsetse flies, placed on the skin of mice in order to allow the flies to feed, resulting in natural disease transmission. Using intradermal needle injection can mimic some aspects of the natural bite transmission, but is only useful when the early onset of infection is being studied or when specific immunological skin features are being addressed. To study longer-term systemic host-parasite interaction events, the IP injection of parasites has been shown to deliver satisfactory results.

Fundamental understanding of trypanosomiasis-associated B cell destruction has been understood mainly from experimental murine infections with *T. b. brucei*.

Life Cycle of Trypanozoon Trypanosomes

Hosts

Primary Host

Tsetse fly (*Glossina* spp.)

Intermediate Host

Humans, domestic cattle and game animals like antelopes and wild buffaloes.

Infective Stage

Metacyclic form of the parasite.

Transmission of Infection

The life cycle of salivarian trypanosomes is completed in two hosts. Both *T. b. rhodesiense* and *T. b. gambiense* need a primary insect host of the genus *Glossina*, the tsetse fly, which is only present in Africa. Humans and some other animals get the infection from the insect vectors (Fig. 3).

Metacyclic stage of the parasite is introduced into the body of humans when the tsetse fly of the

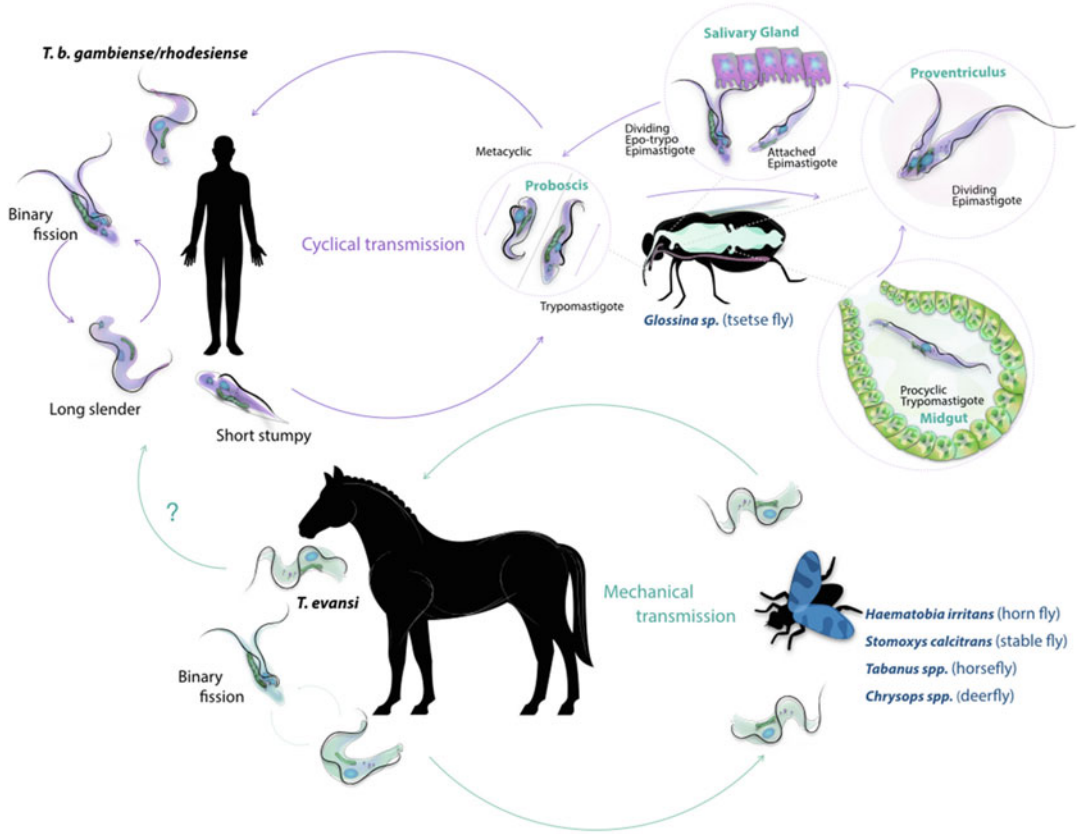

Fig. 3 The *Trypanozoon* life cycle. *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* both cause human African trypanosomiasis (HAT) and rely on tsetse fly-mediated cyclic transmission. The main host reservoir for *T. b. rhodesiense* consists of livestock and game animals. *T. b. gambiense* parasites are split into two groups. Group 1 is rather homogeneous and mainly anthroponotic. Group 2 parasites are much more heterogeneous and considered zoonotic. *Trypanosoma evansi* is an animal parasite that has lost the capacity to complete its life cycle in the tsetse fly. It can be passed between hosts by mechanical transmission. Several different biting fly species are responsible for disease transmission

genus *Glossina* takes a blood meal. The saliva of the fly contains this infective form coming from the salivary gland of the insect. The saliva also contains substance that inhibits blood clotting at the bite site. In the blood, the aflagellate metacyclic forms transform into flagellated trypomastigotes and start multiplying by longitudinal binary fission. The division starts at the kinetoplast followed by nuclear and cytoplasmic divisions. The long slender trypomastigotes are actively motile in the blood and lymph. In chronic infection, many invade the central nervous system where it continues to multiply. In the event of cessation of glycolysis and through quorum sensing, further division of trypomastigotes stops, and they transform into the short stumpy form after passing through a brief intermediate stage.

During the blood meal, the short stumpy forms enter the posterior section of the midgut of the fly where it multiplies into procyclic trypomastigote forms for about 10 days. Subsequently, they penetrate the peritrophic matrix that covers the gut epithelium and migrate to the ventriculus. The parasites can resist digestive enzymes and a strongly alkaline environment of the fly's gut. Then the slender forms migrate to the foregut, where they are found between the 12th and 20th days. They then move up to the oesophagus, pharynx and hypopharynx. Finally, they enter the salivary glands where they are transformed

into epimastigote form. After further asexual multiplications, they finally transform into metacyclic trypomastigotes. When feeding, a tsetse fly may inject up to several thousand parasites into the host. In the fly the whole cycle is completed in 15–35 days. During the saliva infection stage, the parasite downregulates the capacity of the fly to inject saliva containing anticoagulants and anti-platelet aggregation into the bite site. This results in a decreased feeding efficiency and in turn increases the likelihood of the tsetse feeding on multiple hosts, resulting in an increased chance of parasite transmission.

In comparison to *T. brucei*, *T. evansi* has a much simpler life cycle having lost the capacity to adapt to life within the tsetse fly vector. Hence, the long slender morphology is the only form seen in the bloodstream of the mammalian host. *T. evansi* efficiently relies on mechanical transmission. This non-tsetse fly-mediated transmission has allowed the parasite to move out of Africa and infect most parts of the rest of the world.

Pathogenesis and Pathology

In humans, trypanosomiasis is characterized by a first phase, the haemolymphatic stage, where the parasite invades the host's circulatory and lymphatic systems and causes immune-dysfunction. Initial infection is characterized by fever, weakness, enlarged lymph nodes and joint pains. If the parasite passes through the blood-brain barrier, the meningo-encephalitic stage begins, causing neuropsychiatric symptoms such as daytime sleepiness and nocturnal insomnia, due to the fragmentation of the circadian rhythm. Later symptoms lead to the death of the individual if left untreated. Those symptoms are also related to the popular name of human African trypanosomiasis, as sleeping sickness.

Symptoms of gambiense HAT are very similar to those for rhodesiense HAT, but the main difference is that it takes in general much longer for the disease to progress into the second stage. Both infections result in mild anaemia, but not to the extent that is being observed in animal trypanosomes causes by non-brucei trypanosomes.

Immunology

In contrast to many protozoan parasites that ensure optimal survival by hiding from the immune system inside host cells, salivarian trypanosomes remain extracellularly localized throughout the mammalian stage of their life cycle. Hence, these parasites are continuously exposed to attacks by the host innate immune system, as well as the adaptive immune system. To survive these attacks, salivarian trypanosomes have acquired multiple evolutionary strategies to evade and even destroy the host immune system. Evasion relies mainly on the system of antigenic variation exhibited by the parasites. Immune destruction involves the diversion and destruction of the host B cell response, resulting in an impairment of effective antibody production.

Immunity against animal trypanosomes in humans is provided by an innate system, the trypanosome lytic factors TLF1 and TLF2. Humans share this trypanolytic serum activity with gorillas and certain Old World monkeys. Human TLFs contain apolipoprotein A1, the primate-specific ion channel-forming protein apolipoprotein L-1 (APOL1) and the haemoglobin-binding protein haptoglobin-related protein (HPR). Uptake of TLF1 is mediated by the *T. brucei*-specific receptor TbHpHbR (haptoglobin-haemoglobin receptor). Interestingly, baboon APOL1 is much more potent than the human homologue. Hence, it confers resistant against all trypanosomes, even those causing HAT. Since *T. b. rhodesiense* is a human pathogen, it obviously acquired resistance against the human APOL1. This resistance is linked to the expression of a serum-resistant antigen (SRA), with SRA inhibiting the pore-forming capacity of APOL1 inside the acidic environment of the endocytic system of the trypanosome. In contrast, Group 1 *T. b. gambiense* parasites acquire their resistance by a more complex mechanism. This involves reduced uptake of TLF1 due to reduced expression of the HPHBR gene and a reduced ligand binding through mutations in the receptor protein sequence. Additional data has shown that in these parasites, the TgsGP molecule aids in APOL1 resistance by reducing trypanosomal

membrane fluidity. Finally, a third factor, not fully elucidated yet, relates to the action of a cysteine protease. For the heterogeneous Group 2 *T. b. gambiense* parasites, as well as for the human-infective *T. evansi* parasites, the APOL1 resistance mechanism remains to be elucidated.

Infection in Humans

The HAT is characterized by a first haemolymphatic stage phase, progressing towards a second meningo-encephalitic stage. If infections are left untreated, HAT most likely results in death.

First-stage HAT is not characterized by any specific symptoms, but may be accompanied by intermittent headache, fevers and joint pains. These symptoms may correspond with successive waves of parasitaemia and B cell and/or immune activation. Hepatomegaly, splenomegaly and lymphadenopathy are other manifestations. A range of other nonspecific symptoms that may be present include skin rash, weight loss and facial swelling. Neuroendocrine disturbances leading to amenorrhoea in women or impotence in men have been documented. The first stage of *T. b. gambiense* infections can last several years.

Second-stage HAT is characterized by CNS inflammation and an increase in cerebrospinal fluid IgM tires and white blood cell counts ($\geq$20 cells µl). This stage is characterized by the disturbance of the sleep cycle, resulting in night-time insomnia and daytime somnolence.

Infection in Animals

Domestic cattle as well as a wide range of wild animals including buffaloes and antelopes are the main reservoir hosts of *T. b. rhodesiense*. Cattle is a reservoir for the zoonotic transmission of Group 2 *T. b. gambiense*. *T. evansi*, because of the wide geographic spread of the parasite, as well as the increased virulence as compared to *T. brucei*, which is an important parasite of animals. In many host species such as horses, camel, cattle, dogs and even rats, *T. evansi* infections are characterized by anaemia, loss of appetite, weight loss, oedema, fever, salivations, lacrimation and abortion. Neuropathological features including paralysis of the hind limbs are observed with *T. evansi* infections particularly in horses.

Epidemiology and Public Health

In 2018, *T. b. rhodesiense* trypanosomiasis was reported in six sub-Saharan countries, including Kenya, Malawi, Uganda, Tanzania, Zambia and Zimbabwe (Fig. 4/Table 1). The same year, no cases were reported in Burundi, Ethiopia, Mozambique and Rwanda, all countries that in the past were considered endemic for the disease. Together, only 24 cases were reported to the WHO and its partners, representing only 2% of the overall HAT burden for that year. HAT cases were reported in South Africa, the Netherlands, China (each two cases) and France, Germany and India (each one case for the 2017–2018 period). In addition, rhodesiense HAT account for two-thirds of all tourist HAT cases.

T. b. gambiense HAT is still considered the major HAT infection problem, making up for 98% of all cases reported to the WHO and its partners. In total, 953 infections were reported in 2018, in 15 sub-Saharan countries (Fig. 4). Eight countries that are considered to be endemic for gambiense HAT did not report any cases in 2018, and two countries (Gambia and Liberia) did not report any surveillance activities. Cases of gambiense HAT have been dramatically reduced over the last 10 years, as back in 2009, there were still nearly 10,000 case reports. The important epidemiological features of salivarian trypanosomes are shown in Fig. 4.

As *T. evansi* is a mechanically transmitted animal parasite, it has moved out of Africa and can be found in South and Central America, various regions in Africa, the Middle East, China, the Indian subcontinent and the Southeast Asia. The main mammalian host in Africa and the Middle East is considered to be camels. In South America, the main host reservoir is found in horses and local animals such as capybaras. In Asia, *T. evansi* is mainly found in water buffaloes where it serves as a reservoir for parasite transmission to cattle, pigs and goats. More ‘exotic’

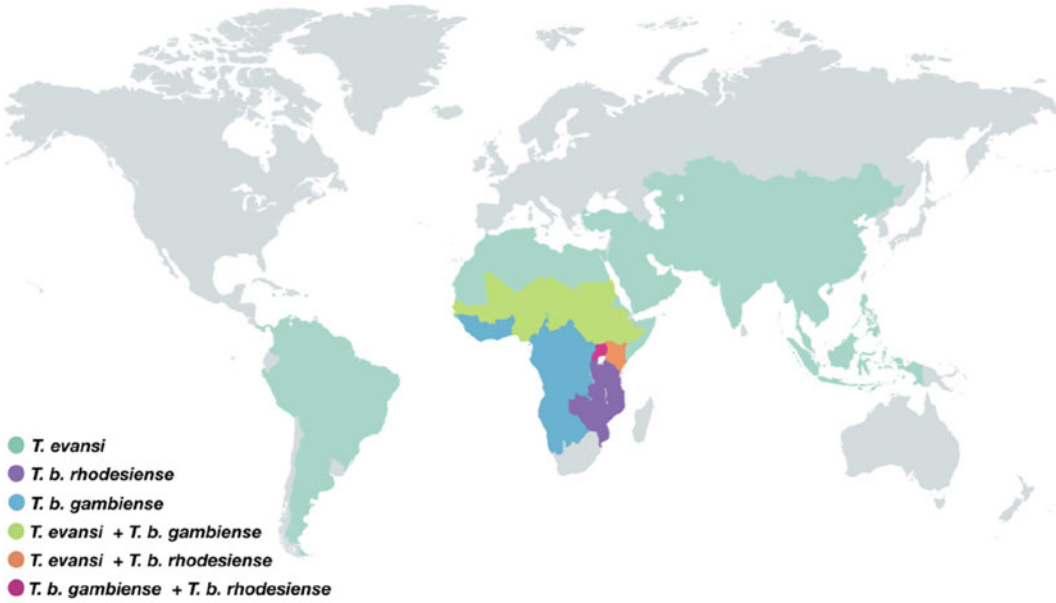

Fig. 4 Geographic distribution of *Trypanosoma brucei rhodesiense*, *Trypanosoma brucei gambiense* and *Trypanosoma evans*i. Due to the wide range of vectors involved in the spread of *T. evansi*, this parasite has moved out of Africa and is now present in large parts of the world. In contrast, the two human-infective *Trypanosoma brucei* subspecies are found only in the sub-Saharan African tsetse belt

animals such as elephants and deer are known to serve as a parasite reservoir in the wild. One of the problems with *T. evansi* is the fact that many infected animals hardly show any symptoms, resulting in the spread of infection through the transport of seemingly healthy animals. This has led to occasional outbreaks such as those reported in Spain and France, after the introduction of infected camels originating from the Canary Island.

Table 1 Epidemiological features of important salivarian trypanosomes

SN	Species	Host	Vector	Geographic distribution	Human infection
1.	*Trypanosoma brucei* subsp. *gambiense*	Humans, bovines	Tsetse fly (*Glossina* spp.)	West and Central Africa	Most common
2.	*Trypanosoma brucei* subsp. *rhodesiense*	Humans, bovines	Tsetse fly (*Glossina* spp.)	East and Southern Africa	2% of all HAT cases
3.	*Trypanosoma evansi*	Equines, bovines, camelids	Tsetse fly *Glossina* spp., stable fly (*Stomoxys* spp.), horse fly (*Tabanids* spp.), deer fly (*Chrysops* spp.)	Central and South America, North Africa, the Russian territories, the Indian subcontinent, China and Southeast Asia	Infrequent
4.	*Trypanosoma vivax*	Bovines, ovines, caprines, equines	Tsetse fly *Glossina* spp., stable fly *Stomoxys* spp., horse fly *Tabanids* spp.	Africa, South America	Extremely rare, lack of reliable reporting
5.	*Trypanosoma congolense*	Bovines	Tsetse fly *Glossina* spp.	Sub-Saharan Africa	Extremely rare, only reported as mixed infection with *Trypanosoma brucei gambiense*

Diagnosis

As the clinical signs of HAT in general are rather unspecific, the first-line diagnosis that relies on symptoms and epidemiological assessment is inefficient. Hence laboratory diagnosis (Table 2) is essential for the treatment of the condition.

Microscopy

Microscopic detection of the parasite is the definite technique for the diagnosis of HAT (Fig. 5). However, since the trypanosome concentration in blood is often below the detection limit of conventional microscopy, concentration techniques, such as buffy coat preparation, are necessary for high yield of parasites. Mini ion exchange chromatography (mAECT) is also used to evaluate parasites from blood samples, prior to microscopy. The use of fluorescent dyes that intercalates nucleic acids can result in high-sensitive detection of parasites by fluorescent microscopy. Microscopy analysis of aspirate fluid from swollen cervical lymph nodes is an alternative tool, used when parasites cannot be detected in the blood. Cerebrospinal fluid can be analysed to confirm the neurological second stage of the infection.

Serodiagnosis

As trypanosomes induce a strong humoral response in their mammalian host, antibody-based diagnostic tests are considered a primary screening tool. Their use has in large contributed to the recent successes in the control of *T. b. gambiense* infections.

The card agglutination test for trypanosomiasis (CATT) is used for the detection of *T. b. gambiense* and *T. evansi* infections (Fig. 5). No such test is available for rhodesiense HAT. HAT/CATT is based on the detection of antibodies that cross-react with particular VSG molecules of laboratory-cultured trypanosomes, i.e. the *T. b. gambiense* LiTat 1.3 and LiTat 1.5 clones. The test is characterized by high sensitivity and specificity, but a lower positive predictive value. This means that often a majority of CATT-positive individuals scores negative in a parasitological assay. However, the very high negative predictive value allows to exclude vast numbers of people from further microscopy screening, a technique that requires skilled analysts and is time-consuming. When found positive by both CATT and microscopy screening, patients have to undergo a 'staging' screening, meaning that the analysis of cerebrospinal fluid is needed to determine whether or not parasite has crossed the blood-brain barrier.

In recent years, several efforts have been undertaken to transform the principle of the CATT into more user-friendly lateral flow formats. These tests are currently still being improved and evaluated under various field conditions. In the end however, antibody-based test will always suffer the drawback of measuring exposure, rather than actual infection. Hence, there are several reasons why most antibody-based trypanosome tests will always have a low positive predictive value. First, there seem to be many individuals with cross-reacting anti-LiTat antibodies that have never suffered HAT, but might have other underlying conditions such as allergies that generate poly-reactive antibodies. Second, it is quite possible that individuals who live in *T. b. brucei* endemic areas are regularly exposed to bites by infected tsetse flies. There is no reason to assume that these infected bites cannot cause a cross-reactive host antibody response, upon efficient lysis of the *T. b. brucei* parasites by the human TLFs. Finally, anti-trypanosome treatment of infected individuals will result in the induction of anti-trypanosome antibodies that will remain in the circulation long after the parasite has been eliminated.

A diagnostic CATT test specifically targeting *T. evansi* has been available since the 1990s. It targets the RoTat1.2 VSG. Nevertheless, the test is unsuitable for *T. evansi* detection in regions where RoTat1.2-negative parasites occur, the so-called *T. evansi* Type B, or the RoTat1.2-negative *T. evansi* Type A, described in Kenya.

Molecular Diagnosis

With the prevalence of HAT decreasing due to the surveillance and treatment successes, there is a need for diagnostic tools that can directly detect the parasite or components released/secreted by

Table 2 Diagnostic methods for human African trypanosomiasis

Diagnostic approach	Samples/methods	Target	Remarks
Microscopy	Blood, bone marrow, CSF, lymph node aspirate. Concentration techniques in blood (mini ion exchange chromatography; micro-haematocrit concentration). Giemsa stain, fluorescent stain. Unstained preparation for motile forms	Trypomastigote form	Most definitive method Low detection limit
Immunological tests	Card agglutination test	Antibodies against variable antigen type LiTat 1.3/1.5	Useful for mass screening of whole blood for control/elimination for *Trypanosoma brucei gambiense* and *Trypanosoma evansi.* Not available for *Trypanosoma brucei rhodesiense*
Molecular diagnosis	PCR, LAMP	18S ribosomal RNA, RoTat1.2 VSG gene	No large-scale field application yet

the parasite. PCR is now increasingly evaluated and used for species-specific diagnosis of *Trypanosoma* species causing trypanosomiasis, both in humans and animals. While PCR is a sensitive technique for direct pathogen detection, this technique has significant limitations in point-of-care (POC) resource-poor field settings. As alternatives, isothermal PCR diagnostic solutions such as LAMP have been developed at experimental level but are still not being implemented on a large scale. Currently, POC tools with a very high positive predictive value are however becoming crucial when taking into account that in a zoonotic setting, asymptomatic animals can

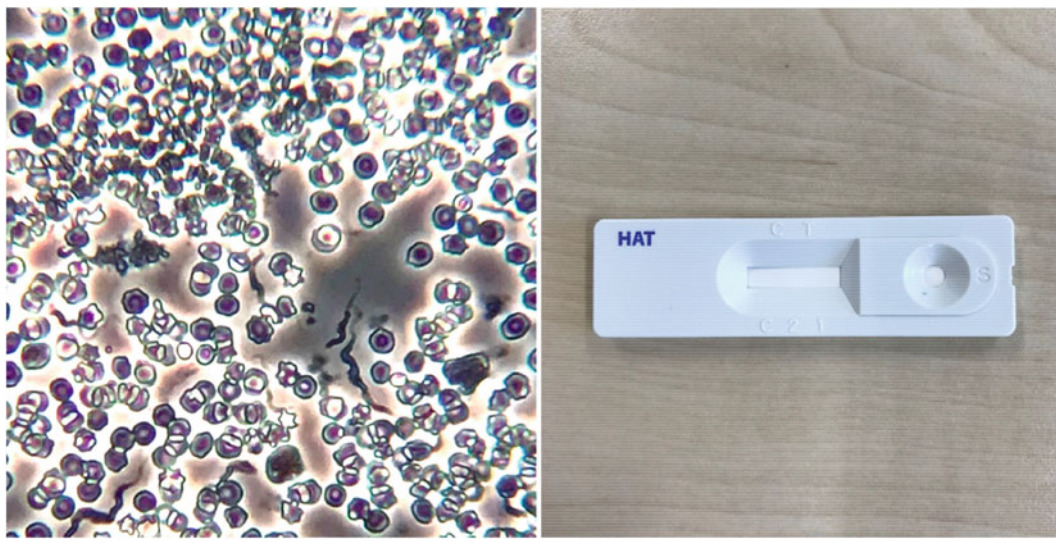

Fig. 5 Phase-contrast microscopy image of *Trypanosoma brucei rhodesiense*, parasite (unstained) as observed with a regular field microscope, using a 20× magnification and a cell phone adaptor for image capturing (left). Microscopy validation is still the only accepted method for true positive case determination. Rapid diagnostic tools, such as this SD Bioline HAT prototype, are rapidly replacing the CATT assay (right). These tests are based on anti-parasite antibody detection; hence they score trypanosome exposure rather than active infection

serve as an everlasting reservoir for human-infective parasites.

For *T. evansi* diagnosis, there is a drive to implement genetic testing that allows direct parasite detection. Here, PCR detection of the gene encoding the RoTat1.2 VSG allows for accurate detection of *T. evansi* in most geographic locations, as does LAMP. Recently, an alternative diagnostic test based on recombinase polymerase amplification (RPA) has been developed. It has been combined with lateral flow detection for easy and rapid result interpretation. The detection of *T. evansi* Type A is achieved through isothermal amplification at 39 °C of the *T. evansi* RoTat1.2 VSG gene. Results of the test are obtained within 20 min. For the detection of *T. evansi* Type B, which does not express RoTat1.2 VSG, loop-mediated isothermal amplification (LAMP) of DNA has been shown to be a more sensitive tool compared to classical PCR tests. The current RPA technology, however, has not been adapted yet for the detection of *T. evansi* Type B.

Treatment

Despite being used for nearly a century and despite inducing a string of severe negative side effects, suramin is still being applied as a major curative treatment for early stage *T. b. rhodesiense* HAT.

Melarsoprol is used as first-line curative treatment of *T. b. rhodesiense* HAT, irrespective of actual disease staging. Being an arsenical compound, the drug has high toxicity and severe side effects, inducing reactive encephalopathy resulting in fatal outcome in up to 10% of patients. Hence, in optimal circumstances, this drug needs to be restricted in its use for treatment of second-stage *T. b. rhodesiense* infection only.

Pentamidine has for long been the most used drug for the treatment of first-stage *T. b. gambiense* HAT. For the treatment of second-stage *T. b. gambiense* HAT, a combination of nifurtimox and eflornithine (NECT) has been introduced in 2009. This combination therapy reduces the complexity of the previously used eflornithine therapy. Both drugs are provided free by the WHO to endemic countries. A free-of-charge kit with all administration necessities is available.

Most recently, in 2018, fexinidazole has been made available as an oral therapy for *T. b. gambiense* HAT and has been incorporated in the WHO interim guidelines as one of the first-line treatments for HAT. The drug is also used to cure non-severe second-stage patients.

No standardized treatment strategy for the treatment of *T. evansi* HT is available so far. However, successful cure of *T. evansi* HT has been achieved by using the rhodesiense HAT treatment scheme using suramin.

Treatment of animal trypanosomiasis relies on the use of diminazene diaceturate. This compound is used effectively for the treatment of *T. evansi* infections in animals, but has not been registered for use in human due to severe side effects of the treatment in animal, including dogs. Diminazene does not cross the blood-brain barrier; therefore it is not effective in the case of CNS infections.

Prevention and Control

Currently, no universal methodology to control HAT is available. Nevertheless the 'National Sleeping Sickness Control Programmes' (NSSCPs) are supported by the WHO focusing on implementing control activities and capacity building through training. Control and surveillance rely on active and passive case finding, diagnosis, treatment, follow-up and control of the animal reservoir.

Controlling the spread of HAT also relies on vector control. Indeed, the control of the tsetse fly population by using nets and insecticidal spraying has helped reduce the number of HAT cases. However, control of the spread of *T. evansi* infections is much more difficult, due to the wide range of biting insects involved in parasite transmission. To avoid the spreading of surra, it has been suggested that equines should be bred several kilometres apart from cattle, which usually act as a reservoir. Monitoring of international

trading and quarantine measures are both essential to avoid introducing infected animals in non-infected countries. Failure to implement these rules can lead to unexpected disease outbreaks such as those that happened in Southern Europe in the recent past. Prevention of aHT by *T. evansi* is difficult. This is because these infections are rare; most are under-reported and occur in situations where humans live in close proximity to infected animals such as water buffaloes that can serve as asymptomatic trypano-tolerant reservoirs. In resource-poor areas, this is a risk factor that is very hard to control, in particular when multiple different insect vectors can be responsible for zoonotic transmission. Here, large-scale animal surveillance efforts and herd-treat livestock animals are crucial to limit the risk of disease transmission.

No vaccine is currently available for the prevention of either human or animal trypanosomiasis. One reason for this is the presence of the inexhaustible VSG gene repertoire encoding the major surface protein. However, in between the VSGs, there are a number of invariant surface glycoproteins present that have been the target of several alternative vaccination approaches. None of these, however, has resulted in any success.

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Case Study

As there are many typical case reports of both rhodesiense and gambiense HAT available, one report that stands out is the identification of an atypical *T. evansi* HT infection in 2015. This was the first HT infection diagnosed at both serological and molecular level in Southeast Asia. The report covers the case of a 38-year-old woman who presented to a healthcare facility in southern Vietnam. Her symptoms included non-species issues such fever, headache and joint pain. Interestingly, the report included the APOL1 measurement in the patient's blood, showing that there was no genetic deficiency that could easily explain the susceptibility to infection. This report followed a decade of APOL1 research, where a consensus grew that this molecule was indeed the most important factor in the trypanolytic activity of human serum. Hence, with full trypanolytic activity being present in this case, it remains to be discovered how some *T. evansi* trypanosomes survive in human blood, while being devoid of the known *T. b. rhodesiense* and *T. b. gambiense* resistance factors.

Research Question

1. Can anti-trypanosome immunity be induced by vaccination, and can vaccine-induced memory against any trypanosome target be recalled upon infection fast enough to stop the imitation of immune destruction by the parasite?
2. Does host pathology and inflammation contribute directly to the signals that drive quorum sensing during peak parasitaemia?
3. Which mechanisms allow *T. b. gambiense* Group 2 and *T. evansi* to avoid APOL1-mediated trypanolysis in aHT?
4. What is the mechanism of the uptake of the major trypanolytic factor, i.e. TLF2, and which resistance mechanism is operated by *T. b. gambiense* Group 1 parasites that allows survival in human serum?

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American Trypanosomosis

V. C. Rayulu and V. Gnani Charitha

Learning Objectives

1. To have knowledge about alternate modes of disease transmission apart from the classical vector transmission through inoculation.
2. To know various forms of clinical manifestations depending on the routes of infection.
3. To have an understanding of the importance of microscopic examination in diagnosis and strain identification by molecular techniques.

Introduction

The protozoan parasite *Trypanosoma cruzi*, responsible for causing American trypanosomosis, was discovered by the Brazilian scientist Carlos Chagas in the year 1909. The disease is endemic to large parts of Latin American countries, with the exception of the Caribbean islands. However, in recent decades, it has progressively been diagnosed worldwide highlighting its growing significance in the USA, Europe, Canada, Eastern Mediterranean and Western Pacific countries. Out of all, people mostly from Latin America are more prone to be infected with *T. cruzi*, and it is considered one of the neglected diseases. Chagas disease is mainly communicated to human beings through contact with faeces/urine of infected blood-sucking bugs, viz. kissing bugs or conenose bugs (belonging to subfamily Triatominae). Among these *Triatoma infestans*, *Triatoma dimidiate*, *Rhodnius prolixus* and *Panstrongylus megistus* are considered as being the most important vectors.

History

Approximately, 7–10 million years ago, *T. cruzi* ancestors were probably introduced to South America via bats. Several travellers and physicians documented records of patients with disease symptoms similar to American trypanosomosis during the sixteenth century. Nevertheless, the critical role of triatomine bugs as vectors in transmitting Chagas disease remained unexplored until 1909. Identification of *T. cruzi* and triatome bugs as the transmission vector of Chagas disease came into limelight only at the beginning of the twentieth century. The disease was first described by Carlos Ribeiro Justiniano Chagas in a 2-year-old baby named

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Berenice suffering from fever and swollen lymph nodes and with hepatosplenomegaly. Trypanosomes which were identical to those seen in the gut of triatomine bugs were seen in the patient's blood. He named the parasite *Trypanosoma cruzi* in honour of Oswaldo Cruz. As a tribute to his remarkable discovery, the World Chagas Disease Day was established to be celebrated on 14 April in memory of the date of the year 1909 when Carlos Chagas diagnosed the first human case of the disease.

Taxonomy

Trypanosoma cruzi taxonomic classification is based on *An Illustrated Guide to the Protozoa*, 2000, by John J. Lee. The genus *Trypanosoma* belongs to the family Trypanosomatidae, order Kinetoplastida, subphylum Mastigophora and phylum Sarcomastigophora in the subkingdom Protozoa and kingdom Protista.

T. cruzi are stercorarian trypanosomes which undergo posterior station (hindgut) development in vectors and are transmitted via faecal contamination of bite site to infect blood and tissues of vertebrate hosts.

Genomics and Proteomics

T. cruzi consists of mitochondrial genome composed of 30 copies of 20–50 kb maxicircles and thousands of copies of ~1 kb minicircles, which together comprise the kinetoplast DNA or kDNA. The whole genome sequencing was done in 2005. It has revealed that the diploid genome contains a predicted 22,570 proteins encoded by genes, of which 12,570 represent allelic pairs. Over 50% of the genome consists of repeated sequences that comprises retrotransposons and genes for large surface molecules. It has a highly plastic genome, an unusual gene organization and complex mechanisms for gene expression such as polycistronic transcription, RNA editing and trans-splicing.

T. cruzi belongs to heterogeneous population comprising a pool of strains that shift between the domestic and sylvatic cycles involving human beings, vectors and animal reservoirs of the parasite. Extensive study on *T. cruzi* populations from different origins demonstrated the presence of a variant strain with marked characteristics. At present, six distinct genealogies of *T. cruzi* are classified into Tc-I, II, III, IV, V and VI discrete typing units that vary in geographical distribution, host specificity and pathogenicity. Completion of the genome sequence of the *T. cruzi* CL Brener strain 31 possibly opens prospects for the development of novel therapeutic and diagnostic techniques.

A total of 2784 proteins in 1168 protein groups from the annotated *T. cruzi* genome in its life cycle have been identified by peptides mapping. Protein products were identified from 91,000 genes annotated as "hypothetical" in the sequenced genome. The four parasite stages appear to use different energy sources like histidine for stages present in the insect vectors and fatty acids by intracellular amastigotes.

The Parasite Morphology

T. cruzi is characterized by three morphological forms, viz. trypomastigote, epimastigote and amastigote.

Trypomastigote

It is found in the peripheral circulation measuring about 20 μm in length and generally slender and exhibits pleomorphism. They are present as elongate slender dividing forms (with long free flagellum) or stumpy non-dividing infective (metacyclic) forms with no free flagellum. They have a thin, irregularly shaped membrane, with centrally positioned nucleus and a posteriorly situated kinetoplast (Fig. 1). A flagellum arises at the kinetoplast and traverses the entire length of the parasite and extends beyond it. A single mitochondrion is present inside the kinetoplast that drives the flagellum. In stained preparations, trypanosomes generally are seen in a C or U shape. The identification of the parasite is usually

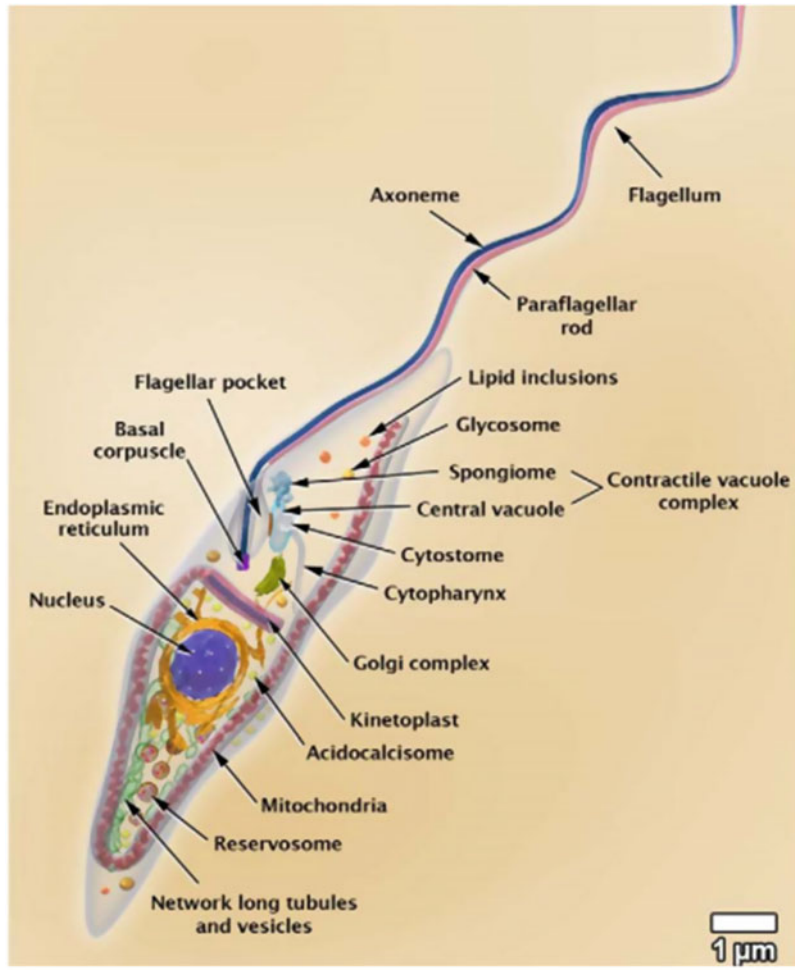

Fig. 1 Schematic representations of *Trypanosoma cruzi* trypomastigote organelles – 3D model (Source Teixeira et al. 2012)

made by its morphological features and needs to be invariably different from *Trypanosoma rangeli*, a non-pathogenic flagellate that infects humans in Central and South America and is transmitted by the same vectors that transmit *T. cruzi.*

Epimastigote

This stage is more or less similar to the trypomastigote stage except that the kinetoplast is located anterior to the nucleus (Fig. 2). Size of the epimastigote measures 10–35 µm in length by 1–3 µm in width.

Amastigote

These are present within the host cells. They are generally round in shape, and the flagellum becomes nearly unapparent (Fig. 3).

Cultivation of Parasite

Specialized systems are available to grow the epimastigotes in axenic culture media. The parasite count is carried out by haemocytometer or automated methods. This helps in assessing the rate of growth or killing potential in drug assays.

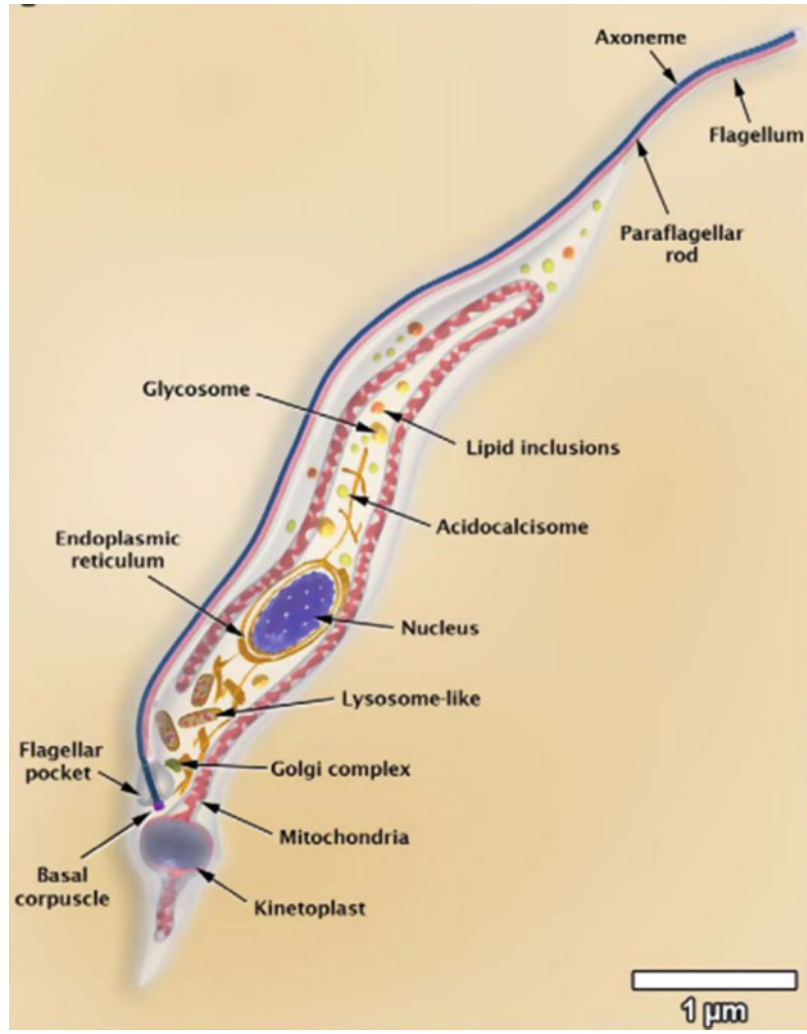

Fig. 2 Schematic representations of *Trypanosoma cruzi* epimastigote organelles – 3D model (Source Teixeira et al. 2012)

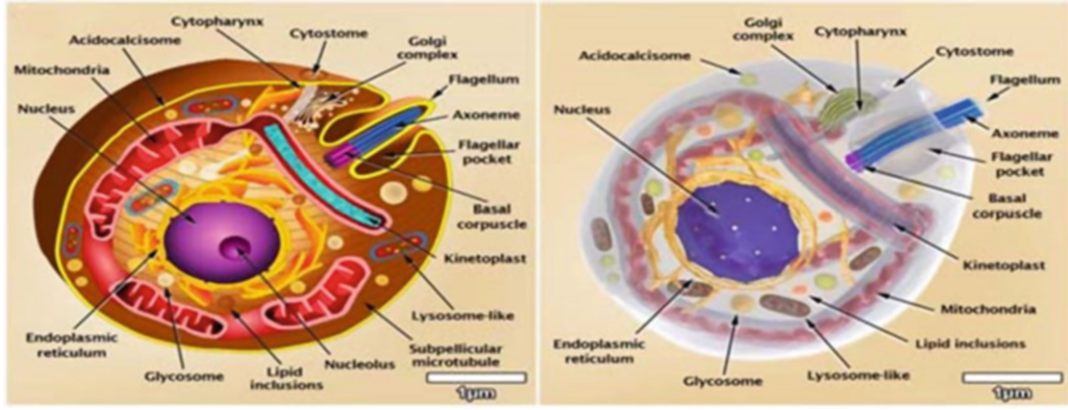

Fig. 3 Schematic representations of *Trypanosoma cruzi* amastigote – 2D and 3D models (Source Teixeira et al. 2012)

Laboratory Animals

Mouse is an excellent model for the study of both acute and chronic *T. cruzi* infections. Thus the murine model is most commonly used to assess the activity of new drugs against *T. cruzi*. Other laboratory animals include rodents, dogs, guinea pigs and primates.

Life Cycle of Trypanosoma cruzi

Hosts

Primary Host

Humans, animals living in close proximity to humans (cats, dogs, wood rats, opossums).

Intermediate Host

Triatomine bugs (*Triatoma infestans*, *Rhodnius prolixus*, *Triatoma dimidiata* and *Panstrongylus megistus*).

Infective Stage

Metacyclic trypomastigotes are the infective stage.

Transmission of Infection

T. cruzi infective form present in reduviid bug faeces enters through the bite wound or scratch wounds but does not invade intact skin. Infective forms are also transmitted to humans by blood transfusion, organ transplantation and contaminated food and drink, through breast milk and congenitally through the placenta (Figs. 4 and 5).

The life cycle of *T. cruzi* includes both vertebrate and invertebrate hosts comprising three well-defined developmental stages (trypomastigotes, epimastigotes and amastigotes). These developmental stages have evolved so that they are conditioned to their individual surroundings which serve multiple purposes which include improved transmission potential, evasion of host immune system and long-term survival. The circulating trypomastigotes in the blood are non-dividing forms which can infect new cells of various tissues in the body. In the cytoplasm of the host cells, trypomastigotes are transformed into aflagellate amastigotes which are the dividing form of *T. cruzi* in mammals.

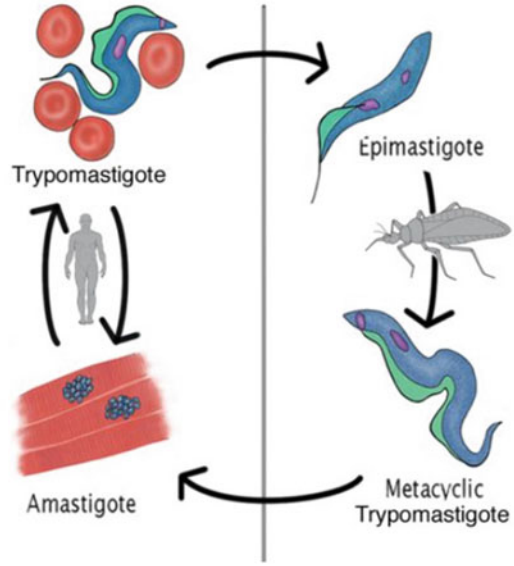

Fig. 4 Developmental stages of *Trypanosoma cruzi* in vertebrate and invertebrate. (Adapted from: Jimenez 2014)

These amastigotes undergo repeated multiplication over a period of 4–5 days and are again transformed into flagellate trypomastigotes and in the process cause death of the infected host cell. The trypomastigotes are released in circulation which can infect new host cells, or they may be taken up by the reduviid bug during their bite. In the gut of the insect, the trypomastigotes metamorphose into rapidly dividing epimastigotes. After a period of a few weeks, these epimastigotes become the metacyclic form, which is the infective stage for mammalian hosts.

Transmission Pathways

In Latin America, *T. cruzi* parasites are mainly transmitted to the host through contact with faeces/urine of infected blood-sucking triton bugs. The primary vectors to humans are the species that inhabit human dwellings, viz. *T. infestans*, *R. prolixus*, *T. dimidiata* and *P. megistus*. The triatomine bugs typically live

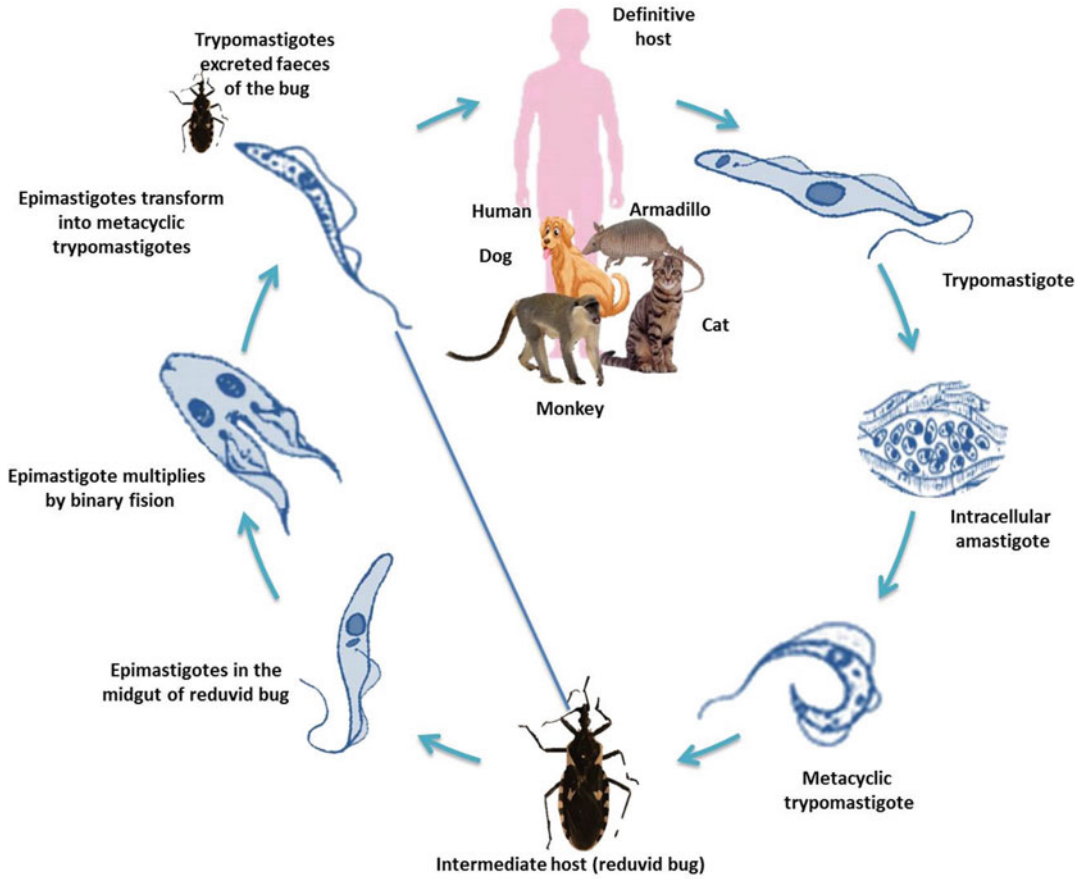

Fig. 5 The life cycle of *trypomastigotes cruzi*

in the walls or roof cracks of homes and peridomiciliary structures. The bugs are nocturnal in nature and are active during nights as and when they feed on mammalian blood. They usually bite on the face/near eyelids and are habituated to defecate or urinate close to the bite site. The parasite enters the body when the individual scratches the bite area causing microabrasions and thus facilitating the entry of contaminated bug excreta.

In addition to classical transmission by the vector, Chagas disease is also transmitted through consumption of food contaminated with *T. cruzi.* Food material may be contaminated with bug faeces, and ingestion of such food is a cause of food-borne transmission, associated with more severe morbidity and high mortality. Other transmission pathways include blood transfusion and organ transplantation or across the placenta during pregnancy. According to recent reports, 22.5% of new infections occurred through congenital transmission. It has also been reported that the infection is capable of being transmitted sexually.

Pathogenesis and Pathology

Pathogenesis of the disease during the early phase is reflective of parasite multiplication and immunological reactions of the host to the parasite. The progression of the infection and parasite replication is controlled by a combination of innate response in the form of NK cells and macrophages and the adaptive response by the proliferation of parasite-specific antibodies. This response is triggered by various pro-inflammatory cytokines like TNF-α and IFN-γ. Chronic phase of the disease is associated with progressive

multiplication of the parasite and concomitant tissue injury and damage along with immunopathological mechanisms. Up to 30% of infected people develop cardiac anomalies and 10% exhibit digestive, neurological or mixed anomalies. As the disease advances, the heart becomes enlarged with cardiac muscle fibres being replaced by scar and fat tissues. Parasites are rarely detected in the heart tissue since they are present at very low levels particularly at later stages of the disease. There may be a massive loss of nerve endings in the heart, colon and oesophagus in the chronic stages of the disease. This condition may contribute to arrhythmias and cardiomyopathy, while in the colon and oesophagus, loss of nervous system control leads to organ dysfunction, blockage of the oesophagus or colon and finally the enlarged organs.

Immunology

The recognition of *T. cruzi* by the immune system depends on both innate and adaptive immune responses of the body. At the outset, the pathogen-associated molecular patterns get recognized by Toll-like receptors of B and T cells which play an important role in bridging humoral and acquired immunities. The innate and adaptive immune responses are characterized by the recruitment of macrophages, dendritic cells, NK cells and B and T lymphocytes along with the cytokines produced by these cells. IFN-γ has an important role as it enhances the production of nitric oxide by macrophages that can destroy the intracellular *T. cruzi*. The key mechanism for systemic protection against *T. cruzi* infection is attained by CD4+ Th1 lymphocyte. It stimulates the production of IL-2 and IFN-γ that in turn trigger the proliferation of cytotoxic CD8+ T lymphocytes. CD8+ T cytotoxic cells produce IFN-γ which in turn activate macrophages, and these activated macrophages along with the perforins produced by CD8+ T cells are instrumental in killing parasite-infected cells. Thus, Th1 response plays a crucial role in *T. cruzi*, while humoral immunity does not play a considerable role. Effective immune evasion mechanisms adopted by the parasite include modulation of the complement system and exerting inhibitory effects on the monocyte-macrophage cells which leads to the chronic phase of the Chagas disease.

Infection in Humans

The initial acute phase lasts for about 2 months after infection. During the long-term chronic phase, the parasites are hidden mainly in the heart and digestive muscles.

The acute stage of the disease is often mild that includes nonspecific manifestations like pyrexia, headache, lymphadenopathy and hepatosplenomegaly. A nodule may appear, and if it is on the eyelid, the condition is known as *Romaña's sign*, and if it is in any part of the body on the skin, it is termed a *chagoma*. Severe acute disease may occur in fewer than 5% infected persons and may turn fatal due to inflammation and fluid accumulation in the heart or brain.

The *indeterminate* chronic Chagas disease is often asymptomatic. However, in 14–45% of people, the disease is manifested in cardiac form with cardiomegaly with cardiac failure and abnormalities in the microvasculature. Further, in 10–21% of people, digestive system involvement is associated with mega-oesophagus or mega-colon. Mega-oesophagus predisposes to odynophagia/dysphagia and acid reflux. Mega-colon may result in constipation or even blockage of intestinal blood supply. About 10% of cases develop neurological manifestations like numbness and altered reflexes or movement.

Symptoms may differ for people infected with *T. cruzi* through other modes of transmission. Persons infected through ingestion of contaminated food and water with faeces of reduviid bug develop severe signs within 3 weeks of consumption. This may include severe nausea and vomiting and dyspnoea, with acute abdominal and chest pain. In infections due to blood transfusion or organ transplantation, the features are similar to those of vector-transmitted disease. Immune-compromised individuals (HIV patients) or those receiving immunosuppressive therapy suffer from severe symptoms associated with inflammation in the brain and surrounding tissue or even brain abscesses.

Infection in Animals

The clinical signs of American trypanosomosis are variable and nonspecific in animals. Dogs acquire infection through faeces of infected reduviid bugs. The bugs often defecate on or near the wounds of the animals and dogs ingest the faeces when licking their wounds. Dogs are also infected by eating infected insects or eating rodents that are infected with *T. cruzi* parasites.

Most infected dogs demonstrate lethargy, decreased appetite and weight loss. In more severe cases, dogs develop signs of heart failure and arrhythmias. Pet owners can observe signs such as fainting, exercise intolerance, vomiting and diarrhoea. Sudden death may occur due to heart failure. Other animals including non-human primates do not typically show any signs of illness.

Epidemiology and Public Health

In the past two decades, Chagas disease has spread to more uninfected areas compared with its evolution since over 9000 years ago. Human activities leading to environmental changes like deforestation are the main culprit for the spread of Chagas disease. Infection caused by *T. cruzi* existed among wild animals but later spread to domestic animals and humans, with relative intensification, since beginning of the twentieth century. *T. cruzi* has been isolated from more than 100 species of wild and domestic mammals. Racoons, wood rats, opossums, non-human primates and dogs are typical mammalian reservoirs. The wide variety of mammalian hosts that *T. cruzi* can infect and the fact that chronically infected animals have persistent parasitaemia result in an enormous sylvatic and domestic reservoir in enzootic regions. This in turn contributes to establishment of the domiciliary cycle of transmission of the parasite in human dwellings.

Chagas disease is a burning public health problem in South America, causing more than 10,000 deaths per year (Fig. 6). Current situation clearly emphasizes that the disease is increasingly becoming a global health concern due to migration of people infected with *T. cruzi* from endemic countries to other parts of the world (Fig. 7 and Table 1). The total estimated number of Chagas patients outside Latin America is more than 4 lakhs with the USA being the most affected country accounting for three-fourths of all cases. Vectors are important in endemic areas, while in non-endemic countries, the main routes of transmission are blood and congenital transmission.

Diagnosis

Detection of *T. cruzi* infections is carried out by conventional parasitological, serological and molecular techniques (Table 2).

Microscopy

The trypomastigotes are most abundant in the peripheral blood during bouts of fever, but may be difficult to detect during the chronic stage of the disease. Fresh specimen of unstained blood or CSF should be examined to observe the motile parasites. Both thick and thin blood films are prepared like malaria parasite and stained by Giemsa or a similar stain. In microscopy, the trypomastigotes are slender and 15–20 μm in length with pointed posterior ends, and they typically appear C or U shaped in appearance. Free flagellum and an undulating membrane may be visible. The kinetoplast is subterminal in position. The sensitivity of blood specimen detection with microscopy ranges from 50% to 95% and is influenced by several factors, ranging from the quality of the microscopic equipment to the expertise of the observer. Amastigotes can be detected in biopsy specimens.

Blood screening for parasite is vital to prevent infection through transfusion and organ transplantation. Conventional microscopy may not detect the infection when the parasitaemia is exceptionally low.

Serodiagnosis

Serological methods are based primarily on the detection of *T. cruzi* circulatory antibodies in the serum. The most commonly used methods are ELISA, indirect haemagglutination, immunoblotting technique and immunochromatographic and

Fig. 6 Endemic zones of Chagas disease (Source: *Wikimedia Commons*)

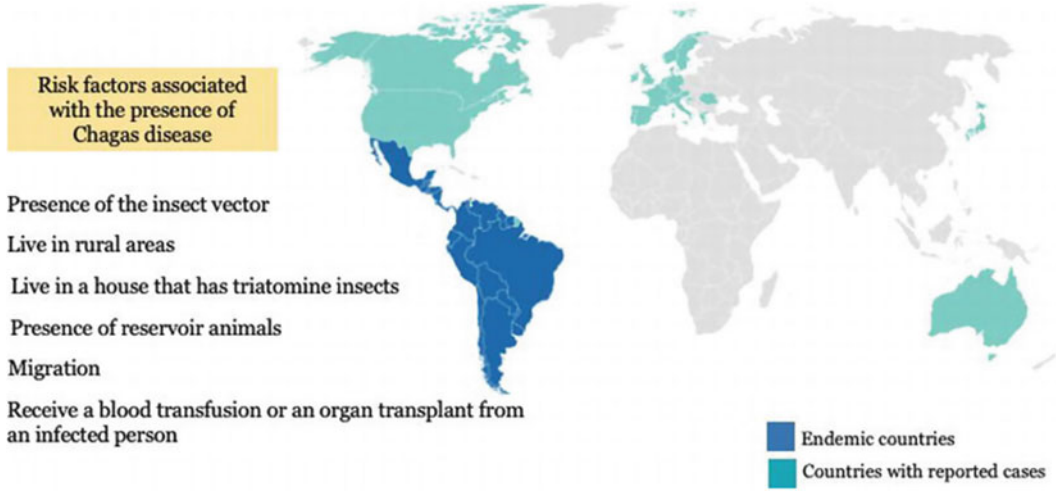

Fig. 7 Distribution of Chagas disease (WHO 2010) (DOI: https://doi.org/10.5772/intechopen.86567)

Table 1 Global distribution of Chagas disease (American trypanosomosis)

Estimated global cases	Distribution	Transmission pathway
Less than 1000 cases	Portugal, Norway, Germany, Austria, Greece	Immigrants
1001–10,000 cases	Australia, Japan, Canada, France, UK, Italy	Immigrants
10,001–100,000 cases	Spain, Costa Rica, Guatemala	Immigrants, blood transfusion
100,001–1,000,000 cases	USA, Bolivia, Peru, Chile, Colombia, Ecuador, Venezuela	Immigrants, blood transfusion, vertical transmission
1,000,001 and above	Bolivia, Peru, Chile, Colombia, Ecuador, Venezuela, Brazil, Argentina, Mexico (endemic countries)	Majority by vector bites (*Triatoma* bugs)

Table 2 Diagnostic methods for American trypanosomosis

Diagnostic approaches	Methods	Targets	Remarks
Parasitological methods (blood screening)	Optical microscopy and microhaematocrit	Aims to visualize the presence of trypomastigotes	Sensitivity varies depending on the stage of infection *Limitations:* Cannot detect chronic phase infections due to low parasitaemia
Immunodiagnostics	Indirect haemagglutination ELISA, IFAT and Western blot	*Trypanosoma cruzi* epimastigote antigens and recombinant proteins (rTc24) are used to target IgG anti-*T. cruzi* antibodies in the blood of infected patients	Best suitable for the diagnosis of the disease even in chronic phase where the parasitaemia is very low *Limitation:* Significant cross-reactivity with *Leishmania* spp.
Molecular assays	Conventional PCR, real-time PCR	Satellite DNA of *Trypanosoma cruzi* and IAC plasmid DNA	High sensitivity and specificity *Limitations:* PCR is not helpful in routine diagnosis

indirect immunofluorescence using crude lysates of the parasite as antigen, recombinant protein or synthetic peptides. These tests despite being highly sensitive and specific show cross-reactivity with *Leishmania* spp. Western blot technique is specific for the detection of *T. cruzi* antibodies using excretion-secretion antigens and/or recombinant proteins. Flow cytometry is useful particularly for differential diagnosis between *T. cruzi* and *Leishmania* infections. The immunochromatographic rapid tests have shown sensitivity and specificity values from 97 to 100% and employ the use of recombinant antigens like H49 and 1F8. The ease of performance and interpretation makes it very useful in field studies.

Molecular Diagnosis

Molecular diagnosis is useful for the accurate detection and characterization of different strains of *T. cruzi*. Several types of PCR including conventional PCR, nested PCR and real-time PCR have been evaluated in the recent past. However, their application is not extensive due to certain limitations. PCR may not be overly sensitive in chronic cases due to exceptionally low level of circulating parasites. Sensitivity of PCR is also influenced by the method of DNA extraction. The low level of parasites in chronic disease results in the PCR-based methods to have sensitivities only of about 45–65%, while specificity remains close to 100%.

Xenodiagnosis

This method is more sensitive than traditional methods. In this, laboratory bred triatomine bugs which are maintained in birds are allowed to feed on the suspected patient. The bug faeces are then examined for the metacyclic forms.

Combination of PCR and xenodiagnosis is much useful to diagnose Chagas disease especially in disease-endemic areas with low parasitaemia.

Treatment

The primary objective of the treatment of Chagas disease is to eliminate *T. cruzi* parasites in the infected host and to prevent the conditions to progress to irreversible lesions associated with the disease. The outcome of treatment by antiparasitic agent often depends on the phase of the disease and the age of the infected individual.

In the acute illness, benznidazole and nifurtimox are highly effective if given soon after infection. Treatment of the chronic phase may not be successful in most of the cases. However, symptomatic treatment of chronic patients is often lifesaving and the sole alternative for this disease. Target-specific treatment for cardiac or digestive or neurological manifestations becomes the need of the hour in critical complicated cases.

Both benznidazole and nifurtimox are contraindicated in pregnant women or in individuals with kidney or liver complications. Nifurtimox is also contraindicated in the backdrop of neurological or psychiatric disorders.

Prevention and Control

Chagas disease is a complex socio-economic and environmental health problem. Till date no vaccine is available for Chagas disease. In endemic areas, vector control has been the most effective method of prevention. Screening of blood and organs for the parasite is mandate to prevent infection through transfusion and organ transplantation. The World Health Organization (2005) recognized Chagas disease as one of the neglected tropical diseases and recommended the following approaches to prevent and control the disease: (1) spraying residual insecticides in and around peri-domiciliary; (2) using bed nets to prevent bite wounds from bugs; (3) maintaining hygiene in food preparation, transportation, storage and consumption; (4) screening of blood, tissue and organ samples before transfusion from donors and recipients; (5) starting antiparasitic treatments in children and women of childbearing age before pregnancy; and (6) screening newborns and other children of infected mothers and providing treatment in early stages.

Case Study

An adult immigrant from El Salvador went to the emergency room of a US hospital with fever and confusion that did not respond to antibiotic treatment. CT scan showed a brain lesion. The patient was HIV-positive, and his last visit to El Salvador was 1 year earlier. A spinal tap showed low glucose and high protein levels in the CSF. In addition, organisms were found in the CSF of size approximately 20 μm. Immunofluorescence assay for antibodies to *T. cruzi* in the CSF was negative but positive in the serum at 1:128.

1. Which other trypanosome is endemic in South and Central Americas, and how it can be differentiated from *T. cruzi*?
2. What are the factors which cause reactivation of chronic Chagas disease?
3. What are the precautions needed to be taken in laboratory while handling the specimen of blood from a patient with suspected Chagas disease?

Research Questions

1. How to develop highly specific and sensitive serological diagnostic tests for *T. cruzi*?
2. How to formulate specific treatment regimen for chronic trypanosomosis?
3. What are the vaccine targets which have been identified for *T. cruzi*?

Further Readings

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Malaria

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Learning Objectives

1. To make the reader aware of the new malaria parasite, *Plasmodium knowlesi*, and its clinical significance and severity of infection.
2. To avoid the pitfall of wrong diagnosis of *P. knowlesi* during microscopy as *Plasmodium falciparum* or *Plasmodium malariae*.
3. To know the importance of molecular diagnosis in species identification.

Introduction

Malaria is a parasitic disease that has plagued mankind for many millennia and has inflicted much health burden and many deaths. It continues to be a major cause of morbidity and mortality particularly in parts of the tropical and sub-tropical regions. This mosquito-borne disease is caused by the sporozoan of the genus *Plasmodium* and transmitted by the infected mosquitoes of the genus *Anopheles*. Sporozoans have a complex digenetic life cycle which requires two hosts to complete: a definitive host in which the sexual reproduction occurs (*sporogony*) and an intermediate host in which the asexual division occurs (*schizogony*). In human malaria, the definitive host and the vector are both the *Anopheles* mosquito, while the intermediate host is the human. In 2018, about half of the world population was at risk of acquiring malaria. However, the global malaria map has continued to shrink during the recent years with more and more countries achieving better malaria control and some even reaching the target of elimination.

Several species of *Plasmodium* are known to cause malaria in humans. Of these, four species (viz. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malaria*) are natural parasites of humans that cause disease with infected humans as reservoirs for further spread. The fifth species, *Plasmodium knowlesi*, is a natural parasite of primates and has been identified as the causative agent of a zoonotic malaria in humans having non-specific malarial symptoms and even a fatal outcome, but if diagnosed early it is treatable. Naturally acquired *P. knowlesi* in a human was first reported in 1965 and thereafter has been reported from South-East Asian countries where primates (e.g. long-tailed and pig-tailed macaques) and the forest-dwelling vector mosquitoes belonging to the *Anopheles leucosphyrus* group are commonly found. Currently *P. knowlesi* is considered as an emerging *Plasmodium* species causing malaria in Asia.

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History

Evidence of fossilised early lineage anopheline mosquitoes has been found in amber dated back to about 100 million years ago, but whether they were vectors of the malarial parasites at the time needs further research. It is believed that malaria may have originated in the West and Central Africa and entered humans from the great apes via mosquitoes. After the end of last glacial period, by about 10,000 years ago, agriculture popularised in Africa, and this Neolithic agrarian revolution led to adaptations of the anopheline mosquitoes to be more anthropophagic in Africa, while they were more zoophilic outside of Africa. Descriptions of fever similar to paroxysmal fevers have been mentioned in ancient Chinese, Indian, Sumerian and Egyptian literature in the last 5000 years. Malaria reached its worldwide spread to have more than half of the world's population at risk of malaria by the nineteenth century. Miasma theory was used to explain the aetiology of malaria till the mid-nineteenth century. By the 1880s the germ theory of diseases challenged the miasma theory. The word *malaria* meaning 'bad air' is believed to be derived from the Italian words *mal* and *aria*.

Robert Knowles and B. Dasgupta at the Calcutta School of Tropical Medicine first described *P. knowlesi* in 1932. They showed in experimental monkey models that this new malaria parasite was capable of causing severe infection in *Macaca mulatta*, which is commonly found in India, but the natural host *Macaca fascicularis* monkey had asymptomatic or mild infection. Quotidian malaria could also be induced in human volunteers.

They also described the life stages of the parasite. The parasite was named *P. knowlesi* in honour of Robert Knowles. At one time, *P. knowlesi* was employed in the treatment of neurosyphilis and was known as fever or malaria therapy. The discovery of penicillins made this therapy obsolete. A large number of malaria cases in the Sarawak region of Malaysia were diagnosed as due to *P. malariae* in 1999. Interestingly, in contrast to the classical *P. malariae* infection, which normally causes low parasitaemia and mild disease, these new cases had moderate to severe manifestations with high parasite loads. Later it could be shown that these cases were due to *P. knowlesi*, a simian malaria parasite.

Taxonomy

The genus *Plasmodium* belongs to family Plasmodiidae, order Haemosporida, class Aconoidasida and phylum Apicomplexa in the subkingdom Protozoa and kingdom Protista. The genus *Plasmodium* has five species, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*, which cause human malaria, of which *P. knowlesi* is the zoonotic species being transmitted from macaque monkeys.

Genomics and Proteomics

Parasites of the genus *Plasmodium* have a digenetic life cycle requiring two hosts, viz. *Anopheles* mosquito and human, for its completion. Sexual reproduction occurs in the mosquito where the parasite is found to be diploid. Asexual reproduction occurs inside the human where the parasitic genome is haploid. The first malaria parasite to be sequenced was *P. falciparum*. A reference sequence of *P. knowlesi* was first published in 2008. The nuclear genome was described as 24.1 Mb, a G + C content of 37.5% and 5188 predicted genes. It contained 190 gaps within the core regions of 14 chromosomes. In a more recent whole genome sequencing study, the sequences covered 5228 genes, including genes and gene fragments annotated as genes of unknown function. Data from 605 genes were excluded because coverage was zero at one or more base positions leaving 4623 genes in subsequent analyses. Of these 2180 genes were annotated as genes with unknown function. More than half of the *P. knowlesi* genes in the genome, 2801 of 4623 genes (60.8%), appear to be dimorphic.

Plasmodium genome encodes more than 5000 proteins, majority of which are hypothetical. Due to the complex life cycle that involves several morphological stages, the gene expression in malaria is dynamic. Use of global proteomic profiling to identify gene expression pattern specific to each stage enables the development of drugs and vaccines that are stage specific. *Plasmodium* can undergo post-translational modifications by which it changes its surface proteins to evade immune responses. Proteomics can be used to identify these post-translational modifications and to quantify relative protein expression, which may be helpful in vaccine production, drug discovery and studies related to drug resistance. Interrelationships between the humans, parasite and the drugs can be interpreted at proteomics level, which will help in deciphering the mechanism of action of drugs and parasitic factors inducing an immune response of the host. *P. knowlesi* proteomic studies have focused on different aspects concerning its applied usefulness. In one study, several immuno-reactive proteins in malarial-infected subjects were identified which included serotransferrin and haemopexin that can be useful as biomarkers of infection. Other *P. knowlesi*-specific antigens were also detected in the study. In another approach, *P. knowlesi* schizont-infected cell agglutination (SICA) antigens were analysed. As many as 40 *P. knowlesi* SICA peptides showed identity with a particular *P. falciparum* erythrocyte membrane protein-1.

The Parasite Morphology

Gold standard for the diagnosis of malaria is microscopic visualisation of parasitic stages in peripheral blood. Morphological features characteristic to different stages vary with the *Plasmodium* species (Fig. 1). *P. knowlesi* shows the following morphological features:

1. **Ring stage/young trophozoite**:
   Red cells: Infected red cell is not enlarged.

   Parasite: Thin, delicate cytoplasm. One or two nuclei. Occasional Accolé forms seen.

2. **Mature trophozoite**:
   Red cells: Infected red cell is not enlarged. Sinton and Mulligan's stippling can rarely be seen with special stains.

   Parasite: Compact cytoplasm with coarse, dark brown pigment. Single large nucleus. Occasional band forms seen.

3. **Mature schizont**:
   Red cell: Red cell is not enlarged. Sinton and Mulligan's stippling can rarely be seen with special stains.

   Parasite: A coarse dark brown pigment mass, with up to 16 merozoites with large nuclei clustered around it. Sometimes rosette formation is seen. Mature merozoites appear segmented.

4. **Female gametocyte**:
   Red cells: Red cell is not enlarged. Sinton and Mulligan's stippling can rarely be seen with special stains.

   Parasite: Round/oval in shape. Occupies almost the whole cell. Compact eccentric nucleus.

5. **Male gametocytes**:
   Red cell: Red cell is not enlarged. Sinton and Mulligan's stippling can rarely be seen with special stains.

   Parasite: Round/oval in shape. Parasite occupies almost the whole cell. Scattered malarial pigment. Diffuse nucleus.

Morphology of *P. knowlesi* in microscopic examination of blood films could be confused with *P. falciparum* or *P. malariae* due to similarities in appearance during some stages of life cycle.

Cultivation of Parasites

In vitro culturing of malaria parasites has been a challenge due to their digenetic life cycle. A successful continuous in vitro culture was designed for the first time by Trager and Jensen in 1976 for cultivating *P. falciparum*. Even though *P. falciparum* culture was a success, efficient in vitro culture systems have not yet been

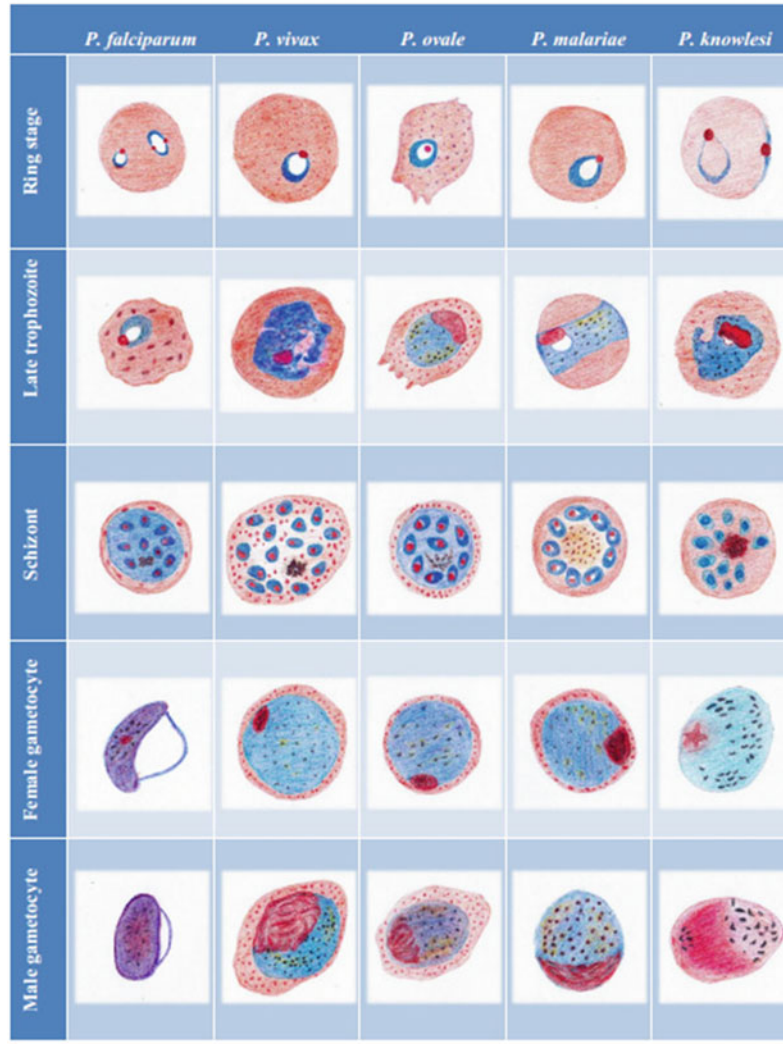

Fig. 1 Schematic diagram of developmental stages of malaria parasites

established for other species causing human malaria. The first successful in vitro culture was achieved in 1945 when Ball and colleagues were able to maintain *P. knowlesi* in culture in rhesus RBCs for periods of up to six erythrocytic cycles. Butcher in 1979 described a system which was modified in a recent study. In this, *P. knowlesi* were added to freshly drawn *M. fascicularis* RBC at 2% haematocrit in a modified RPMI medium 1640 containing 0.5% (wt/vol) Albumax II and 10% (vol/vol) human serum in static cultures at 37 °C. It was later adapted to grow in human RBC in a continuous culture system.

Laboratory Animals

The rhesus monkey (*Macaca mulatta*) is the most studied animal model for *P. knowlesi.* The animal develops high parasitaemia in a short period of time and is almost always lethal for this host. Apart from this animal, the olive baboon (*Papio anubis*) has also been suggested as a model for severe malaria and human cerebral malaria. In *M. fascicularis*, the long-tailed macaque, *P. knowlesi* produces low parasitaemia.

Life Cycle of *Plasmodium knowlesi*

Hosts

Definitive Host

Forest-dwelling mosquitoes of *Anopheles leucosphyrus* group.

Intermediate Host

Macaca nemestrina (pig-tailed macaques), *M. fascicularis* (long-tailed macaques). Humans are the incidental hosts.

Infective Stage

The sporozoites present in the salivary glands of infected mosquitoes are infective.

Transmission of Infection

Bite of *Anopheles* mosquitoes.

The life cycle of *P. knowlesi* in humans and primates is similar and resembles that of *P. vivax* (Fig. 2). The sporozoites present in the salivary glands of infected mosquitoes are introduced into the bloodstream of susceptible host during a mosquito bite. These infective stages are then carried to the liver, where they invade liver parenchyma cells. They undergo a process of multiple nuclear divisions, followed by cytoplasmic division (*schizogony*) leading to the development of extra-erythrocytic or hepatic schizonts. This is followed by the rupture of infected liver cells, releasing several thousand individual parasites (merozoites) into the bloodstream. The merozoites penetrate red blood cells and adopt a typical 'signet-ring' morphology.

In the bloodstream, the young ring forms (trophozoites) develop further within the red cells and start to undergo nuclear division (erythrocytic schizogony) to form schizonts. Depending on the species, about 8–24 nuclei are produced before cytoplasmic division occurs, and the red cell ruptures to release the individual merozoites, which then infect fresh red blood cells. The erythrocytic cycle in *P. knowlesi* is completed in 24 h compared to 72 h in *P. malariae*, while for the other three species, it is about 48 h, thus making it the fastest growing malaria parasite among all the five *Plasmodium* spp. Instead of entering the cycle of erythrocytic schizogony, some merozoites develop into male or female gametocytes within the host red cell. These do not develop further in the human host, but enter the mosquito when the insect vector ingests the blood.

In the mosquito vector, the nuclear material and cytoplasm of the male gametocytes differentiate to produce several individual gametes, which give it the appearance of a flagellate body (exflagellating male gametocyte). The gametes become detached and penetrate the female gametocyte, which elongates into a zygotic form, the ookinete. The ookinete penetrates the mid-gut wall of the mosquito and settles on the body cavity side and develops into an oocyst, within which numerous sporozoites are formed. When mature, the oocyst ruptures, releasing the sporozoites into the body cavity of the mosquito, from where some find their way to the salivary glands.

Pathogenesis and Pathology

P. knowlesi is similar to *P. falciparum* in its ability to produce severe malaria which may even be fatal. The severity in *P. knowlesi* malaria can be explained by its ability to produce hyperparasitaemia and possibly parasite sequestration.

Hyperparasitaemia

Because of the short erythrocytic cycle of 24 h, the infection is characterised by daily increment in parasite load if left untreated. Genotype differences between the strains can cause differences in disease severity.

Parasite Sequestration

Parasite sequestration is a well-known phenomenon in *P. falciparum*-induced cerebral malaria, but similar manifestation has not been documented in *P. knowlesi*-affected patients, but this needs further exploration. Cytoadherence of parasitised RBCs to human endothelial cells has been demonstrated in one experimental study.

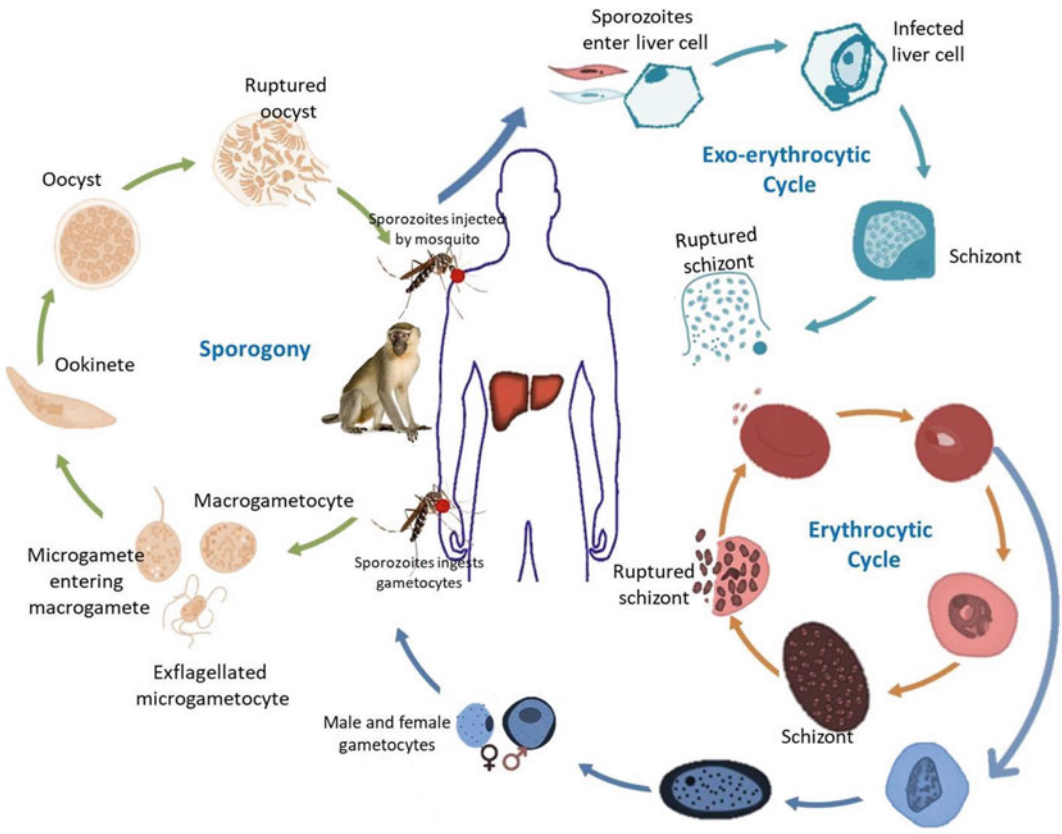

Fig. 2 Life cycle of *Plasmodium knowlesi*

Various putative virulence factors have been studied in *P. knowlesi*, and some of them are also being explored as possible vaccine targets. One such molecule is an adhesin known as the reticulocyte binding-like (RBL) family which has been found to be involved in host cell erythrocyte binding and helps in merozoite invasion. NBPXa protein of RBL family is found in microneme organelles and is an important infection determinant. Another group of molecules belong to the erythrocyte binding-like (EBL) family which can bind to RBC receptors. Duffy binding protein α (PkDBPα) is considered an important member in *P. knowlesi* EBL family which binds with Duffy antigen receptor and helps in the invasion of erythrocytes. Genetic polymorphisms in PkDBPα can increase the disease severity. Tryptophan-rich antigens help in resetting formation and merozoite invasion, thus also contributing to disease severity.

Immunology

Immunity in malaria is complex and is generally short term unless it is boosted through frequent exposure. Broadly the immunity is of two types: innate immunity and acquired immunity.

Innate immunity is the inherent host defence mechanisms such as age of the red cells, type of haemoglobin, enzymes in red cells and other factors such as Duffy antigen. G6PD deficiency provides some form of protection against *P. knowlesi*. Another study tried to find out the relationship of different Duffy genotypes among the patients and controls in the endemic areas and the susceptibility to *P. knowlesi*. However the study was inconclusive because of the extreme homogeneity of the Duffy distribution in the region.

Acquired immunity is characterised by both cell-mediated immunity and humoral immunity.

Cell-mediated immunity is mediated through natural killer cells and macrophages with a complex network of cytokines. Humoral immunity is achieved by IgM, IgG and IgA antibodies. Antibodies against asexual blood stages may protect against invasion of red cells by parasites, while antibodies against sexual stages may reduce disease transmission.

Malaria in a non-immune individual would be an acute infection, and in a repeatedly exposed individual, it manifests in a more modified or milder form due to the concomitant immunity or *premunition* immunity maintained through repeated exposure to the parasites. Thus, host immunity is a determining factor of the severity of malaria. In areas with high transmission of parasites, repeated infections may lead to the development of either partial or complete immunity against disease, though they remain susceptible to infection. Such patients may be asymptomatic or will have mild symptoms. Host immunity is the highest in areas where the disease transmission is the highest. However, such acquired immunity will gradually wane if the person leaves that high malaria transmission area. Ability of the malaria parasite to undergo post-translational modifications to change the surface antigens from time to time is a mechanism to evade host immune response.

Infection in Humans

Once an infected *Anopheles* mosquito bites a human, it may take about 2–4 weeks for first symptoms to appear. The clinical spectra of malaria in humans vary from asymptomatic parasitaemia, uncomplicated malaria, severe malaria to even death. Clinical manifestations depend on the host immunity and the timing and efficacy of treatment.

Generally initial symptoms consist of non-specific fever with prodromal signs such as headache, malaise, myalgia and anorexia. This is followed by malarial paroxysms (chills with rigors, high fever and sweating), associated with signs and symptoms of anaemia, thrombocytopenia and splenomegaly. *P. knowlesi* infections are characterised by daily, symptomatic episodes because of the unique 24-h erythrocytic life cycle.

Febrile episodes occur shortly after the rupture of schizont-infected red cells, which explains the characteristic periodic fevers. Malarial fever episodes are generally associated with short periods of chills and rigors and are commonly referred to as paroxysms of malaria. However, with *P. falciparum*, the cycles of different broods of parasites remain asynchronous unlike in other forms of malaria. Therefore, typical tertian fever pattern is not usually observed in falciparum malaria. In recent years many atypical fever patterns have been observed which may not point towards a specific species, and many overlaps have been described.

Severe infection has been documented in 9–39% of cases in various studies with fatality rates of 2–10%. This is primarily due to short erythrocytic cycle and the ability to infect mature as well as immature erythrocytes. The net result is high parasitaemia, which is achieved in a short duration of illness. This is also accompanied with thrombocytopenia. When complications develop in severe infections, they manifest in various forms which include acute kidney failure, hypotension, jaundice and acidosis. Parasitaemia of $>35{,}000$ parasites/μL and a platelet count of $<45{,}000/\mu L$ have been defined as severe *P. knowlesi* malaria by some workers.

Infection in Animals

Simian malaria has been described and studied for years. A study has shown that *M. fascicularis* experimentally infected with *P. knowlesi* erythrocyte stage parasites from humans developed pre-patent infection on day 7 and demonstrated diurnal sub-periodic pattern. Both *M. fascicularis* and *M. nemestrina* develop low parasitaemia, and the disease is not fatal for them.

Epidemiology and Public Health

P. knowlesi, the new malarial parasite, was first reported from Malaysia, and subsequently it was

found to cause substantial malaria cases in that country. *P. knowlesi* has also been reported from other neighbouring countries like Thailand, Indonesia, Cambodia and Myanmar. Malaria among travellers from non-endemic areas to these countries have also been documented.

Anopheles mosquitoes of the Leucosphyrus group are the important vectors for *P. knowlesi*, and the same mosquitoes carry *P. vivax* and *P. falciparum* in the endemic areas. They inhabit the forest areas of South-East Asian countries which are also the natural habitats of the *M. fascicularis* (long-tailed monkey) and *M. nemestrina* (pig-tailed monkey) species. Thus the population staying in the forest or forest-fringe areas are vulnerable to *P. knowlesi* infection. Recent ecological changes including deforestation can alter the balance of parasite-mosquito-host in these regions resulting in the conversion of this zoonotic into an anthroponotic species.

Evolutionary studies have recorded that *P. knowlesi* may be as old as or even older than *P. vivax* or *P. falciparum* with its most recent ancestor appearing about 98,000–478,000 years back. Thus it is an ancient parasite which was present in some of the Asian countries even before the human migration into these areas about 70,000 years ago.

Human-Vector-Human Transmission: Although still considered a zoonotic disease, the possibility of human-vector-human transmission cannot be entirely rejected, and mathematical modelling supports this hypothesis. It is possible that human-to-human transmission is taking place in some situations, although not very efficiently as yet.

Diagnosis

Early, if not immediate, laboratory confirmation of malaria diagnosis is important, since early and effective treatment of malaria saves lives. None of the symptoms or signs of malaria are specific, which makes clinical diagnosis unreliable. Therefore, laboratory confirmation of malaria through demonstration of parasites, its antigens or products in patient's blood plays an important role in patient management (Table 1).

Microscopy

Examination of thick and thin blood smear remains the gold standard for all the malaria parasites except *P. knowlesi*. Experienced microscopists can determine the species of malaria from a blood film, but differentiation of

Table 1 Diagnostic methods for *Plasmodium knowlesi* malaria

Diagnostic approach	Methods	Target	Remarks
Microscopy	Thin and thick blood film examination after staining with Leishman/Wright's/ other Romanowsky techniques	Early and late trophozoites, schizonts	Gold standard but not conclusive. Ring forms resemble *Plasmodium falciparum*, and trophozoites and schizonts resemble those of *Plasmodium malariae*. Low parasitaemia can confuse the diagnosis
Immunological tests	Immuno-chromatographic rapid diagnostic tests	Antigen detection. Aldolase-/ histidine-rich protein II/parasite lactate dehydrogenase	Low sensitivity and misdiagnosis. No specific antigen detection test exists for *Plasmodium knowlesi*
Molecular diagnosis	Nested PCR, real-time PCR, LAMP	18 s small-subunit ribonucleic acid (SSU rRNA); PkF1150-PkR 15,560; apical membrane antigen 1 (AMA-1); β-tubulin gene	Nested PCR is considered to be the reference test for species differentiation. Can detect *Plasmodium knowlesi* as low as 1 parasite/μL of blood

P. knowlesi from other species is difficult. The morphology of *P. knowlesi* resembles that of *P. falciparum* or *P. malariae* at different stages of erythrocytic schizogony. The early trophozoites may appear similar to *P. falciparum*, while the late developmental stages may be mistaken for *P. malariae* because of the band forms (Fig. 1). In view of the potential serious nature of infection due to *P. knowlesi* as compared to *P. malariae*, a WHO Consultation Meeting recommended that all *P. malariae* cases diagnosed on microscopy should be reported as *P. malariae*/*P. knowlesi.*

Serodiagnosis

Antigen Detection Tests: The simple 'dipstick' tests (RDTs), which are sufficiently reliable to be used by inexperienced staff with minimal level of training or under field conditions where microscopy is not available, have become extremely popular. They are particularly useful in low malaria transmission settings and where zero levels of local transmission have been achieved, due to the relative ease in performance and interpretation. Some RDTs detect a single species (either *P. falciparum* or *P. vivax*), some detect multiple species (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) and some further distinguish between *P. falciparum* and non-*P. falciparum* infection or between specific species.

Comparison of various RDTs as to their usefulness in diagnosing *P. knowlesi* has been reported in a number of studies. Aldolase-based tests have a low sensitivity of 23–45% in different studies, while LDH-based tests were found to be even inferior with a sensitivity of mere 25%. Histidine-rich protein II (HRP-2)-based tests used for *P. falciparum* can misdiagnose *P. knowlesi* as *P. falciparum* because of cross-reactivity to the monoclonal antibody used in the test. Evaluation of three different RDTs against PCR and microscopy-positive samples has found all RDTs to have overall low sensitivities compounded with the problem of cross-reactivity with other malaria parasites. These problems with the existing kits superadded with the continuing use of these kits in endemic areas have created logistic and diagnostic confusions.

Molecular Diagnosis

Molecular diagnosis that has superior levels of sensitivity also plays a role in malaria diagnosis in selected settings. However, such ultra-sensitive methods are generally not used in routine diagnosis of malaria in endemic areas due to the high cost and the need for established laboratories.

PCR and other amplification tests are the only means for definitive diagnosis for *P. knowlesi*, as microscopy and antigen detection tests are inadequate in species detection. The gene target which has been commonly used for species differentiation is 18S-SSU rRNA.

Nested PCR is considered to be the reference test for species differentiation. PmK8 and Pmkr9 primers were used in nested PCR but are no longer used because of cross-reactivity with *P. vivax*. Hence, the more specific PkF1150-PkR 15,560 primers are preferred. Nested PCR has got some serious drawbacks including the use of five to six reactions to differentiate the five malaria parasite species and, hence, longer turnaround time and chances of contamination. The real-time PCR technique is also being used for diagnosis, and it has a higher sensitivity as compared to nested PCR. The loop-mediated isothermal amplification (LAMP) technique has also got good sensitivity. Originally β-tubulin gene was used and found to be 100-fold more sensitive (up to 100 copies of DNA) when compared to single-round conventional PCR. Later on apical membrane antigen 1 (AMA-1) gene target was used with good results. The important gene targets for the diagnosis of *P. knowlesi* are summarised in Table 2.

Treatment

For many years the standard treatment for acute malaria was chloroquine. Clinically, chloroquine and other antimalarials have been found to be active for *P. knowlesi* because of its zoonotic origin and hence no selection pressure for the development of resistance. But, keeping in mind the rapid development of parasitaemia and attendant risk of severe malaria, the WHO has recommended artemisinin-based combination therapy similar to falciparum malaria. The

Table 2 Important molecular detection methods for *Plasmodium knowlesi*

Assay type	Gene target	Primers	Sensitivity
Nested PCR	SSU rRNA (S type) csp	Pmk8 + Pmkr9 PkF1060 + PkR1550 Kn1f + Kn3r	1–6 parasites/μl
Hexaplex PCR	SSU rRNA		Detects all five malaria parasites simultaneously and mixed infections up to two species
Real-time PCR	SSU rRNA	PK1 + PK2 NVPK-P PKe'F, PKg'R Pk	10 copies/μl 5 copies/reaction 100 copies/μl 10 copies/μl
LAMP	Apical membrane antigen 1 β-Tubulin	F3, B3, FIP, BIP, FLP, BLP	10 plasmid copies/sample 100 plasmid copies/sample

artesunate-mefloquine (AM) combination has been found to be a better drug compared to chloroquine due to faster parasite clearance. Intravenous artesunate is used in complicated cases.

Prevention and Control

There remains a clear risk of continuous *P. knowlesi* infections with changing ecological structure which results in closer contact of humans with the vectors and the reservoirs. The WHO Global Malaria Program has been effective for other malaria parasites with pure anthroponotic behaviour, but in countries of South-East Asia, these strategies may not be fully effective because of ongoing passage of *P. knowlesi* in the macaque population. Culling of monkeys is an impractical measure since it can affect the biodiversity of the region. Use of insecticide-treated bed nets and residual spraying of houses with insecticides can be effective but may not be useful for some mosquitoes which are outdoor feeders. Understanding of the vector biology and transmission of knowlesi malaria is necessary for the implementation of successful control programme.

Other Zoonotic Malaria

Plasmodium cynomolgi infection had been demonstrated to be transmitted to human volunteers in experimental studies in the last century. It is a common parasite of macaque monkeys (*M. fascicularis*) in Asia transmitted by *Anopheles freeborni* mosquitoes. The first case of naturally acquired human infection was reported in 2014 from Malaysia. *P. cynomolgi* is similar in morphology to *P. vivax*, and hence there may be many cases which are misdiagnosed, particularly in endemic areas.

P. simium and *P. brasilianum* are natural parasites of platyrrhine monkeys in South and Central America. Transmission of *P. simium* infection from a monkey to human transmitted by *Anopheles cruzi* has been reported. This parasite is genetically closely related to *P. vivax* and is morphologically similar. On the other hand, *P. brasilianum* is similar to *P. malariae* both genetically and in morphological features. Human volunteer study has shown the potential for transmission of the parasite from the simian host to human through *Anopheles freeborni* vector.

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Case Study

A 35-year-old male was admitted to the emergency department with severe abdominal pain and vomiting. He had an unremarkable medical history and was a wildlife photographer by

profession with no history of alcoholism. He has returned from a tour to Malaysia and Vietnam 1 month back and has taken malaria prophylaxis drugs. He has taken treatment for fever from a general practitioner during the past 5 days but complained of not getting better with the medication given. On examination, he was alert and febrile (39 °C) and had blood pressure 100/60 and pulse rate 80 beats/minute. His lung signs were normal, with epigastric tenderness and mildly distended abdomen. Investigations revealed a haemoglobin level of 9 g/dl and leucocyte count of $13x10^9$/l with 70% neutrophils. Upon further enquiry, patient revealed he has defaulted the prophylactic drug course. Further laboratory tests revealed ring and band forms suggestive of *P. malariae* in a thin blood film. The travel history of the patient and the symptoms not fitting with *P. malariae* infection prompted nested PCR using markers for the four malaria parasites together with Pmk8 and Pmkr9 primers for *P. knowlesi*. The patient was found to be having *P. knowlesi* infection, which was successfully treated with artemisinin-mefloquine combination therapy.

1. What are other areas of the world where *P. knowlesi* has been detected in humans?
2. What is the reason for severe malaria in case of *P. knowlesi*?
3. What is the rationale of using artemisinin-based therapy if the parasite is sensitive to chloroquine and other antimalarials?

Research Questions

1. Is human-to-human transmission possible or going on in endemic areas for *P. knowlesi*? How genetic studies and molecular epidemiology will help in elucidating the mystery?
2. Can mapping of vectors of *P. knowlesi* and overlay on human *P. knowlesi* incidence/prevalence maps help in elucidating the epidemiology and resultant control measures?
3. How to design a serological diagnostic kit for *P. knowlesi* which can also be used in field conditions?

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Babesiosis

Jayanta Bikash Dey

Learning Objectives

1. To know how to differentiate between *Babesia* spp. and malaria parasite during microscopical examination.
2. To have the knowledge about the similarity in presentation of babesiosis and malaria and the risk factors for the development of severe disease.

Introduction

Babesiosis is a zoonotic disease caused by an apicomplexan parasite of the genus *Babesia*. *Babesia* is transmitted from a vertebrate reservoir to human via an invertebrate vector which is a tick. The organism invades and eventually lyses red blood cells. It produces malaria-like syndrome including fever, haemolysis and haemoglobinuria. *Babesia* has a worldwide distribution but is predominantly found in the USA. It is endemic in certain parts of the USA. Majority of infections are caused by *Babesia microti*, but *Babesia divergens* and *Babesia duncani* are also responsible for sporadic cases detected but infrequently in Europe. In Asia a few cases have been documented in Japan, Taiwan and China.

History

Victor Babes, a Hungarian pathologist, first observed the intraerythrocytic organism in 1888. In 1893 Smith and Kilborne described a similar piroplasm in erythrocytes from Texas cattle with fever. Initially named *Pyrosoma*, the organism was later identified as *Babesia bigemina*. It was the first arthropod-borne disease to have been identified. The first well-documented case of human babesiosis was a splenectomized Yugoslavian farmer whose death was reported in 1957 by Skrabalo and Deanovic. Originally identified as *Babesia bovis*, the causative agent was later reported to be *B. divergens*. In 1969, a 59-year-old resident of Nantucket Island of the USA presented with fever and headache, and eventually the causative agent was identified by Spielman and co-workers as *B. microti*. It is a parasite of white-footed mice transmitted to humans by the tick *Ixodes scapularis*. Thereafter, 100 cases have been reported in the USA alone.

Taxonomy

The genus *Babesia* belongs to the family Babesiidae, order Piroplasmida, class

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Aconoidasida and phylum Apicomplexa in the kingdom Chromista. *B. microti*, *B. divergens*, *B. duncani* and *Babesia venatorum* are species that have been identified as being infectious to humans worldwide.

Genomics and Proteomics

The first complete genome sequence of a *B. microti* isolate was reported in 2012 and showed that the parasite is significantly distant from other apicomplexan taxa, including *B. bovis* and *Theileria* species. *B. microti* genome has four chromosomes, one mitochondrial gene and one circular apicoplast. The nuclear genome is ~6.5 megabase (Mb), the smallest apicomplexan genome sequenced to date, while the mitochondrial and apicoplast genomes are 11.1 and 28.7 kb, respectively.

Proteomics in *Babesia* is still in a developmental stage. A combination of nanotechnology and mass spectrometry is used to get a proteomic profile of *B. microti*. More than 500 parasite proteins have been identified which have role in transport, carbohydrate and energy metabolism, proteolysis, DNA and RNA metabolism, signalling, translation, lipid biosynthesis, and motility and invasion. Some surface antigens have been identified which have a role in immune response to the parasite. Two such antigens, BmSA1 and BMR1_03g00947, have been found to elicit host immune response. Invasive potential of merozoites is encoded by two proteins, namely, merozoites surface antigen 1 (MSA-1) and rhoptry-associated protein 1 (RAP-1).

Morphology

Three different stages of *Babesia* that are found inside human body are sporozoite, trophozoite and merozoite.

Sporozoite

Sporozoite stage of *Babesia* is introduced inside human body during the bite of infected tick. Sporozoites are pyriform in shape with a broad apical pole showing organelles such as rhoptries and micronemes which are typical of apical complex. The sporozoites enter the red blood cells.

Trophozoite

Trophozoites are cyclical ring-like structure. On the basis of the morphology of trophozoites, *Babesia* are divided into two groups: (1) small babesias (1.0–2.5 μm long) which include *B. microti* and *B. divergens* and (2) large babesias (2.5–5.0 μm long) like *B. bigemina* and *B. canis*. The size of the parasite determines its alignment inside the erythrocytes. Thus the large babesias meet each other at their pointed ends at an acute angle, while small babesias meet at an obtuse angle.

Merozoite

The transformation of the trophozoites into merozoites results in the formation of a tetrad structure resembling the Maltese cross, which is a special characteristic of *Babesia* spp. (Fig. 1). Because of great similarity between trophozoites of *Babesia* and *Plasmodium falciparum*, they must be distinguished during a blood smear examination. Major distinguishing features are thus: (1) *Babesia* trophozoites have variable shape and size, (2) trophozoites of *Babesia* do not have pigment, (3) *Babesia* trophozoites contain vacuoles and (4) trophozoites appearing in tetrad formation within red blood cells are also indicative of *Babesia*.

Cultivation of Parasites

A few *Babesia* spp. are cultured in bovine erythrocytes. Blood sample from infected bovine with parasitaemia of 0.1–0.2% is collected. Then defibrination of blood cells is done with shaking with glass beads. Thereafter erythrocytes are suspended to packed cell volume of 5–10% in HEPES-buffered medium 199 (60%) and bovine serum (40%). pH is adjusted to 7 with 1NHCL. Then it is transferred to culture vessel at 37 °C in

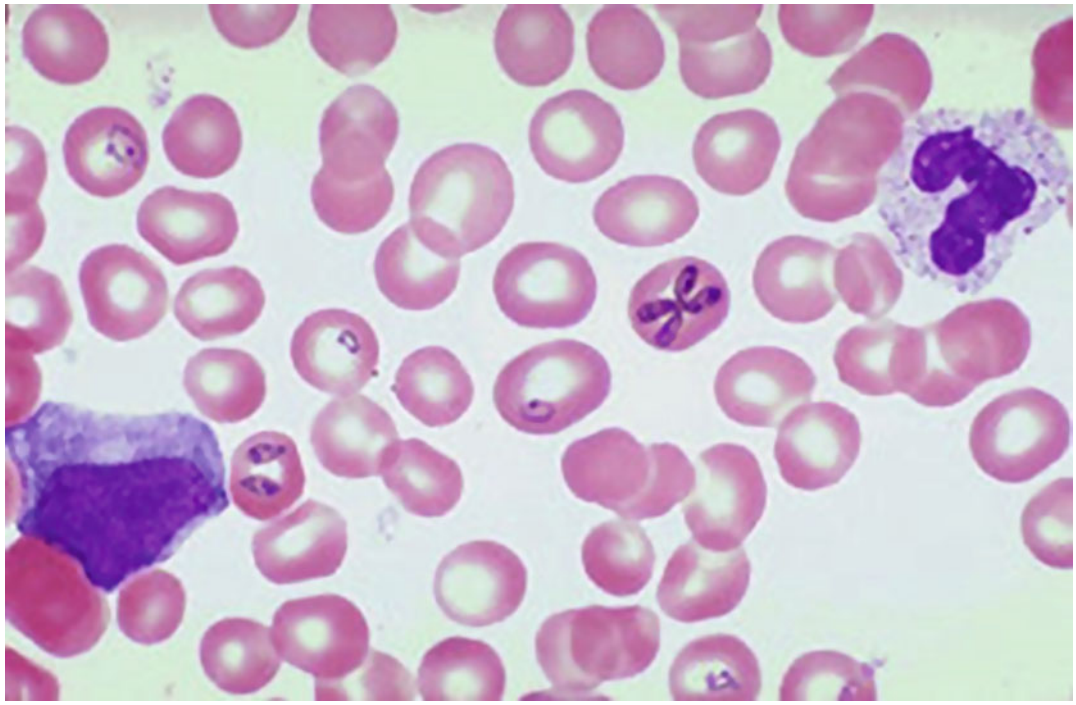

Fig. 1 *Babesia microti* in Giemsa-stained thin smear showing the Maltese cross form. (Courtesy: Spencer S Eccles Health Sciences Library)

atmosphere of 5% CO_2/95% humidified air. At an interval of 48–72 h, culture is diluted from 3- to 25-fold, adding medium containing freshly collected uninfected bovine erythrocytes.

Laboratory Animals

Laboratory animals are widely used for the study of pathological and immunological reaction of host against *Babesia* spp. Various models of the mice like BALB/C mice, immunosuppressive BALB/C mice, SCID mice and NOD SCID mice have been used and are inoculated with *B. microti*-infected red blood cells by intra-peritoneal injection. The infection rate of erythrocytes is related to the immune status of host mice.

Life Cycle of Babesia spp.

B. microti is the most common species that infects humans.

Hosts

Definitive Hosts
Ixodes ticks.

Intermediate Hosts
White-footed mouse (*Peromyscus leucopus*), other mammals.

Accidental Host
Humans.

Infective Stage

Sporozoites.

Mode of Transmission
Bite of the nymphal stage of *Ixodes* ticks.

The sporozoites enter the mouse during a blood meal of the tick vector, and in the mouse, asexual reproduction takes place inside the RBCs. Newly formed trophozoites are released during RBC lysis and invade new cells. Some of the merozoites

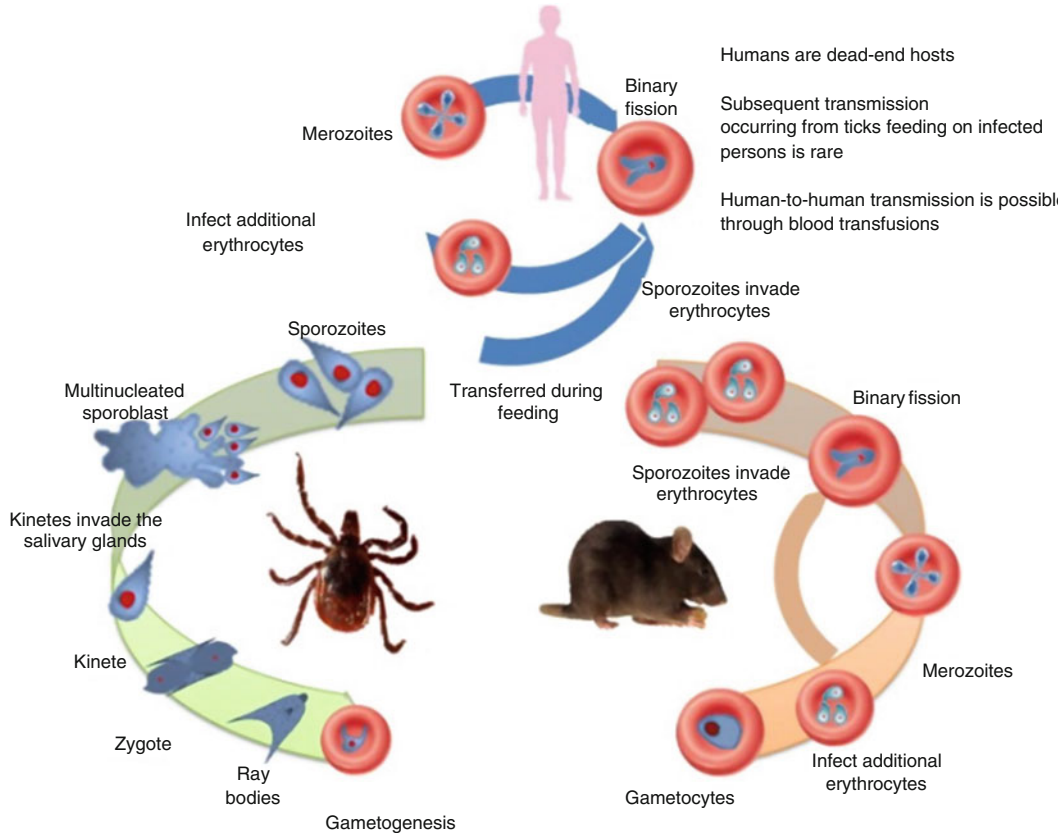

Fig. 2 Life cycle of *Babesia microti* (Courtesy CDC)

transform into male and female gametes which are then ingested by the tick (Fig. 2).

Female ticks get infected while taking a blood meal. In the digestive tract, fertilization of gametes takes place. The resulting zygote passes from the intestine to the haemolymph and then to the salivary glands. In the salivary acini, a multinucleate sporoblast is produced. The production of sporozoites from sporoblast starts when the tick begins feeding.

Humans are the accidental dead-end hosts when they come in contact with the ticks containing the sporozoites. The sporozoites enter the RBCs and undergo asexual reproduction which is responsible for disease manifestations.

Pathogenesis and Pathology

The haemolysis which occurs due to the asexual multiplication of the parasite is responsible for the clinical features and complications in babesiosis. The complications include haemolytic anaemia, haemoglobinuria, jaundice and even acute renal failure. There is production of pro-inflammatory cytokines which act as double-edged swords. On the one hand, the nitric oxide intermediates which are generated as a response to these cytokines kill the parasites, but on the other hand also cause damage to the RBCs.

Autopsies have found parasites in erythrocytes concentrated in congested capillaries of many organs and especially in the hepatic sinusoids. There may be cholangiohepatitis, irregularity in hepatic chords, congestion in central and interlobular vein, areas of focal necrosis and leucocyte infiltration in perivascular area. These indicate strong inflammation in the liver.

Immunology

Babesia infection induces both antibody-mediated and cellular immunity, but

cell-mediated immunity predominates and is important in immunity since the parasite is an intracellular pathogen. Antibodies are effective only for clearing extracellular parasites in bloodstream. Thus protection is primarily mediated by CD4+ T-cell response, apart from associated help from innate immune mechanisms like macrophages and NK cells. In the initial stage, the IgG seems to prevent the infection by binding with the sporozoites. The innate immune response is responsible for inhibiting the merozoites and to check the severity of infection. In this respect, the NK cells and the macrophages are key players by the production of IFN-√, TNF-α and reactive nitrogen and oxygen species. CD4+ T-helper cell-produced IFN-√ is thought to be responsible for clearance of infection.

Infection in Humans

Clinical presentation of babesiosis can be asymptomatic or may have severe manifestations. Those at most risk for both being infected and developing severe symptoms include people over 40 years of age and immune-compromised patients.

Incubation period varies between 1 and 4 weeks. Healthy immune-competent individual usually remains asymptomatic or shows mild symptoms such as fever, chill, sweating, headache, loss of appetite or nausea. But it can produce life-threatening condition in splenectomized patient, immune-compromised patient or patient with liver or kidney disease. In these patients, it can lead to the development of severe haemolytic anaemia, thrombocytopenia, disseminated immune coagulation (DIC) and malfunction of vital organs.

Infection in Animals

B. bigemina, *B. bovis* and *B. divergens* cause infection in cattle. The disease caused by *B. divergens* is also known as red water fever. The disease is characterized by fever, weakness, ataxia, haemoglobinuria and anaemia. The acute disease generally runs a course of 3–7 days. Anaemia and jaundice develop especially in more protracted cases. Muscle wasting, tremors and CNS involvement develop in advanced cases followed by coma and death.

Babesia canis, *Babesia rossi*, *Babesia vogeli*, *Babesia gibsoni*, *Babesia conradae* and *Babesia microti*-like species are the species that cause infection in canines. The clinical manifestations found in dogs can range from subclinical infections to multi-organ failure, with a risk of death. Clinical signs associated with canine babesiosis are apathy, weakness, anorexia, pale mucous membranes and a poor general condition. All *Babesia* species can cause fever, enlarged lymph nodes and spleen, anaemia, thrombocytopenia, jaundice and pigmenturia.

Epidemiology and Public Health

Transmission of babesiosis mainly occurs through the bite of infected *Ixodes* ticks. Nymphal stage of tick is mainly responsible for transmission. *Ixodes scapularis* and *Ixodes ricinus* are the two species which are commonly associated with human transmission. Human-to-human transmission occurs through contaminated blood transfusion. Transplacental or perinatal transmission may occur but rarely.

Most cases of babesiosis are reported in the USA with sporadic reports from Europe. Very few cases have been reported from Asia. CDC has notified of a total of 2161 cases of babesiosis by 28 of the 40 states in the USA in 2018, and a total of 14,042 cases of babesiosis were reported between 2011 and 2018. The blacklegged ticks are main vectors in the Northeast and the second involving other unidentified aetiologic agents in the Midwest and Northwest USA.

The risk of contracting babesiosis is directly dependent on the population of *I. scapularis* ticks in the region. A study of ticks in different regions of the USA has identified the two highest density clusters of ticks in the Northeast and upper Midwest which correspond to the two endemic areas of *Babesia*. Table 1 depicts the distribution of important *Babesia* spp. and their tick vectors.

Table 1 *Babesia* species of importance in humans

Species	Distribution	Intermediate host	Definitive host (ticks)
Babesia microti	North-eastern region of the USA	White-footed mouse, white-tailed deer, occasionally humans	*Ixodes scapularis*
Babesia divergens	Europe	Cattle, occasionally humans	*Ixodes ricinus*
Babesia duncani	Canada, USA	Mouse, occasionally humans	*Ixodes scapularis*
Babesia venatorum	Asia, Europe	Sheep, roe deer, occasionally humans	*Ixodes ricinus*

Table 2 Laboratory diagnosis of *Babesia* infections

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Examination of stained peripheral blood smear	Different intraerythrocytic forms	Gold standard test Drawback: Multiple smears have to be examined
Immunodiagnostics	Antibody detection (IFA test)	IgM and IgG	Good sensitivity and specificity Limitation: Cross-reacting with *Plasmodium* species
Molecular assays	PCR, RT-PCR	18SrRNA	High specificity and sensitivity

Most human transmission that depends on the tick vector is contracted during the summer time. The risk for acquiring babesiosis through transfusion of blood or blood components is low.

Diagnosis

For definitive diagnosis and appropriate treatment, detection of the pathogen is necessary. Among various methods microscopic detection, serologic test and molecular techniques are used (Table 2).

Microscopy

Thick and thin blood smears stained with Giemsa stain have to be examined. Ring forms are found which have to be differentiated from ring form of *P. falciparum*. Ring forms are round or oval and may appear as singles or pairs or rarely in tetrads. The tetrad or Maltese cross is more often seen in *B. microti* and *B. duncani*. Babesia can be differentiated from *P. falciparum* due to the absence of haemozoin pigment, the typical Maltese cross pattern and presence of extracellular forms. Parasitaemia in babesiosis can range between 1% and around 80% in severe infection in asplenic patients. The percentage of parasitaemia needs to be calculated in all positive cases. *Babesia venatorum* cannot be distinguished morphologically from *B. divergens*.

In Vitro Culture

Several in vitro culture methods have been used so far. A microaerophilous stationary phase (MASP) culture technique, where parasites proliferate in a settled layer of blood cells, has been found more convenient. The medium consists of HEPES-buffered medium 199, bovine serum and infected and normal erythrocytes at pH 7. The suspension is placed in culture vessel and incubated in 5% CO_2. Cultures supporting the growth of parasites turn red to black.

Serology

The indirect fluorescent antibody test (IFAT) uses *B. microti* parasites as antigen and can detect antibody in 88–96% of patients. The antigen used is washed parasitized RBCs from infected hamsters. The IFA titres may go up to 1:1024 in the first week of illness and come down to 1:16–1:256 over a period of 6 months, but may persist for

more than 1 year. Low-titre cross-reactivity may be seen with malaria-infected patients.

Molecular Diagnosis

Presence of *Babesia* DNA within erythrocytes can be detected by PCR. This is helpful in low parasitaemia and in cases where it is not possible to distinguish babesia from malaria parasites by microscopy. These assays usually employ the highly conserved sequences such as nss-rDNA as amplification targets. Analytical sensitivity has been shown to be 100 fg of parasite 18srDNA equivalent to 0.0000001% of infected erythrocytes. A colorimetric *B. bigemina* DNA probe could detect parasitaemias as low as 0.001%. Several genes are commonly used to discriminate among *Babesia* species, like nuclear ribosomal RNA genes and the two internal transcribed spacer (ITS1 and ITS2) genes.

Treatment

Asymptomatic persons do not require treatment. Patients of babesiosis are usually treated with a combination of atovaquone and azithromycin or clindamycin and quinine for 7–10 days. Atovaquone is given 750 mg orally thrice a day along with azithromycin 500–1000 mg orally daily. Other regime includes clindamycin, 600 mg orally three times a day, along with quinine 650 mg orally three times a day.

Prevention and Control

Preventive measures are needed to reduce the risk for babesiosis and other tick-borne infections. People who live, work or travel in tick-infested areas need to follow simple steps to protect themselves against tick bite and tick-borne infection. During outdoor activities in tick habitats, precautions need to be taken to keep ticks off the skin. Walking should be done in cleared trails and to stay in the centre of the trail. The amount of exposed skin should be minimized. Insect repellent can be used on skin and clothing. After outdoor activities, daily tick checks should be conducted, and any ticks found need to be promptly removed.

Case Study

A 56-year-old man presented with complaints of generalized weakness, night sweats, fever, myalgia, decreased appetite and mild nausea. The patient stays in a tick-endemic area in the north-eastern part of the USA. Approximately, 2 months ago he noticed an engorged tick while in the shower for which he completed a prophylactic course of doxycycline. Blood smears were ordered for review.

On the thin blood smear, there were multiple vacuolated, pleomorphic, ring-form-like organisms seen in multiple infected red blood cells. No extracellular organisms were identified. The organism was identified as a species of *Babesia* with 0.8% parasitaemia and confirmed by PCR as *B. microti.*

1. What are the similarities and the differences in the morphology of *Babesia* spp. and malaria parasite in blood film microscopy?
2. What is the definitive method for diagnosis?
3. What are the treatment options for babesiosis?

Research Questions

1. What are the characteristics of various *Babesia* spp. and different genotypes in individual cases?
2. How to improve our understanding about the epidemiology including the tick vectors and the animal reservoirs for babesiosis?
3. What rapid diagnostic tests can be helpful in the quick diagnosis of babesia infection at field levels and better management of cases?

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Cryptosporidiosis

K. Vanathy

Learning Objectives

1. To understand the significance of T-helper cells and innate immunity in protection against *Cryptosporidium* infection.
2. To stress the importance of immunosuppression in causing severe and protracted infection.
3. To make the reader aware about the multiple methods available for diagnosis apart from microscopy.

Introduction

Cryptosporidiosis caused by the coccidian *Cryptosporidium* spp. is one of the most common causes of food and waterborne outbreaks since 2004. The first human case of cryptosporidiosis was reported in 1976. Since then many cases have been reported in both immunocompetent and immunosuppressed hosts. The infection occurs following the ingestion of food and water contaminated with the oocyst of *Cryptosporidium.* The acid-fast sporulated oocysts are the diagnostic form of the parasite. The infection can be prevented by strict hygienic measures.

History

Cryptosporidium was first described in the stomach of mice in 1907. Later E. E. Tyzzer described the motile merozoites of *Cryptosporidium muris* in mouse's gastric epithelium. He named it as genus *Cryptosporidium* (*Crypto* = hidden sporocysts) because unlike other coccidia they did not have sporocyst surrounding the sporozoites. Tyzzer described the morphology and life cycle of another species, *Cryptosporidium parvum*, in 1912. He also described in detail about the developmental stages of the coccidia. The morbidity and mortality due to *Cryptosporidium* diarrhea was first related to the severe diarrheal illness in poultry, caused by *Cryptosporidium meleagridis,* a new species that was first described by Slavin in 1955. It was in the year 1971 that *C. parvum* was reported as a new species causing diarrhea in cattle and lambs. The first case of human cryptosporidiosis was reported in 1976, after which many more cases were reported in both immunocompetent and immunocompromised hosts.

Taxonomy

The genus *Cryptosporidium* belongs to the family Cryptosporidiidae; order, Eimeriidae;

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subclass, Cryptogregaria; class, Gregarinomorphea; phylum, Apicomplexa; and subkingdom, Neozoa, in the kingdom Protozoa.

Cryptosporidium is similar to other coccidia in that they are *monoxenous* (*mon*o, one; *xenous*, host). They are found in the microvillus of epithelial cells in the small intestine but not inside the host cells. They have different stages of development, of which endogenous stage has an attachment organelle, and they are found intracellular but are extra-cytoplasmic.

Genus *Cryptosporidium* has many species that infects a wide range of hosts. These include *C. parvum* (ruminants and humans), *C. hominis* (humans), *C. muris* (rodents and some other mammals), *Cryptosporidium andersoni* (cattle), *C. meleagridis* (birds and humans), *Cryptosporidium baileyi* (chicken and some other birds), *Cryptosporidium canis* (dogs), *Cryptosporidium felis* (cats), *Cryptosporidium galli* (birds), *Cryptosporidium molnari* (fish), *Cryptosporidium wrairi* (guinea pigs), *Cryptosporidium saurophilum* (lizards and snakes), and *Cryptosporidium serpentis* (snakes and lizards). Among all these species, *C. parvum* causes most infections.

Genomics and Proteomics

C. hominis genome is about 9 Mb in size with eight chromosomes. The eight chromosomes range from ∼0.9 to ∼1.4 Mb and exhibit 31.7% GC content compared with 30.3% for *C. parvum.*

C. parvum was previously known as bovine genotype or genotype 2. The previously designated species of *C. parvum* human genotype or genotype 1 or H was currently renamed as *C. hominis*. This was similar in size to *C. parvum* oocyst measuring 4.6–5.4 by 3.8–4.7 μm. There had been a difference in the ribosomal gene expression of *C. hominis* and *C. parvum*, of which the latter expresses two types of rRNA genes (type A and type B), whereas in *C. hominis* more than two transcripts were detected. The genus *Cryptosporidium* has more than 22 species, of which zoonotic *C. parvum* affects both human and animals, while the anthroponotic *C. hominis* also affects both humans and animals. The genotyping is based on SSU rRNA. *C. parvum* and *C. hominis* are further subtyped based on DNA sequence analysis of 60 kDa glycoprotein (gp60 or gp40/15). The subtypes Ia, Ib, Id, Ie, If, and Ig are subtypes of *C. hominis*, and IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh, IIi, IIk, and III are of *C .parvum*. The purpose of knowing the subtypes is to understand about the biological character of the parasite and their difference in clinical presentation.

Protein expression in the soluble fractions of excysted and non-excysted oocysts of *C. parvum* has been reported from one study. A total of 142 proteins were detected in soluble fractions of both excysted and non-excysted oocysts, and ribosomal proteins constituted a significant proportion. Six heat shock proteins and 17 secreted proteins were also expressed. It was noted that many of the detected proteins are involved in infection/pathogenesis, energy pathways, cellular division and replication, and DNA modification.

The Parasite Morphology

Different stages of the coccidian parasites are as follows:

Asexual stage (*sporogony*-inside host cell), sporozoites, Type I meront (8 merozoites), Type II meront (4 merozoites).

Sexual stage *(gametogony*), micro- and macrogametes, zygote, thin- and thick-walled oocysts.

The morphology of the parasite varies according to the stage of development.

The Oocyst

The oocyst which is the infective form is of two types: thin walled and thick walled. They measure about 4–6 μm in size, are round in shape, and are surrounded by a cyst wall. They bear four sporozoites inside. The sporozoite is crescentic in shape with pointed anterior end and blunt posterior end and a nucleus located posteriorly.

Thick-walled oocyst: Thick-walled oocyst is the infective form. It is oval in shape with a smooth surface. It consists of thick and coarse outer wall, fine granular inner wall, and an oocyst membrane in between these two layers. It has a suture point at one end where the sporozoites are released. Electron microscopy shows a double-layered oocyst, outer and inner layers. The oocysts are very resistant to chlorine and other disinfectants.

Thin-walled oocyst: It is similar to the thick-walled oocyst but surrounded by a thin-walled membrane. This stage is responsible primarily for causing autoinfection in humans.

The Sporozoite

The sporozoite measures about 5 × 0.5 μm. They have a rough surface with pointed apical region and rounded posterior end.

The Merozoite

Trophozoite measures about 1–2.5 μm in length, and they have smooth surface. Type I and II meronts vary in size from 1.5 to 3.5 μm, respectively, and the merozoites released by them are of similar in size 0.4 × 1 μm. Type I merozoites are rod shaped with pointed apical region with rough surface, and Type II merozoites have round, rough surface. Microgametes from Type II merozoites measure around 0.1 μm with spherical, rough surface, while macrogametes measure around 4 × 5 μm with oval, rough surface.

Cultivation of Parasites

There has been advancement in in vitro cultivation of *Cryptosporidium* in cell lines. COLO-680 N cell lines infected with two different species (*C .parvum* and *C. hominis*) tend to produce a greater number of infective oocysts, as identified by various microscopic and molecular methods. In vitro culture of *Cryptosporidium* also paves the way for further studies and the development of drugs.

Laboratory Animals

Many animals such as turkeys, chickens, mice, rabbits, rats, guinea pigs, cats, and dogs were used for animal experimental studies. But none of them exhibited symptoms similar to that of cryptosporidiosis.

Life Cycle of *Cryptosporidium* spp.

Cryptosporidium spp. complete their sexual and asexual life cycles in a single host (man or other animals) (Fig. 1).

Hosts

The genus *Cryptosporidium* has more than 30 species, of which around 20 have been identified from humans. *C. parvum* and *C. hominis* cause most human infection. *C. meleagridis*, although an avian coccidian, is considered as the third most important species causing human infections.

Infective Stage

Thick-walled sporulated oocyst causes transmission of infection from person to person, while thin-walled oocyst causes autoinfection in the same infected host.

Transmission of Infection

As few as ten infective *Cryptosporidium* oocysts can cause infection. Humans acquire infection by consumption of food and water contaminated with thick-walled oocyst. The human and animal excreta used as manure for crops contaminate the surface water, ground water, and drinking water sources. Thin-walled oocyst causes autoinfection in the same individual. The thin-walled auto-infective stage contributes to an overwhelming life-threatening infection in immunocompromised hosts.

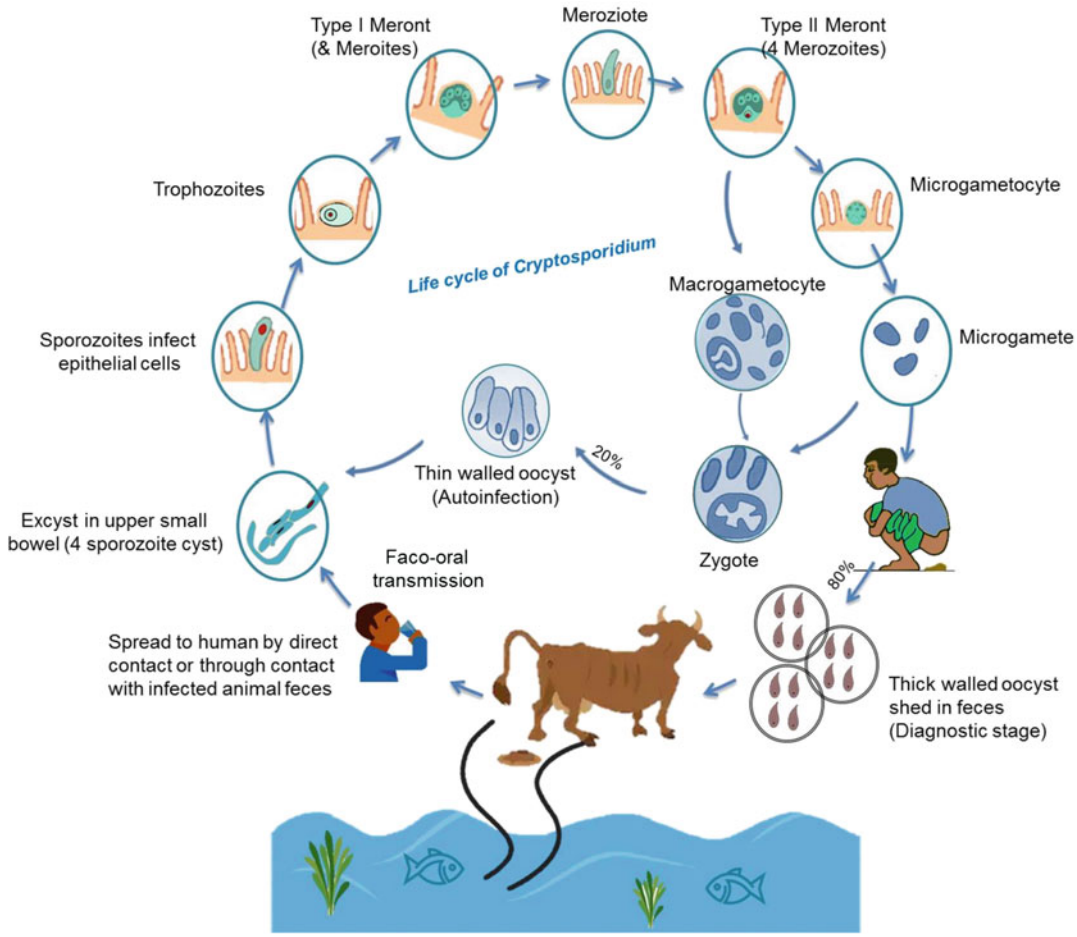

Fig. 1 Life cycle of *Cryptosporidium*

Cryptosporidium completes its life cycle in two different stages: asexual stage (*schizogony*) and sexual stage (*gametogony*) within a single host. Both the stages are intracellular and are surrounded by a host cell membrane which is extra-cytoplasmic.

Asexual Stage (Sporogony)

On ingestion of thick-walled oocysts, the sporozoites are released from the oocyst in the small intestine. Within the enterocytes, the sporozoites develop into intracellular trophozoites, which are the transition stage of the parasite. They multiply asexually by nuclear division to give rise to two types of meronts: Type I and Type II. These meronts in turn produce eight and four merozoites, respectively.

Sexual Stage (Gametogony)

Type II merozoites invade new host cell and undergo sexual reproduction to differentiate into male and female gametocytes. They further divide into macro- and microgametes, respectively, which undergo fertilization to form oocysts or *zygot*e. They form four sporozoites inside the sporulating oocyst. The oocyst is of two types: thin- and thick-walled oocysts. The thin-walled oocyst releases the sporozoites within the lumen of the intestine and causes autoinfection, thus repeating the cycle of sporogony and gametogony. The thick-walled oocysts are excreted in feces and can cause infection in humans, and thus the cycle is repeated.

The life cycle of *Cryptosporidium,* among the coccidian parasites, is different in that they do not

invade deep inside the host cells but are intracellular although extra-cytoplasmic inside the cell. The coccidia are found within the parasitophorous vacuole of the host cell, and the vacuole which contains the organism is present in the microvillus region of the cell surface. The suture wall in the oocyst ruptures, and sporozoites are released inside the small intestine. The sporozoites are released along with the stool in contrast to other similar coccidian parasites, *Cyclospora* and *Cystoisospora.* These parasites undergo excystation only in soil where the temperature is below 37 °C and in the presence of oxygen. Nearly 20% of the oocysts formed are thin walled. After *excystation*, they penetrate the brush border epithelium of small intestine and lie inside the parasitophorous vacuole of microvillus region. They are capable of causing severe and chronic infection in immunocompromised patients even though they are not exposed to environmental oocyst. Around 80% zygote develop into thick-walled oocysts which are resistant to chlorination, and 20% develop into thin-walled oocysts that cause autoinfection.

Pathogenesis and Pathology

Cryptosporidium produces several virulence factors that play important roles in parasite life cycle.

The oocysts in the intestine undergo *excystation* during reducing conditions on exposure to pancreatic enzymes and bile salts. The sporozoites attach to epithelial cells by means of parasite protein, CP47, a 47 kDa *C. parvum* protein. Both sporozoites and merozoites have apical organelles like rhoptries, microneme, dense granules, microtubules, apical rings, and pellicle. Inside the parasitophorous vacuole, there is a change in the apical organelle resulting in attachment to host cells. They cause villous atrophy, mucosal erosion, and depression of the surface mucosa, followed by infiltration of lymphocytes, neutrophils, and plasma cells. The villous atrophy results in D-xylose malabsorption.

Infection of intestinal epithelial cells leads to the activation of nuclear factor kappa B(NF-κB). This activates target genes like antiapoptotic genes such as osteoprotegerin. They allow the parasite to release merozoites before cell death and also activate pro-inflammatory molecules like cytokines and chemokines. The oocysts after causing the infection alter the normal functioning of the intestinal barrier. This in turn causes an increase in the intestinal permeability, absorption, and secretion of fluid and electrolytes resulting in chronic, profuse watery diarrhea especially in immunocompromised hosts. The activation of kinase pathway by the host cells helps in the release of pro-inflammatory cytokines like TNF-α and IL-8. This in turn attracts the phagocytes and leukocytes which can liberate the soluble factors. This results in intestinal secretion of chloride and water and decreases sodium absorption along with glucose transport.

Immunology

Both innate and adaptive immunity play a role in controlling the infection. CD4+ T cells play a major role in AIDS patients.

CD4 cells play an important role in acquired immunity in cryptosporidiosis. The severity and chronicity of *Cryptosporidium* infection depend on the immune status of the individual. Cryptosporidiosis is self-limiting in immunocompetent hosts and in patients with CD4 count >150/μl, chronic in <100/μl, but is life threatening and fulminant in immunocompromised hosts, especially with CD4 count <50/μl. In patients with HIV, the severity of *Cryptosporidium* infection depends on the CD4 cell count.

The role of antibody-mediated immunity in cryptosporidiosis is associated with an increase in IgG, IgM, and IgA to Cp23 in infected individuals compared to healthy, and it gives them longer immunity. IgA production in mothers was found to reduce the rate of infection in children. Monocytes and macrophages which secrete IL-15 and IL-18 were also found to control the infection. IL-18 stimulates IFN-γ production, NK cell activation, and production of

defensins which help in controlling the infection. Interferon γ is also responsible for acquired immunity.

Innate immune response plays an important role in the initial phase of infection. In innate immunity, mannose-binding lectin (MBL) helps in stimulating the immune response. Those children and HIV-infected individual who have MBL deficiency are more susceptible to *Cryptosporidium* infection. Polymorphism in MBL gene results in recurrent infection. The presence of Toll-like receptors on the surface of host cell helps in immune response to the organism. There is an increase in the production of antimicrobial peptides like LL-37 and human β-defensin 2.

Infection in Humans

Incubation period is 1 week. Most of *Cryptosporidium* infections are asymptomatic.

Cryptosporidium infection in immunocompetent individuals with an intact immune system is self-limiting. They usually present with low-grade fever, abdominal cramps, nausea, and anorexia. They have 5–10 times watery, frothy diarrhea with mucous flecks. The symptoms usually subside in 2–3 weeks (Table 1).

Cryptosporidium infection is found to be more severe and persistent in immunocompromised patients with HIV (AIDS); those on chemotherapy and radiotherapy, solid organ, and bone marrow transplant recipients; and patients on immunosuppressive drugs, primary immunodeficiency, etc. The symptoms may last for 7 days to 1 month depending on the severity of illness. It results in significant fluid loss, even up to 25 L. Not only the symptoms involve the gastrointestinal tract, but extra-intestinal sites like the respiratory tract, pulmonary tract, liver, and pancreas may also be involved. These patients do not recover from the illness over a period of time resulting in worsening of their symptoms leading to many complications and even death. The only treatment option for these individuals is to reverse their immunocompromised state rather than with any specific drugs. Survival of patients with CD4 count <200/μl is less compared to those with CD4 count >200/μl. Table 2 compares *Cryptosporidium* infection in immunocompetent and immunocompromised hosts.

Infection in Animals

Cryptosporidium infection is prevalent in a wide range of animals including chicken, mice, turkeys, cattle, dogs, pigs, and sheep. Bovine cryptosporidiosis is distributed throughout the world. Gastrointestinal symptoms are more prominent in cattle. Diarrhea is profuse, watery, and yellow. Fomites such as clothes and footwear used in livestock farm which have been exposed to feces of infected animals can transmit the infection.

Epidemiology and Public Health

Cryptosporidiosis is endemic in the developing countries than in the developed countries. The

Table 1 Clinical presentation of *Cryptosporidium* in immunocompromised and immunocompetent hosts

Characters	Immunocompetent host	Immunocompromised host
Host	Children	Adult
Mode of transmission	Person-person, animal to humans	Person-person
Clinical presentation	Mild to moderate diarrhea, 2–10 times per day	Severe diarrhea, as many as 70 times with fluid loss of up to 12 L/day
Duration of illness	Short, <20 days	Long, months to years
Weight loss	10% of body weight	50% of body weight
Prognosis	Recovery is complete and spontaneous	Usually fatal

Table 2 Distribution of *Cryptosporidium* infection in the global population

Cryptosporidium spp.	Host	Location	Distribution
Cryptosporidium andersoni	Bovines	Abomasum	Malawi
Cryptosporidium felis	Felids, human	Small intestine	India, Kenya
Cryptosporidium hominis	Human	Small intestine	India, Kenya, Malawi, Malawi, South Africa
Cryptosporidium meleagridis	Birds, human	Intestine	India, Kenya, Malawi
Cryptosporidium muris	Rodents, human	Stomach	India, Kenya
Cryptosporidium parvum	Ruminants, human	Intestine	India, Kenya, Malawi, Malawi, South Africa

meta analysis study reported global pooled prevalence of *Cryptosporidium* to be 7.6%. The highest prevalence of infection was reported in Mexico (69.6%), Nigeria (34%), Bangladesh (42.5%), and the Republic of Korea (8.3%) (Table 1). In India, the prevalence of cryptosporidiosis is found to vary from 4% to 13%. The prevalence is higher in semi-urban and rural areas due to poor sanitation. Small, environmentally resistant oocysts, a large number of livestock animals and human reservoirs, low infective dose of <10–100 oocysts, increased multiplication capacity $>10^{10}$, and resistance to available drugs and disinfectants contribute to higher prevalence of cryptosporidiosis in the community. A large waterborne outbreak of diarrhea affecting nearly 4,03,000 people was reported in Milwaukee, Wisconsin, in 1993.

Individuals with HIV, who are immunocompromised due to malignancy, or those on immunosuppressive drugs are more susceptible to infection by *Cryptosporidium* species. Cryptosporidiosis is considered as an AIDS-defining illness (clinical category C) and a category B pathogen by CDC and National Institute of Health. Improper hand hygiene after touching the farm animals is considered an important cause of zoonotic transmission from animals to humans. Nosocomial infection and mechanical transmission through soil and insects are the other modes of transmission.

Laboratory Diagnosis

Microscopy

Stool sample is collected in a wide mouth leak-proof universal container. Three stool samples are collected over a period of 10–15 days since the oocyst shedding in feces is intermittent. Five to six smears per sample are examined to detect *Cryptosporidium* oocysts in infected hosts. Duodenal aspirate, bile, sputum, bronchoalveolar lavage, and biopsy tissues are the other samples examined for the oocysts.

Floatation methods including Sheather's sucrose floatation, zinc sulfate and saturated salt solution, and the sedimentation techniques including formalin-ether and formalin-ethyl acetate methods are various concentration methods used to increase oocyst yield in the stool. Of all these methods, Sheather's sucrose floatation is the most recommended method because it increases higher yield of *Cryptosporidium* oocysts in stool specimens compared to other techniques.

Cryptosporidium oocysts appear as round, double-walled, and refractile bodies, in the saline, iodine, or lactophenol cotton blue wet mount preparations of the stool. Modified acid-fast stain, safranin-methylene blue, negative staining, dimethyl sulfoxide-modified acid-fast staining, immunofluorescence stain, hematoxylin and eosin, and Giemsa and Jenner staining are various permanent staining methods used to detect and identify the coccidian in stool specimens.

The modified acid-fast stain using 1% concentrated sulfuric acid as a decolorizer is the most frequently used method. In the stained smear, the acid-fast oocysts appears as a round, pink-colored structure with four sporozoites and of 4–6 μm in size (Fig. 2). Nearly 50,000–500,000 oocysts/gm of the stool need to be present in order to be visible in the acid-fast stain of the stool smear. The modified acid-fast stain is 83.7% sensitive and 98.9% specific. Table 3 summarizes the differentiating features of *Cryptosporidium*, *Cyclospora*, and *Cystoisospora* in stool specimens. Direct

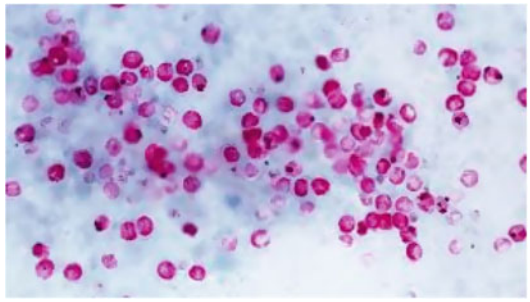

Fig. 2 Acid-fast stain showing oocyst of *Cryptosporidium*

immunofluorescent (IF) stain using monoclonal antibodies specific to cryptosporidial antigen is a highly sensitive method for the diagnosis of cryptosporidiosis (Table 4). Direct IF is considered the gold standard for *Cryptosporidium* in stool microscopy.

Serodiagnosis

Coproantigen Detection

Many serological tests using either native antigen or recombinant antigen of *Cryptosporidium* are available for the detection of serum antibodies in cryptosporidiosis. The antibodies are increased during 6–8 weeks of infection. Antibody detection is mainly useful for sero-epidemiological studies.

Initially, the ELISA was done using crude *C. parvum* extracts; hence, it was less specific than immunoelectrotransfer blot, but now the availability of recombinant *Cryptosporidium* antigens has increased its specificity. ELISA has shown high sensitivity and specificity in studies with various recombinant proteins. ELISA using

Table 3 Differential features of intestinal coccidian parasites

Features	*Cryptosporidium*	*Cyclospora*	*Cystoisospora*
Oocyst size	4-6 μm	8–10 μm	23–36 μm
Shape	Round	Round	Oval
Number of sporozoites	Four sporozoites	Two sporoblast with two sporozoites in each	Two sporoblast with four sporozoites in each
Acid fastness	All oocyst appears as pink acid-fast structures	Variable—some are pink, few oocysts are colorless	All oocyst appears as pink acid-fast structures
Sporulation of oocyst	Occurs inside the host	In soil (environment)	In soil (environment)
Autofluorescence	No	Yes	Yes
Infective form	Sporulated oocyst	Sporulated oocyst	Sporulated oocyst
Diagnostic form	Sporulated oocyst	Unsporulated oocyst	Unsporulated oocyst
Treatment	Nitazoxanide	Cotrimoxazole	Cotrimoxazole

Table 4 Diagnosis of cryptosporidiosis

Diagnostic approaches	Methods	Target	Remarks
Microscopy	Wet mount	Stool saline and iodine wet mount	Similar in size to yeast. Often misinterpreted. Need expertise
	Staining methods	Acid-fast staining, immunofluorescence stain, hematoxylin and eosin, Giemsa and Jenner staining	High parasite load is required to identify Sensitivity – IF>fluorescent stains>AF stain. Direct IF currently a gold standard for stool examination
Serodiagnosis	Immunochromatographic test, ELISA	Oocyst cell wall antigen	Good sensitivity with ELISA
Molecular techniques	Polymerase chain reaction-restriction fragment length polymorphism, multiplex real-time polymerase chain reaction, loop-mediated isothermal amplification	SSU rRNA, gp60, hsp70, 18S rRNA, COWP, and TRAP (C1 and C2	High sensitivity and specificity. Require skilled personnel

a recombinant 23 kDa antigen (Cp23, recombinant form of *C. parvum* 27-kDa antigen) has shown results to the ELISA using native 27 KDa antigen. Serum antibody to Cp23 correlates with past infection while that to Cp17 suggests recent infection. Cp23 has been used to determine longitudinal infection trend and age-specific seroprevalence in cryptosporidiosis. Recombinant rCP41 (cloned and expressed in *Escherichia coli*) has also been used in the ELISA for the seroprevalence studies in cryptosporidiosis.

Rapid immunochromatographic methods are now increasingly used to detect the oocyst cell wall coproantigen in stool specimens for the diagnosis of cryptosporidiosis. The coproantigen is detected by using *Cryptosporidium/Giardia* or triage panel containing *Cryptosporidium/Giardia/Entamoeba*-specific antigens. It is a popular procedure practiced in various laboratories due to its higher specificity (98–100%) and rapid results. ELISA for coproantigen detection has variable detection limits of 3×10^5 to 10^6, which is similar to microscopy. The ELISA, although has the low sensitivity, has the advantage of high specificity of 98%–100%, and large number of samples can be processed in a short time. These tests are suitable to be performed even on fresh, frozen, or formalin-preserved stool samples

Molecular Diagnosis

Molecular methods show high sensitivity by detecting 1 to 10^6 oocysts in stool and other specimens. PCR-restriction fragment length polymorphism (PCR-RFLP), multiplex allele-specific-PCR (MAS-PCR), and quantitative real-time PCR are different nucleic acid-based molecular methods in cryptosporidiosis. 18S rRNA, Hsp 70, TRAP C1, COWP, and DHFR genes are the target genes for *Cryptosporidium* species identification. Further subtype determination can also be done using subtyping tools such as glycoprotein (GP) 60 gene, minisatellite, and microsatellite markers and also by the analysis of extra-chromosomal double-stranded RNA elements.

Nested assay detects most of the common pathogenic *Cryptosporidium* species with the small-subunit rRNA-based PCR-RFLP using external primers of 1325 bp and internal of about 826 bp. Nested assay is the most popular method that has been validated in numerous laboratories globally. The MAS-PCR, which is based on dihydrofolate reductase gene sequence, can differentiate between *C. hominis* (357 bp) and *C. parvum* (190 bp) in a single step and is also useful for detecting small number of oocysts (<100) in the sample. Multiplex assays such as Luminex xTAG gastrointestinal pathogen panel, BioFire FilmArray GI panel, Nano CHIP GI panel, and BD Max parasitic panel are available currently as highly sensitive and specific assays in the detection and identification of *Cryptosporidium* species.

Treatment

Cryptosporidiosis in immunocompetent hosts is a self-limiting disease. The treatment, therefore, is based on fluid replacement with oral rehydration therapy (ORS) or parenteral therapy for seriously ill patients. Chemotherapy by nitazoxanide, paromomycin, and combination of paromomycin (1 g twice daily) and azithromycin followed by monotherapy with paromomycin has been evaluated in the treatment of the condition but with varying results.

Decreasing the dose of immune suppression therapy or strengthening the cellular immune response especially in HIV patients by highly active antiretroviral therapy (HAART therapy) forms the mainstay of treatment of cryptosporidiosis in immunocompromised patients. This helps in increasing their CD4 count. Rifaximin and rifabutin have been tried, but still more studies are required to prove its effectiveness.

Prevention and Control

Prevention is by maintaining proper personal hygiene. Preventive measures include washing hands properly before and after food, after using toilets, and after touching farm animals; avoiding swimming in polluted water, public water park, or

river; washing fruits and vegetables before cooking; avoiding eating uncooked food, and using clean water for washing vegetables. Water purification is important by flocculation and filtration method since chlorination is not effective. Zoonotic transmission can be prevented by wearing gloves and washing hands after handling material contaminated with animal feces and avoiding contact with domestic pets and farm animals especially cattle if they have diarrhea. No vaccine is available.

Case Study

A 42-year-old male who was a post renal transplant recipient on immunosuppressants 1 year back came with a history of chronic refractory diarrhea for past 3 months. He was investigated for complete hemogram; stool examination for ova, cyst, trophozoites; and stool culture before on OPD basis. All investigations were negative. He was treated with antibiotics and anti-parasitic drugs. The patient did not respond to any treatment. The stool was again sent to look for parasites. The modified acid-fast stain showed many round pink irregularly stained structures of approximately 4 μm in size.

1. What is the parasite? What are the other acid-fast parasites seen in stool?
2. What is the commonest artifact in stool with which this parasite can be confused, and how to resolve the issue?
3. What helminthic parasite is also commonly seen in the patients described above?

Research Questions

1. What is the role of humoral immune response in preventing the infection process in cryptosporidiosis?
2. How to assess the existing anti-*Cryptosporidium* drugs to control infections since the existing drugs are not very effective?

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Sarcocystosis

Azdayanti Muslim and Chong Chin Heo

Learning Objectives

1. To provide an idea about this rare parasite which has a limited geographic distribution.
2. To understand the risk factors that can help in preventing the infection.

Introduction

Sarcocystosis is a rare zoonotic disease caused by an intracellular coccidian parasite of the phylum Apicomplexa, the *Sarcocystis* spp. The parasite is ubiquitous in nature and is widely found in mammals, reptiles, birds and possibly fishes. The infection is primarily a veterinary problem with high prevalence in livestock, such as cattle, goats, and sheep. Human sarcocystosis was considered rare and mainly discovered as an incidental finding at autopsy or post-mortem. However, the global attention towards this disease has been escalated in recent years following the series of outbreaks reported amongst travellers visiting some islands in Malaysia.

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History

Sarcocystosis was first described in 1843 by a Swiss Scientist, Friedrich Miescher, who discovered the long, thin, thread-like cysts in the skeletal tissue of deer mouse (*Mus musculus*). The cysts were referred as "Miescher's tubules" for about 20 years before Kuhn, who found the similar organisms in the muscles of swine in 1865, had proposed the name of the parasite as *Synchytrium miescherianum.* However, the name *Synchytrium* was already given for another organism. Hence, the parasite remained unnamed until 1882 when Lankester named *Sarcocystis* as its genus, which is derived from the Greek words: sarx (= flesh) and kystis (= bladder). In 1889, *Sarcocystis miescherianum* was documented as species name by Labbe. Since then, many species of *Sarcocystis* have been named based on the discovery of the intramuscular sarcocysts in various animals.

Sarcocystis was also thought as a fungus at the beginning before it was confirmed as a protozoan related to genera *Eimeria* and *Toxoplasma* by electron microscopic study in 1967. The life cycle of the *Sarcocystis* spp. remained unclear

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until 1970s, when bradyzoites from sarcocysts in the muscle of common grackles bird (*Quiscalus quiscula*) were undergone development into a sexual stage called oocysts after inoculation into cultured mammalian cells. Further studies involved transmission of *Sarcocystis* of cattle (which was initially considered as a single species, *Sarcocystis fusiformis*) into potential definitive hosts. Dogs, cats and humans, all definitive hosts of the parasite, revealed three morphologically distinctive species which were then proposed as *Sarcocystis bovicanis*, *Sarcocystis bovifelis* and *Sarcocystis bovihominis*, respectively. Although names of these species were later changed, the life cycle of predator-prey relationship (i.e. definitive and intermediate hosts) was finally established and remained the same.

Taxonomy

Sarcocystis belongs to the phylum Apicomplexa, class Conoidasida, order Eucoccidiorida and family Sarcocystidae, along with genus *Cystoisospora* and *Toxoplasma* coccidian parasites.

The taxonomic studies of *Sarcocystis* is still ongoing, and currently, more than 200 species in various definitive and intermediate hosts have been reported, especially in meat-producing animals. Some wildlife animals also serve as intermediate hosts (e.g. raccoons and rodents) or final definitive hosts (e.g. opossums and snakes) for *Sarcocystis* (Table 1).

Genomics and Proteomics

Knowledge on the genomic and proteomic studies for *Sarcocystis* spp. was largely derived from *Sarcocystis neurona*, the species which cause the equine protozoal myeloencephalitis (EPM). To date, two genomes of *S. neurona* strains have been reported in the GenBank: SN1 and SN3 clone E1 genomes. The first genome sequence of *S. neurona* strain SN1 was reported in 2015. The strain was isolated and sequenced from an infected otter that died from protozoal encephalitis. The size of the genome is ~127 Mbp, which is more than twice the genome size of other coccidian parasites, due to the high amount of repetitive long interspersed elements (LINEs) and DNA elements.

This genome study has provided further understanding on the pathogenesis of the *Sarcocystis*. For example, the sequence analysis identified conservation of the invasion machinery which are present in the coccidia parasites. Two main conservation clusters have been identified: one composed largely of MIC, AMA1 and RON proteins (for cell attachment and invasion) and another consists of a limited set of ROP and GRA proteins (may be involved in the alteration of host immune effector function). The study indicated that many dense granules and rhoptry kinase genes, which are essential for the alteration of host effector pathways in *Toxoplasma* and *Neospora* parasites, are not present in *S. neurona*. Nevertheless, the genome of *S. neurona* has a unique

Table 1 Some of the *Sarcocystis* species and their definitive and intermediate hosts

Species	Definitive host	Intermediate host
Sarcocystis cruzi	Dog	Cattle
Sarcocystis hirsuta	Cat	Cattle
Sarcocystis hominis	Human	Cattle
Sarcocystis suihominis	Human	Pig
Sarcocystis capracanis, Sarcocystis hircicanis	Dog	Sheep
Sarcocystis fayeri	Dog	Horse
Sarcocystis muris	Cat	Mouse
Sarcocystis miescheriana	Dog	Pig
Sarcocystis chalchasi	Hawk	Pigeon

or divergent repertoire of surface-associated SRS adhesin proteins which are essential in the formation of tissue cyst evading mechanism from host immune reaction and establishment as chronic infection in the hosts.

The Parasite Morphology

Oocyst, sporocyst and sarcocyst are three important morphological structures in the life cycle of *Sarcocystis*.

Oocysts and Sporocysts

The sporulated oocysts, each containing two sporocysts with four sporozoites and a refractile residual body each, are colourless and thin-walled (<1 μm). The sporocysts are oval in shape and measure about 8–10 μm in diameter, and 1215 μm wide by 19-20 μm long in size (Figs. 1 and 2). Both oocysts and sporocysts are excreted and detected in the faeces of the infected definitive hosts. Since the wall of the oocyst is fragile and easily breakable, the detection of its individual sporocyst is more common in the faecal samples.

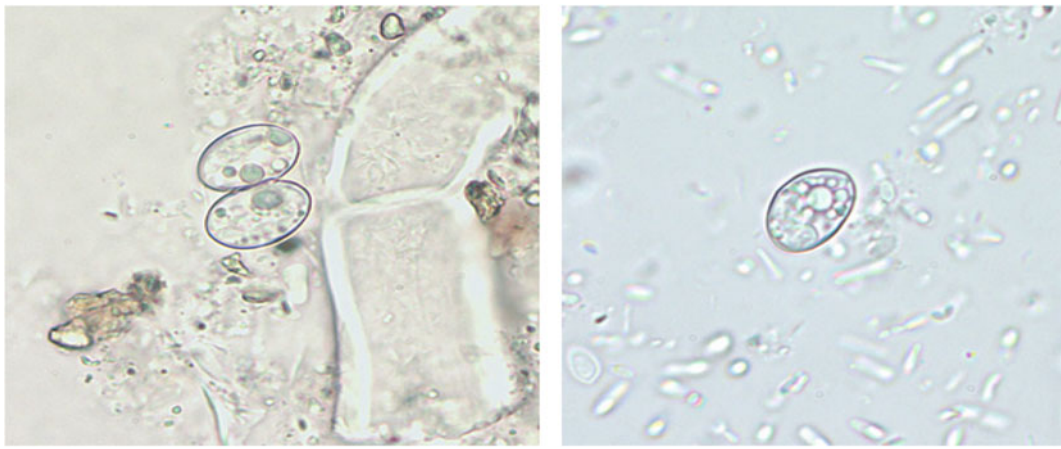

Fig. 1 (Left) The oocysts of *Sarcocystis*. (Right) Disintegrated sporocyst. Image source: https://www.cdc.gov/dpdx/sarcocystosis/index.html

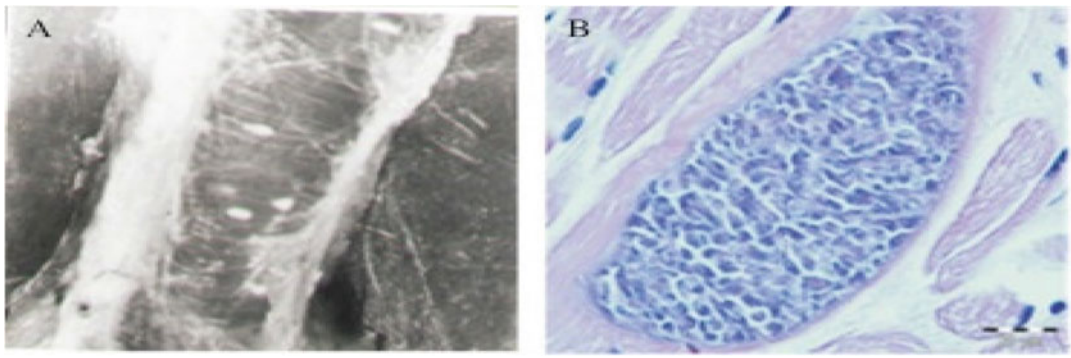

Fig. 2 (Left) Macroscopic sarcocysts in the tissue. (Right) Microscopic sarcocyst containing numerous bradyzoites (100×). Image source: Latif & Muslim 2016

Sarcocysts

Unlike oocysts and sporocysts, the sarcocysts are found in the striated tissue of skeletal or cardiac muscles, tongue, diaphragm and oesophagus. The sarcocysts are spindle, elongated or cylindroid in shape, but irregular structure could also be seen. The size of the sarcocysts differs depending on different stages of development, approximately from 140 to 250 μm to 1cm long depending on the type of host and parasite species. Also, their wall structure varies among *Sarcocystis* species. Each sarcocyst contains the infective stage structure, known as the crescent-shaped bradyzoites.

Cultivation of Parasite

Some *Sarcocystis* species have been successfully cultivated in vitro in the laboratory. These species include *S. neurona, Sarcocystis speeri, Sarcocystis falcatula, Sarcocystis lindsayi, Sarcocystis cruzi, Sarcocystis tenella* and *Sarcocystis capracanis.* Proper isolation of the oocysts or sporocysts isolated from the intestinal scrapings or from the faeces of the definitive hosts is one of the important steps in culturing the *Sarcocystis* parasites in the laboratory.

High volume of sporozoite excystation from sporocysts is prerequisite to achieve a successful in vitro cultivation. The viability of the sporozoite and its survivability after excystation may be affected by the type of storage media and the *Sarcocystis* species. One of the best storage media for the cell culture application for *Sarcocystis* is HBSS (Hanks' Balanced Salt Solution) with antibiotic known as PSFM mixture. The latter contains penicillin G, 10,000 units; streptomycin, 10 mg; Fungizone, 0.05 mg; and Mycostatin, 500 units/ml, in which the sporozoites remain viable for 12 months in 4 °C. Further selection of the cell line depends on the cell or tissue in which the *Sarcocystis* spp. develop in vivo. For example, *S. cruzi* was successfully cultivated in BM (bovine monocytes) cell line but not in mouse macrophages.

Laboratory Animals

Laboratory animals such as mice, rats, cats and monkeys were used mainly in understanding the morphology, life cycle, pathogenesis and immunological reactions in *Sarcocystis* infection. For example, Beaver and Maleckar (1981) had described the morphological characteristics of the cysts for *Sarcocystis singaporensis*, *Sarcocystis zamani* and *Sarcocystis villivillosi* from the tissues of laboratory rats after feeding them with the sporocysts obtained from the faeces of snakes, *Python reticulus*. The life cycle of *Sarcocystis muriviperae* was established in laboratory mice (*Mus musculus*) after oral administration of sporocysts collected from the faeces of a Palestinian viper (*Vipera palaestinae).* In another experimental study, laboratory rat (*Rattus norvegicus*) served as both definitive and intermediate hosts for *Sarcocystis rodentifelis*. A rodent model for the pathogenicity study of *S. neurona*, the causal agent for equine protozoal myeloencephalitis (EPM) in horses, has also been established.

Life Cycle of *Sarcocystis* spp.

Although many species of *Sarcocystis* have been discovered, a complete life cycle is only described for 26 species thus far.

Hosts

The life cycle of *Sarcocystis* is heterogeneous and complex, and their life cycle is based on predator-prey host relationship. The life cycle involves two types of hosts: a carnivorous/omnivorous definitive host (e.g., dog, snake) and a herbivorous intermediate host (e.g,. birds, small rodents and ungulate mammals).

Humans act as both definitive and intermediate hosts for different species of *Sarcocystis*. Two species, *S. hominis* and *Sarcocystis suihominis*, both can cause human infections using humans as definitive hosts. This happens when humans

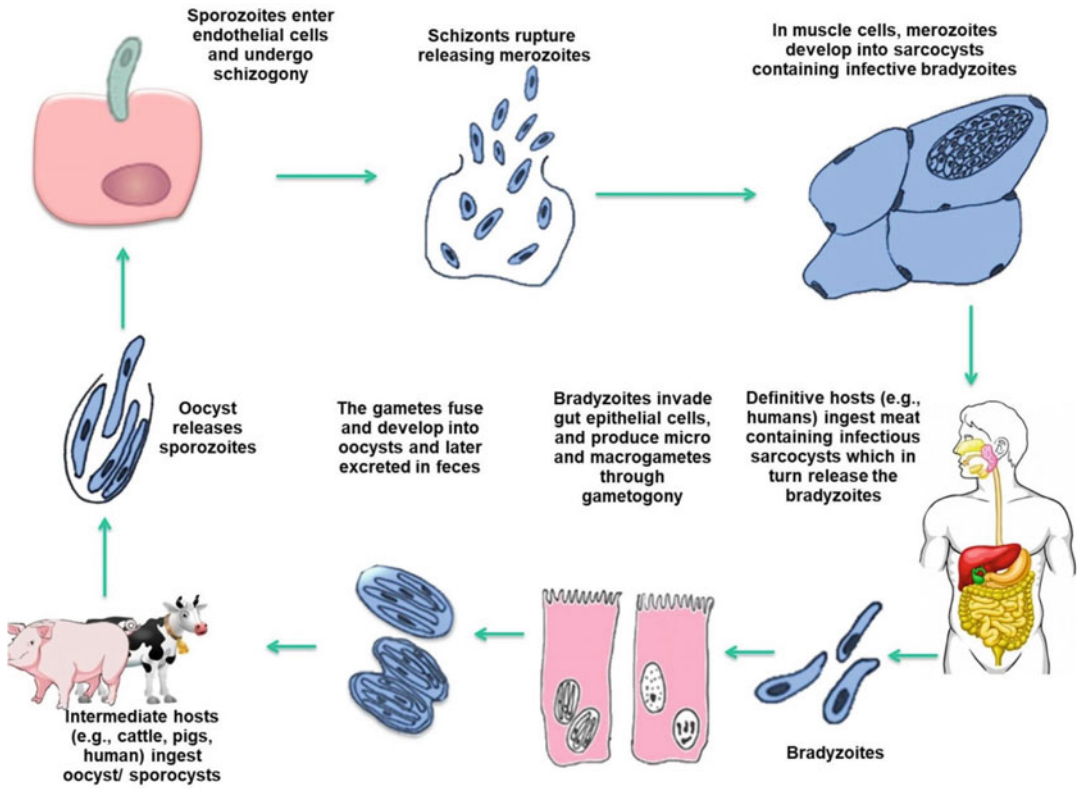

Fig. 3 Life cycle of *Sarcocystis* (image credit: Chong Chin Heo)

ingest raw or undercooked meats from infected cattle or pigs. On the other hand, *Sarcocystis nesbitti* causes human muscular sarcocystosis, for which humans serve as intermediate hosts and reptiles serve as the definitive hosts. Humans become the dead-end hosts after ingestion of oocysts/sporocysts from contaminated water and food (Fig. 3).

Infective Stages

Intestinal sarcocystosis: Bradyzoites from ruptured sarcocysts.
Muscular sarcocystosis: Sporocysts.

Transmission of Infection

The definitive hosts acquire the infection (intestinal sarcocystosis) by eating the infected animals or meats containing sarcocysts. In humans, the infection can be acquired by ingestion of raw or undercooked beef or pork harbouring the sarcocysts. In the intestine, numerous bradyzoites (infective stage) excreted from the ruptured sarcocysts invade the mucosa cells and are transformed into the male (*micro*) and female (*macro*) gametes. The fusion of macrogametocytes and microgametocytes by sexual reproduction or *gamogony* results in the formation of oocyst containing two sporocysts (each with four sporozoites), which are shed in the faeces of the definitive hosts.

Asexual reproduction occurs in the intermediate hosts following the ingestion of oocysts or individual sporocysts (infective stage) in contaminated water or food from the faeces of infected definitive hosts. Sporozoites are excysted from the ruptured sporocysts in the small intestine. The motile sporozoites migrate through the gut epithelium, to the endothelial cells of blood vessels and spread to different areas of the body.

The sporozoites undergo schizogony resulting in the production of large number of merozoites. It takes about 15–16 days for the merozoites to develop from the entry of the sporocysts. These merozoites invade the small capillaries, blood vessels, and subsequently muscle cells. The merozoites then round up to form metrocytes, followed by sarcocysts containing the infective bradyzoites. These are infective for the definitive hosts. The life cycle is repeated when infected animal or meat containing sarcocysts is eaten by definitive hosts.

Pathogenesis and Pathology

In the intestinal sarcocystosis, the sexual stage of life cycle occurs in the lamina propria of the small intestine of the host. The bradyzoites released from infected meats from the sarcocysts become male and female gametes, fuse to produce zygotes and finally develop into oocysts. The reproduction of the parasites leads to the secretion of *Sarcocystis* substances which promote the release of inflammatory mediators. Eosinophilic enteritis with infiltration of polymorph nuclear cells (PMN) may occur. Necrosis is possibly seen as a result of autoimmune reactions.

S. cruzi, *S. hominis* and *Sarcocystis ovicanis* cause muscular sarcocystosis in cattle, pigs and sheep, respectively, the intermediate hosts of the parasite. The pathogenicity of *Sarcocystis* varies depending on the amount of ingested sporocysts, the ability to multiply, the location and proliferation of schizonts, and the probability to reach the central nervous system. Host factors such as the immunity, gestation, stress, lactation and nutritional status also play a role in determining the severity of the *Sarcocystis* infections.

The pathogenesis of muscular sarcocystosis include proliferation phase and stick phase. Proliferation phase occurs after sporozoite penetration and migration from the epithelial cells of small intestine to the endothelial cells of the vascular tunics throughout the body. The asexual replication (*schizogony*) process causes inflammation (endoarteritis) and eventually increases the capillary permeability, leading to extravasation of fluids and bloods. The disrupted cells promote vacuolization and leucocyte infiltration in the tunica media of the blood vessel. The systemic pressure might be elevated due to the partial blockages in the vascular lumen. Cell lesions similar to a late hypersensitivity reaction (type IV) may occur as a result of aggressive immune response and accumulation of mononuclear cells in vascular tissue. If the asexual multiplication takes place in placental cotyledons or myoepithelial cells of the pregnant hosts, abortions, and foetal deaths may occur.

During the cystic phase, numerous merozoites are released from the mature schizonts leading to the next-generation endothelial schizonts. These merozoites invade the skeletal, cardiac myocytes and neurons and become metrocysts where a series of mitotic divisions take place before fully developing into mature sarcocysts containing the infective stage, bradyzoite. During this cystic phase, eosinophilic myositis will occur mainly due to the high level of IgE and intensity of the parasites. After cyst formation, no pathological response is further observed. However, the infected intermediate hosts continue to suffer from growth retardation. The lysed bradyzoite protein has exhibited neurotoxic activity that may cause bleeding, paralysis and even death of the host.

Immunology

An experimental study in human volunteers who repeatedly consumed the sarcocysts in beef and pork revealed no or little immunity in the intestinal sarcocystosis. Protective immunity following an early infection has been demonstrated in the intermediate hosts suffering from muscular sarcocystosis.

Immunoglobulin M (IgM) antibodies appear after 3–4 weeks in animals infected with *S. cruzi* infection but return to pre-infection levels after 2–3 months. IgG antibodies increase after 5–6 weeks post-infection and persist for 5–6 months. However, the persistence of *Sarcocystis* antibodies is different between species and hosts. Cytotoxic antibodies or metabolites from

cell-mediated immunity are known to destroy the second generation of extracellular merozoites. The protective immunity towards muscular sarcocystosis might explain why the prevalence of *Sarcocystis* was lower in older sheep, pigs, and goats reported before. It has been observed that the foreign travellers were experiencing the illness following the outbreaks in some islands in Malaysia, but not the local population. It was presumed that the local population who were infected some time earlier had some protective immunity during the outbreak.

Infection in Humans

Intestinal Sarcocystosis

In humans, intestinal sarcocystosis is commonly caused by *S. suihominis* through infected pork or *S. hominis* through infected beef. The incubation periods are very short. In an experimental study, the onset of diarrhoea occurred in 3–6 h and usually within 48 h after the ingestion of sarcocysts.

Infection is usually mild or asymptomatic. Nausea, vomiting, abdominal discomfort, self-limited diarrhoea, acute and severe eosinophilic enteritis, fever and chills are present in symptomatic cases. The symptoms are usually self-limiting and typically resolved within 36 h of onset. Generally, the severity of the infections depends on the amount of ingested sarcocysts in meats or, perhaps, the species of the *Sarcocystis*. It has been suggested that *S. suihominis* infection from infected pork causes more serious problem than *S. hominis* infection in human.

Muscular Sarcocystosis (Extra-intestinal Sarcocystosis)

Humans may also act as dead-end intermediate hosts for certain *Sarcocystis* species by accidental ingestion of oocysts/sporocysts which can cause muscular sarcocystosis. The condition has a broad spectrum of clinical presentation, ranging from asymptomatic to severe eosinophilic myositis. Acute manifestations including fever, myalgia, and bronchospasm occur within 3 weeks after returning from the field operations. Other infrequent manifestations include elevated eosinophil count, creatine kinase and erythrocyte sedimentation rates, subcutaneous nodules and lymphadenopathy. Sarcocystosis have also been linked with cardiomyopathy glomerulonephritis and even malignancy. Table 2 summarizes the clinical presentations of human sarcocystosis.

Table 2 Intestinal sarcocystosis and muscular sarcocystosis in human

Characteristic	Intestinal sarcocystosis	Muscular sarcocystosis
Route of infection	By consuming/eating raw or undercooked meat from infected animals (intermediate hosts)	By ingestion of water/food and from environments contaminated with faeces from infected carnivore or omnivore (definitive hosts)
Infective stage	Sarcocysts containing crescent-shaped bradyzoites	Oocysts/individual sporocysts containing sporozoites
Symptoms	Nausea, vomiting, abdominal discomfort, self-limited diarrhoea, acute and severe enteritis, fever and chills	Musculoskeletal pain or myalgia, fever, rash, cardiomyopathy, bronchospasm, subcutaneous swelling, eosinophilia
Incubation period	Symptoms may start at 3–6 h, lasting 36–48 h. Sporocyst excretion may persist for months	Weeks to months, lasting months to years
Diagnosis	Detection of oocysts or sporocysts in faeces (beginning 5–12 days after ingestion), molecular investigations	Biopsy specimen containing sarcocyst; antibodies to bradyzoites (serology), molecular investigations
Treatment	None. Co-trimoxazole and furazolidone have been used in some case studies	None approved. Co-trimoxazole, furazolidone, albendazole, anticoccidials, pyrimethamine, anti-inflammatory drugs
Prevention	Avoid eating raw/uncooked meats. Freeze meat at −5 °C for 48 h	Boiling water. Do not drink untreated water. Avoid raw vegetables. Good hygienic practice

Infection in Animals

Sarcocystis infection is usually asymptomatic or mild in animals. Some *Sarcocystis* species cause serious infections such as weight loss, anaemia, muscle, weakness, lymphadenopathy, abortion and even death in meat-producing animals. The severity of the infection depends whether the animal is pregnant, lactating or under stress.

S. cruzi infection in cattle causes fever, anorexia, cachexia, decreased milk yield, muscle spasms, anaemia, loss of tail hair, hyperexcitability, weakness and prostration. Even after recovery from acute illness, the cattle fail to grow and die in a cachectic state. *S. tenella* causes encephalomyelitis in sheep.

Equine protozoal myeloencephalitis (EPM) caused by *S. neurona* is one of the most serious manifestations of sarcocystosis, especially in American horses. Horses are aberrant hosts for the parasite. The parasites are found in neurons and leucocytes of the brain and spinal cord. Clinical symptoms include gait abnormalities such as knuckling, ataxia and muscle atrophy of the hind limb. EPM can also mimic many other neurologic diseases. Pigeon protozoal encephalitis is another serious manifestation, associated with severe brain lesions in pigeons caused by *Sarcocystis chalchasi*.

Epidemiology and Public Health

Muscular sarcocystosis in humans is a rare zoonotic infection. Although *Sarcocystis* species are widely distributed, most of the cases are reported from the South-East Asian countries, particularly Malaysia. Before the series of human outbreaks in islands in Malaysia, less than 100 cases of this infection have been documented worldwide. Majority of the cases were diagnosed by accidental biopsy and at autopsy or necropsy without description of the clinical symptoms or identification of *Sarcocystis* species. In Malaysia, it was observed that the indigenous population was the most at risk, attributed to their poor sanitary conditions and higher consumption of wild animals such as wild boars, deer and lizards.

Two outbreaks of muscular sarcocystosis were recorded in Tioman Island and Pangkor Island during 2012 to 2014 among more than 100 travellers visiting Malaysia from Germany and other countries (Fig.4). Severe myalgia,

Fig. 4 Location of the reported cases of human muscular sarcocystosis in Malaysia. Image source: Latif & Muslim 2016

eosinophilia, elevated serum CK levels and negative *Trichinella* infection serology were observed in 68 cases. Fatigue (91%), acute fever (82%), headache (59%) and arthralgia (29%) were also observed. Muscle biopsies revealed intramuscular cyst-like sarcocysts in six cases. The causal agent was identified as *S. nesbitti*, the parasite that was reported in monkey (*Macaca fascicularis*). Further studies suggested that snakes such as reticulated phytons and monocled cobras served as definitive hosts for *S. nesbitti,* as revealed by molecular studies targeted on 18S rDNA genes of the parasite. It was proposed that the humans acquired the muscular sarcocystosis by the accidental ingestion of water, food or soil contaminated with sporocyst excreted from infected defective hosts such as cats, dogs, and snakes (e.g. pythons).

Sarcocystis spp. causing muscular sarcocystosis are ubiquitous in nature and are found in many animals including primates, rodents, birds and zoo animals. The infected animals, similar to humans, have been found to harbour sarcocysts in their muscle tissues. The sarcocysts were detected primarily at necropsy or in abattoir. *Sarcocystis* infection was a major problem in livestock such as cattle, water buffaloes, sheep, goats and pigs, which were found to be highly infected, even up to 100% with the *Sarcocystis* cysts.

Humans acquire the intestinal sarcocystosis by ingestion of raw or undercooked meats from infected animals. Only two species, *S. hominis* and *S. suihominis* and in some cases *S. heydorni* (in infected cattle), are known to cause zoonotic intestinal sarcocystosis. Human intestinal infections, with the exception of Africa and Middle East, have been reported worldwide. In Europe, the infection has been reported mostly from Netherlands, Germany, and Slovakia. The infection has also been documented in Thailand due to consumption of raw pork and beefs, and also in Laos and Tibet (China). No case of intestinal sarcocystosis has been reported in Malaysia, except one asymptomatic case in indigenous people.

Diagnosis

Intestinal sarcocystosis is suspected in individuals who have a recent history of eating any raw or undercooked meat and complain of abdominal pain, nausea, and diarrhoea.

Microscopy

Definitive diagnosis is based on the detection of oocysts or free sporocysts in the stool of humans or animals (predators/definitive hosts). The diagnosis of human muscular sarcocystosis is made by the detection of sarcocysts in muscle biopsies of skeletal and heart muscles, tongue, oesophagus and diaphragm.

Microscopy of direct stool smear or following concentration by various faecal concentration techniques such as floatation and formalin ether concentration is used for the diagnosis of intestinal sarcocystosis. Ziehl-Neelsen acid-fast staining is used to differentiate apicomplexan parasites including *Sarcocystis* species. However, sensitivity of the test depends on the duration of sporocyst excretion. The long pre-patent periods for *S. hominis* (14–18 days) and *S. suihominis* (11–13 days) may show false negative results because the *Sarcocystis* may not be shed during the acute stage of infection. In animals, diagnosis of intestinal sarcocystosis is usually based on the microscopy of mucosal scrapings during the postmortem, not on stool microscopy.

Direct muscle or tissue compression between two glass slides is the easiest and rapid method for examination by microscopy. Staining by modified methylene blue or periodic acid Schiff may facilitate the detection and identification of sarcocysts to diagnose human muscular sarcocystosis. For the diagnosis of animal muscular sarcocystosis, macroscopic cysts *S. fusiformis* in water buffalo and *S. nesbitti* in long-tailed monkey can be demonstrated by the naked eye examinations. Histological examination by haematoxylin–Eosin (Fig. 5), Giemsa staining and peptic digestion are the other methods. Electron microscopy is useful to identify and distinguish *Sarcocystis* species, but is of academic interest.

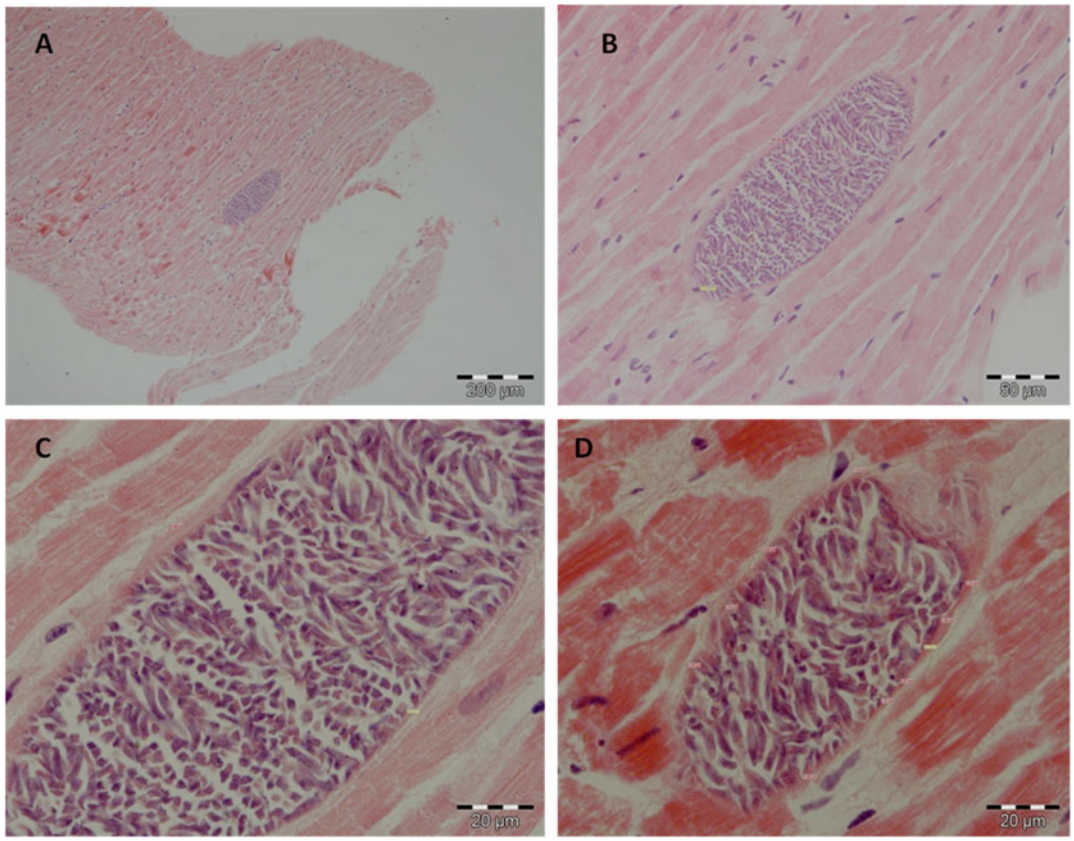

Fig. 5 Sarcocyst in skeletal muscles stained with H&E. (Source: Latif et al. 2015; Chong Chin Heo)

Serodiagnosis

Indirect fluorescence antibody technique (IFAT) and enzyme-linked immunosorbent assays (ELISA) are the frequently used antibody-based methods for the diagnosis of muscular sarcocystosis (Table 3).

Molecular Diagnosis

Molecular-based techniques are especially useful for the accurate identification and detection of *Sarcocystis* spp. Recently, the PCR targeting the 18S rDNA(Fig. 6) and COX 1 have been used for identification of *Sarcocystis* species. The DNA sequence studies may further be useful for the phylogenetic analysis, understanding the pathogenesis as well as development of therapeutic strategies.

Treatment

No prophylaxis or specific treatment for human intestinal sarcocystosis is currently available. However, co-trimoxazole or furazolidone drugs have been evaluated for the treatment of intestinal sarcocystosis in some case studies.

Similarly, there are no established or approved treatment for human muscular sarcocystosis. However, albendazole and ivermectin have been used to reduce the chronic symptoms of the muscular infection. Corticosteroids such as prednisolone are used for severe myalgia and may provide symptomatic relief by the reduction of inflammation-associated muscle involvement. The use of anti-protozoal drugs such as co-trimoxazole and metronidazole has shown some promising results for eosinophilic myositis.

Table 3 Diagnostic methods for sarcocystosis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Biopsy or autopsy/ necropsy	Sarcocysts in muscle tissue (muscular sarcocystosis)	Gold standard test Drawback: Invasive
	Stool collection/mucosal scraping (in animal)	Sporocysts/oocyst excretion (intestinal sarcocystosis)	Limitation: Low sensitivity
Immunodiagnostics	Antibody (ELISA)	IgG; IgM antibodies	High sensitivity especially for the serodiagnosis of muscular sarcocystosis
	Antibody (IFAT)		
Molecular assays	Nested PCR, PCR, RFLP, genotyping, RAPD, AFLP	18S rRNA, Cox-1, ITS-1, ITS-2	High sensitivity and specificity Limitation: Expensive, require skilled personnel

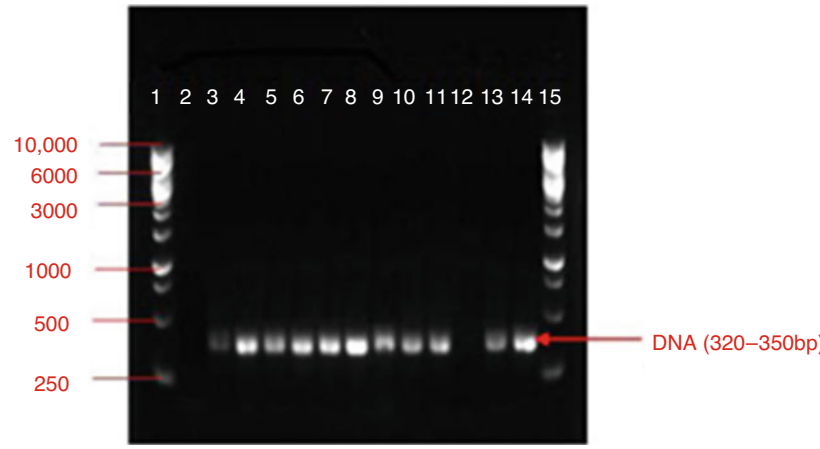

Fig. 6 Electrophoresis of PCR (targeted on D2 region in conserved regions of the 18SrRNA of *Sarcocystis* spp.) product of *Sarcocystis* isolated from meat-producing animals (i.e. sheep, goats and cattle in Malaysia. The DNA fragment size ranged between 300 and 350 bp (Source: Latif et al. 2015)

No standardized protocols have been developed for the treatment of sarcocystosis in animals. Prophylactic administration of amprolium and salinomycin was, however, found to be effective in preventing severe manifestations and death caused by *S. cruzi* in experimentally infected cattle and sheep. Toltrazuril, ponazuril or diclazuril, and a sulphonamide combined with either pyrimethamine or trimethoprim have been used as potentially useful prophylactic agents against *S. neurona*, the species causing equine protozoal myeloencephalitis (EPM). Drug trials using ponazuril, pyrimethamine-sulphonamide or trimethoprim-sulphonamide combinations have shown promising results in the treatment of *Sarcocystis* infections in dogs, cats, birds and other animals.

Prevention and Control

Neither prophylaxis nor vaccines are available against human sarcocystosis. Human intestinal sarcocystosis can be prevented by proper cooking or freezing meat thoroughly at least for 1 day to kill infectious bradyzoites. Cooking meat at 70 °C

for 15 min or freezing at −20 °C for 1 day or −4 °C for 2 days kills the infective muscle cysts. Human muscular sarcocystosis can be prevented by avoiding food, water or contact with soil that are potentially contaminated with faeces of infected definitive hosts. Boiling water, thorough washing and cooking of vegetables and good hygienic practices prevent the transmission of infection to humans.

Surveillance and detection of the *Sarcocystis* spp. in meat-producing animals is essential in the monitoring systems. To reduce the enteric transmission in definitive animal hosts, dogs, or other carnivores living in the same proximity should not be allowed to eat raw or dead animals. Food supplies such as grain used to feed house animals should be covered. Amprolium or salinomycin could be used as prophylaxis in order to reduce illness in infected cattle or sheep.

Case Study

A 32-year-old female visited the Wurzburg general hospital with complaints of persistent severe myalgia during last 5 weeks. She had visited Malaysia about 2 months ago, and the symptoms of fever, fatigue, and muscle pain started a week after returning home. Upon examination, the pain was prominent in the upper arms, back, thighs, and calves. Electrocardiograms were unremarkable. Laboratory findings revealed blood eosinophilia and elevated level of creatinine phosphokinase (CPK) of 1127 U/L. Both trichinellosis and toxoplasmosis serology findings were negative. A muscle biopsy specimen taken from tibial muscle and histopathological examination demonstrated *Sarcocystis*-like cyst filled with nucleated protozoan cells located in the muscle resembling the sarcocysts reported in human and animals in Malaysia. Based on the history, clinical signs and laboratory findings, acute muscular sarcocystosis was postulated. The patient was treated with albendazole (400 mg twice daily) for 14 days and prednisolone for 7 days (80, 40, 20 mg/day in decreasing dosage). The treatment was well tolerated, and improvement with complete subsidence of symptoms was seen during a follow-up examination after 3 weeks.

1. What may be the mode of infection in this case?
2. What travel advice should be given to a person going for a jungle safari in Malaysia and some neighbouring countries?
3. What are the differential diagnoses in a patient presenting with the above signs and symptoms?

Research Questions

1. Is the true burden of the disease, especially human intestinal sarcocystosis, underestimated?
2. Will evaluation of periodic local surveillance in both animals and humans provide a better understanding of *Sarcocystis*-host relationship?
3. How to improve our knowledge about the biology and molecular characterization of *S. nesbitti*?

Further Readings

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Balantidiasis

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Learning Objectives

1. To understand that balantidiasis can result in chronic as well as severe infection which may be potentially life-threatening.
2. To have the knowledge that *Balantioides coli* infection is important not only for humans but also for other animal species.

Introduction

Balantidiasis is a zoonosis caused by the protozoan ciliate *Balantioides coli*, which has worldwide distribution but is mainly detected in developing countries. It is a highly neglected parasitosis that can infect different animal species, especially pigs and nonhuman primates. Humans are considered to be occasional hosts, and infections among humans are mostly associated with proximity to pigs. Transmission of *B. coli* occurs mainly through ingestion of cysts as contaminants of water and food such as fruits and vegetables, or from hands that become contaminated after handling pigs. After becoming infected, humans can present a variety of clinical states, going from asymptomatic to chronic or acute, including fulminating. The parasite inhabits the large intestine and mainly gives rise to situations of diarrhea or dysentery. However, extraintestinal colonization with severe symptoms has also been reported. Although coproparasitological diagnosis under a microscope is used most often, confirmation of the protozoan species is recommended, and this can only be done through molecular techniques. Among the prophylactic measures for controlling this parasitosis, the following can be highlighted: improved basic sanitation; diagnosis and treatment of parasitized hosts; careful washing of fruits and vegetables; and use of protective equipment such as gloves and boots for people who handle animals, especially pigs.

History

The genus *Balantidium* (from Greek; *balanto* = bag) was proposed by Claparède and Lachmann

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in 1858 for a ciliate that they observed in the rectum of frogs. This was then named *Balantidium entozoon*. The species *B. coli* was first discovered by Malmsten in 1857 in two human patients with acute dysentery. He named the parasite *Paramecium coli*. Leukert in 1861 discovered a ciliate identical to that described by Malmsten, in the large intestine of a pig. Stein in 1863 felt that the pig ciliate described by Leukert and the human ciliate described by Malmsten were identical in appearance, and so he named both of them *B. coli*.

Although the parasite is still very well known within the scientific community as *B. coli*, it has now been proposed, in the light of advances in molecular biology techniques, to change the name of the species *Balantidium coli* to *Balantioides coli*. Through molecular analyses, it was observed that the species *B. coli* was grouped in a position that was phylogenetically distinct from *Balantidium entozoon*, the species that was the first to be described taxonomically. This demonstrated that *B. coli* should, therefore, belong to another genus, and for this reason, it was suggested that its name should be changed to *Neobalantidium coli*. In 2014, it was proposed that the species *B. coli* should be reintegrated into the genus *Balantioides*, the nomenclature that had already been attributed by Alexeieff in 1931.

Taxonomy

The genus: *Balantioides* belongs to family: Balantidiidae in the phylum: Ciliophora; class: Litostomatea; subclass: Trichostomatia; order: Vestibuliferida; and superfamily Trichuroidea.

The type species is *Balantioides coli* (Malmsten, 1857) Alexeieff, 1931.

A total of 50 species of *Balantidium* have been described, often according to size differences and the host species that they parasitize, but the validity of most of these species is still unresolved. These unresolved cases include *Balantidium caviae* from guinea pigs; *Balantidium suis* from pigs; *Balantidium wenrichi*, *Balantidium philippinensis*, and *Balantidium cunhamunizi* from nonhuman primates; and *Balantidium struthionis* from ostriches. The taxonomy will ultimately be resolved once the organisms undergo sequencing of their small-subunit rRNAs. After molecular analysis, these species are included within a single gene cluster and are, therefore, considered to be synonyms of *B. coli*.

Genomics and Proteomics

Studies using molecular biology tools have aided in confirming ciliate species and networks and in describing other species. SSrRNA is a gene of 1.5 kb which has been used in the classification of the parasite and is of importance along with morphological characteristics. The SSrRNA sequences of *Balantidium coli*/*Neobalantidium coli*/*Balantioides coli*(synonyms) are now available in GenBank, and comparison between them across various hosts, especially pigs, ostriches, nonhuman primates and humans, has not revealed much variability.

Two variants of *B. coli* have been characterized, A and B, based on 500-bp-sized hypervariable region ITS1-5.8S rRNA-ITS2. These two variants represent two separate micronuclear rRNA genes, both of which may exist in the same strain of *B. coli*. The variants A and B have again been subdivided depending on their ITS1 helix II features into five types (A0, A1, A2, B0, and B1). The epidemiological importance of genetic variants A0, A1, A2, B0, and B1 needs to be further studied through more sequences, including investigations on more ciliates isolated from humans.

Through electron microscopy studies and cytochemical assays, the Skotarczak research group identified production of the enzyme catalase in *B. coli*, mainly from peroxisomes of *B. coli* isolated from symptomatic pigs. Also in *B. coli* isolates from symptomatic pigs, this research group used cytoenzymatic assays to prove the existence of secretory-type activity in mucocysts, consisting of the enzymes ATPase and glucuronidase.

The Parasite Morphology

B. coli is considered to be the largest protozoon and only parasitic ciliate in humans.

Trophozoites

The trophozoites of *B. coli* are an active form of this protozoon and are considered pleomorphic. Overall, the length of the trophozoites can range from 30 μm to 300 μm and its width from 30 μm to 100 μm. Their anterior region is more tapered and their posterior region is more rounded. At the anterior end, they have a funnel-shaped depression known as the *peristome*, which leads to the *cytostome*, also called the mouth, and the *cytopharynx*, that is, the interior oral cavity. The cytoplasm is enveloped in a thin pellicle covered by numerous cilia that extend from the oral cavity. The cilia are organs of locomotion that are embedded in the pellicle, that is, the plasma membrane. They cover almost the entire body of the parasite, including a region of the *peristome* where they serve to propel food particles into the *cytostome*.

The cytoplasm of the trophozoites contains food vacuoles, where digestion of food such as starch grains, bacteria, red blood cells, or fat droplets takes place. The indigestible remains are eliminated through a small groove at the posterior tip of the body of the trophozoite, known as the *cytopyge* or anal pore. The cytoplasm also contains two contractile vacuoles. One of these is in the middle portion of the body and the other is in the posterior region near the *cytopyge*. These organelles are responsible for maintaining a stable osmotic pressure, draining excess fluid in the cytoplasm, and ejecting it to the environment external to the cell. Extrusomes (e.g., mucocysts), peroxisomes, hydrogenosomes (e.g., endoplasmic reticulum), and ribosomes and other cytoplasmic components are also observed. However, no Golgi apparatus has yet been seen.

Trophozoites possess two nuclei, which are comparatively called the *macronucleus* and *micronucleus*. The *macronucleus* is sausage, kidney, or bean shaped, and may be located in any part of the cytoplasm. However, the *micronucleus* has a spherical appearance and is located in the concavity of the *macronucleus.* Occasionally, amicronucleated form may be seen. The *macronucleus* displays a dense rope-like network of chromatin, dispersed in the nucleoplasm. The DNA in the vegetative *micronucleus* occurs uniformly and in a densely packed chromatin. All of the DNA sequences present in the *macronucleus* are derived from the *micronucleus*, but the *macronucleus* sequences are only a subset of the *micronuclear* sequences.

Cysts

The cystic form is the parasite's means of transmission and resistance to the environment, and it remains viable at room temperature for at least two weeks. The cysts may be spherical or slightly ovoid, and measure 40–65 μm in diameter. Their wall is thick and hyaline. They usually have a brownish color, but sometimes appear yellowish or greenish. Inside the cysts, cytoplasmic inclusions, cellular debris, food vacuoles and a *macronucleus* may be seen, but it is rare to observe a *micronucleus* under a microscope (Fig. 1).

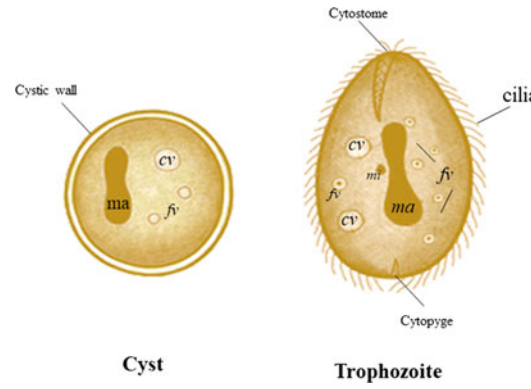

Fig. 1 *Balantioides coli* has two stages in its life cycle: cysts and trophozoites. Cyst: ma, macronucleus. Trophozoite: ma, macronucleus; mi, micronucleus; cv, contractile vacuole; fv, food vacuole

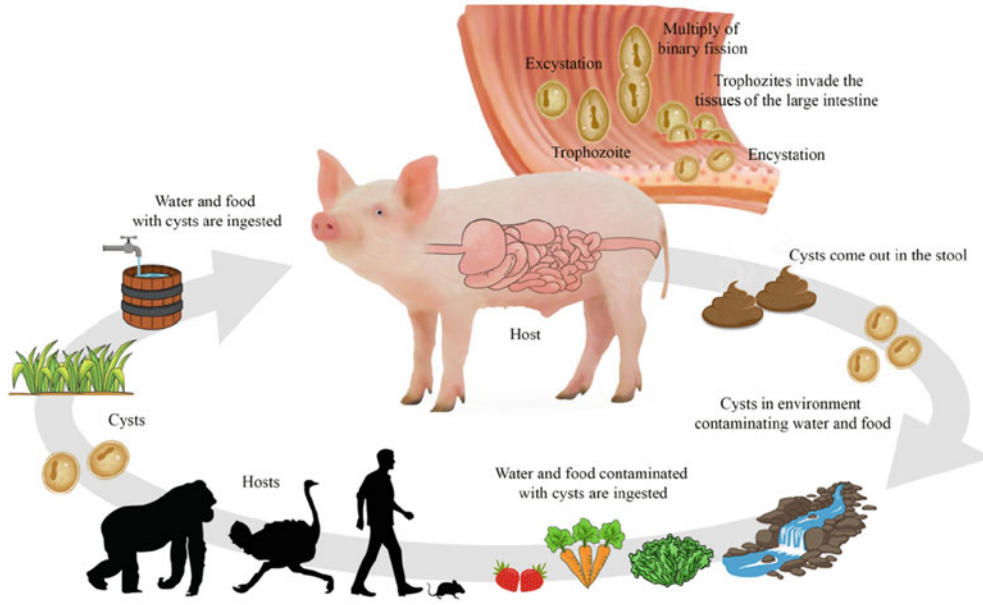

Fig. 2 Life cycle of *Balantioides coli*

Cultivation of Parasite

B. coli can be isolated and maintained in acellular culture media, generally in a *xenic* culturing system, or even in a *monoxenic* system when associated with the bacterium *Escherichia coli*. The culturing media most used for isolation and maintenance of *B. coli* are Pavlova (as modified by Jones), TYSGM-9, and LES (Locke egg serum). Isolation of protozoan strains can be done by simply inoculating an aliquot of the fecal material into the culturing medium plus rice starch. To maintain the culture, subcultures are performed at intervals of 48–72 h, with incubation at 36 °C.

Laboratory Animals

Attempts have been made to infect piglets, monkeys, and guinea pigs with the cysts of the parasite.

Life Cycle of *Balantioides coli*

Host

The life cycle is completed in a single host. *B. coli* can infect different host species (Fig. 2). The main hosts are pigs, nonhuman primates, and large birds, for example, ostriches and rheas. Humans are considered to be occasional or accidental hosts.

Infective Stage

Cysts.

Transmission of Infection

The main mechanism for the transmission of *B. coli* is indirect, through ingestion of cysts that are present in water and/or foods such as raw fruits and raw vegetables, or through direct contact with other people's hands that have become contaminated

with cysts. There have also been reports of human cases of inhalation of cysts, giving rise to pulmonary infection; direct colonization by trophozoites in the urogenital tract, especially in women; besides the occurrences of eye infection.

B. coli mainly inhabit the large intestine, including the caecum and colon. When the cysts come into contact with the gastric and pancreatic juices, breakage of the cyst wall begins, with the start of excystation. However, total excystation occurs only in the large intestine. In the intestinal lumen, the trophozoites multiply by means of transverse binary division. Because of their strong ciliary beat, the trophozoites are not eliminated with the feces into the environment, given that they are able to move against the peristaltic waves. Some trophozoites lose their ciliary mucosa and begin the process of encystation, with the formation of the cystic wall. These structures are then eliminated with the feces to the external environment and, if ingested, restart the cycle.

In some specific situations, the trophozoites invade the intestinal mucosa and are able to colonize diverse tissue layers of the large intestine. In this invasion process, *B. coli* may also colonize extraintestinal sites, through the blood or lymphatic circulation, by means of perforation of the colon and spreading through the peritoneal cavity. The extraintestinal locations colonized by *B. coli* in humans, which have already been reported, include the peritoneum, liver, gallbladder, genitourinary tract, lungs, spine, and eyes.

In addition to asexual reproduction, *B. coli* trophozoites can also reproduce sexually, through conjugation, that is, when two trophozoites come into contact through the cytostome and exchange genetic material.

Pathogenesis and Pathology

Many factors are involved in the pathogenesis of balantidiasis. These include the host's nutritional status, intrinsic virulence of the strain of *B. coli*, intestinal microbiota, enteric infections due to other etiological agents like pathogenic bacteria, parasite load, chronic diseases, compromised immune system, and the host's age.

The tissue damage produced by the parasite to the intestinal mucosa is directly related to its invasive capacity. This invasion occurs due to constant movement of the cilia and through lytic aggression caused by the release of a set of enzymes such as hyaluronidases. These enzymes possibly help in penetrating the intestinal tissues by dissolving the ground substance between the cells. A similar pattern of proteolysis and invasion of the intestinal mucosa by trophozoites has been reported in cases of infection by *Entamoeba histolytica*.

Organelles called mucocysts can be seen just below the plasma membrane of *B. coli* trophozoites. Mucocysts are extrusomes, or specialized secretory vacuoles, and morphologically present as polyhedral structures of different shapes. Extrusomes are thus named because they immediately and explosively discharge their mucoid content (proteins) when they are subjected to stimuli. The secretory activity of mucocysts has been implicated in ciliated invasion, because mucocysts have beta-glucuronidase activity. This enzyme catalyzes the hydrolysis of mucopolysaccharides, thus facilitating penetration of the cilia into the intestinal mucosa of the host.

Tissue invasion is often shown through pathological studies, in which lesions are mainly observed in the large intestine. In the beginning of the invasion, the lesions tend to be superficial with small foci of necrosis where the parasites are found. With the passage of time, the ulcers tend to become larger and gain the presence of blackened necrotic tissue. Parasites can then also be found in blood capillaries, lymphatic channels of infected tissue, and neighboring lymph nodes. Microscopically, the parasite can be observed in all layers of intestinal aggregates. Small clusters are more often seen in the submucosa or around micro-abscesses. The cellular reaction is mainly lymphocytic and eosinophilic. Such cases usually occur when the parasite perforates the intestinal wall and, through anatomical proximity to the tissues or circulatory blood or lymphatic system, colonizes other organs, thus giving rise to extraintestinal infections.

Immunology

Studies proving information on the immune response relating to infection by *B. coli* are scarce. The experiments that have been conducted have nonetheless demonstrated the production of components that form part of the immunological response. Zaman in 1964 showed that live *B. coli* exposed to fluorescein-conjugated antiserum was immobilized in the cell within a few minutes. Sestak et al. in 2003 studied captive rhesus macaques with and without chronic diarrhea and found numerous organisms belonging not only to protozoan parasites but also to bacteria and viruses with bacterial pathogens being predominant. The prevalence of ciliates similar to *B. coli* was around 12%. Monkeys with chronic diarrhea had higher levels of interleukin-1, interleukin-3, and tumor necrosis factor α. This may not be only due to the ciliate protozoan parasites as other organisms were also present.

Infection in Humans

B. coli can give rise to clinical manifestations in infected hosts, ranging from mild to severe. The presentations of the infection are classified as (1) asymptomatic carrier; (2) dysenteric or acute form, which ranges in intensity from mild to (3) fulminant; (4) chronic infection; and (5,6) extraintestinal infections.

1. Asymptomatic hosts of the parasite eliminate cysts into the environment and end up being considered to be sources of infection. Children who come into contact with environments contaminated with pig feces have already been identified as the main asymptomatic hosts.
2. The acute form appears suddenly, with 3–15 diarrheal episodes daily, accompanied by tenesmus, feces containing mucus, blood, and neutrophils. The patients complain of epigastric pain, nausea, and abdominal pain. Weakness is a marked symptom because rapid weight loss may occur.
3. Some patients may lose up to 40 kg over a 3-month period. The acute fulminating form is generally seen in emaciated patients or at late stages of some other severe disease. Overt hemorrhage may occur, and this leads to death through exsanguination and dehydration in 3–5 days.
4. The chronic form is characterized by the presence of loose bowel movements alternating with episodes of constipation. There may be epigastric distress, colicky abdominal pain, and tenesmus. The number of bowel movements ranges from 3 to 20 a day, and mucus is often seen, but pus and blood are only rarely seen. The duration of the infection ranges from 4 months to 26 years in adultsInfection in humans and from 1 week to 4 years in children.
5. Although the large intestine is the most common site for *B. coli* disease, there are extraintestinal sites of infection. When the parasite colonizes the liver, and peritoneum, the main symptoms seen in infected patients are fever, abdominal pain, and diarrhea or dysentery.
6. Respiratory tract infections manifest as fever, in addition to dyspnea and chest pain. In the genitourinary tract, dysuria, frequent urination, and pelvic pain are observed. In spinal infection, the patients' main complaints are fever and limited mobility. In the eyeball, symptoms of keratitis are evident, including blurred vision, foreign-body sensation, and photophobia.

Infection in Animals

In relation to both pigs and other animals, reports on clinical cases caused by *B. coli* are very scarce. Some authors have considered that, in pigs, *B. coli* is a commensal protozoon and that under certain circumstances such as immunocompromised states, nutritional deficiencies, or associations with other pathogens, its presence can cause diarrhea. Death of pigs due to balantidiasis is uncommon, but it can occur one to three weeks after the onset of symptoms. In other recently published reports, authors suggest that the clinical manifestations in pigs are similar to those that occur in human hosts and that these

Table 1 Distribution of *Balantioides coli*

Species	Distribution	Main hosts	Occasional host
Balantioides coli	South America, Central America, Philippines, New Guinea, Micronesia, and ancient Persian region	Pigs and nonhuman primates, ratite birds	Human

can vary from asymptomatic to acute or chronic forms. The factors that influence the pathogenicity of this parasite in infections in nonhuman primates are unclear. This protozoon has been primarily diagnosed in feces from Old World primates and great apes. In these animals, *B. coli* can cause chronic diarrhea or dysentery with mucus and blood.

Epidemiology and Public Health

B. coli is a parasite with worldwide distribution, that is, infections by this ciliate have already been reported in several regions of the world. The prevalence of balantidiasis in humans has been reported to be about 0.02–1%. The highest prevalence rates occur in tropical and subtropical areas. Thus, the endemic focus seems to be in South America, Central America, the Philippines, New Guinea, Micronesia, and ancient Persian region (Table 1). In South America, countries like Brazil and Venezuela stand out prominently (Fig. 3).

The main factors that favor the triggering of human balantidiasis are human contact with pigs, lack of a suitable place to discard feces (especially pig feces), and the climatic conditions of tropical and subtropical countries, which favor viability of the cysts of *B. coli* in the environment. Unlike in humans, infection by *B. coli* is very common in

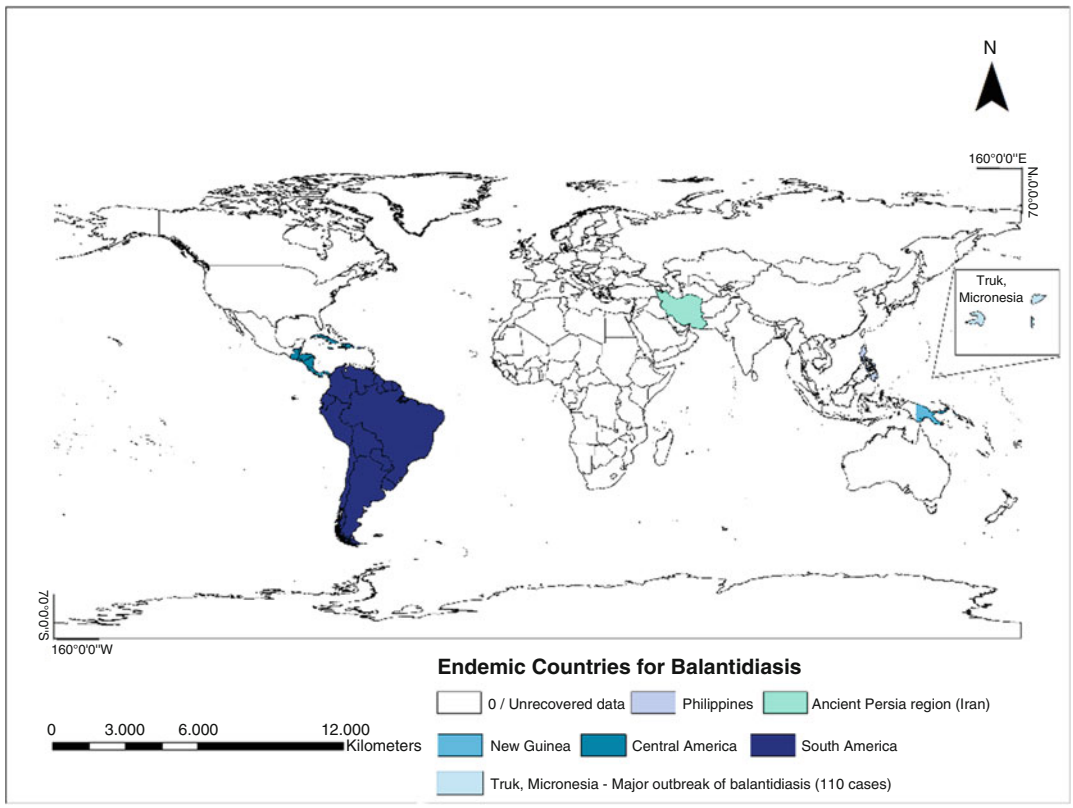

Fig. 3 Countries endemic for balantidiasis. On blue scales are the main locations that reported balantidiasis

pigs, and these animals are considered to be the main reservoirs for human infection. This puts veterinarians and other animal keepers at risk of becoming infected with this parasite. In addition, pig intestine handlers in slaughterhouses and people who use the feces of these animals as fertilizer are also considered to be at risk. *B. coli* is considered to be an opportunistic parasite because immunocompromised individuals and old people appear to be less resistant to infection, such that they usually present severe dysenteric disease.

Water contaminated with cysts of the parasite has been shown to be one of the main risk factors for parasite acquisition. In the biggest ever outbreak of human balantidiasis, the feces of parasitized pigs were considered the main triggering factor. This outbreak occurred in Truk, an island in Micronesia, Oceania, in 1973, where 110 people were infected with *B. coli* after the passage of a typhoon, which destroyed the island's drinking water reservoirs and contaminated rivers with feces from infected pigs.

The proximity between humans and pigs was identified as a determinant of the high incidence of human balantidiasis in Papua New Guinea, where human infection has already reached epidemiological rates of 28%. However, there have also been reports of cases of infection in Muslim countries where people are not in the habit of consuming pork and do not raise pigs, thus highlighting the possibility that other animals may act as reservoirs, including feral pigs, dromedaries and even humans.

Diagnosis

The diagnosis of balantidiasis is achieved by laboratory techniques (Table 2).

Table 2 Laboratory diagnosis of balantidiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Direct examination	Trophozoites mainly and cysts	Cheap and fast technique Material needs to be fresh and without chemical preservative to detect the viable parasite
	Spontaneous sedimentation or centrifugal sedimentation with ether or ethyl acetate	Cysts	Cheap and fast technique They are much more suitable for cysts research than flotation techniques
	Permanent staining	Trophozoites and Cysts	Mark internal structures and help differentiate from other ciliated protozoa
	Biopsies of the colon and other infected organs, followed by staining	Trophozoites	The damage caused by trophozoites in the process of intestinal mucosa invasion is observed
In vitro cultures	The culture media most used for isolation and maintenance of *Balantioides coli* are Pavlova modified by Jones and TYSGM-9	Trophozoites	Allows maintaining the parasite's trophozoites for further studies. These techniques are expensive, time-consuming and tiring
Immunodiagnostics	Immunological tests for the diagnosis of *Balantioides coli* have already been developed by some authors.	Immunoglobulin and coproantigen	There is no standardized immunodiagnosis test for *Balantioides coli* research
Molecular assays	PCR	18S rRNA and ITS1-5.8s rRNA-ITS2	High sensitivity and specificity Determine the species of ciliate and, in the case of *Balantioides coli*, to classify the types of variants

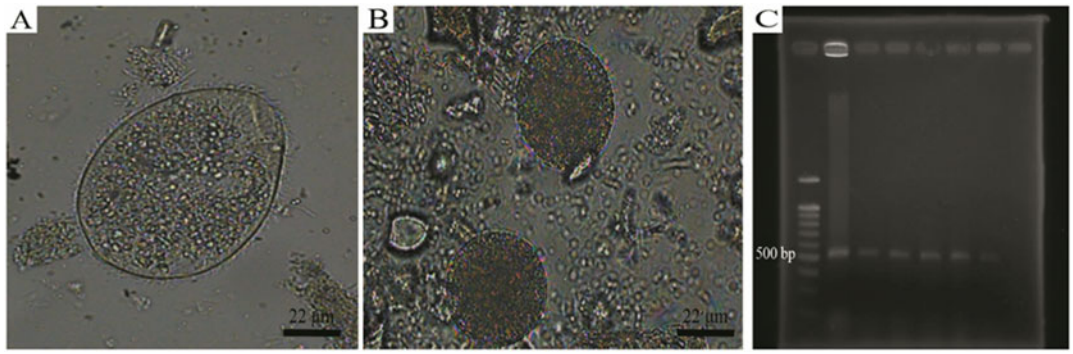

Fig. 4 Diagnosis of *Balantioides coli*. Forms of trophozoites (**a**) and cysts (**b**) detected in microscopic parasitological techniques. Electrophoresis gel with 500 bp (**c**) band compatible with that detected for *Balantioides coli*

Laboratory Diagnoses in Humans and Animals

Microscopy

In relation to intestinal infections, the diagnosis of balantidiasis is routinely made using coproparasitological techniques followed by microscopy, in which trophozoites and/or cysts are observed in fresh fecal samples with or without chemical preservatives (Fig. 4a, b). *B. coli* cysts are released only intermittently into feces, which means that fecal samples need to be repeatedly collected on multiple occasions, in order to make the diagnosis. Material obtained from fresh feces needs to be examined immediately because trophozoites deteriorate within a short time. In diarrheal samples, which predominantly contain trophozoites, direct examination is recommended. The trophozoites display rapid revolving motility. The cystic form is usually found in solid and semisolid stools. To make the diagnosis, direct examination in association with parasitological sedimentation techniques is indicated. The latter can include spontaneous sedimentation or centrifugal sedimentation with ether or ethyl acetate. In human feces, cysts are generally detected in less than 10% of the fecal material, whereas trophozoites are diagnosed in more than 80%.

Permanent staining of the fecal material, for example, with hematoxylin, makes the diagnosis easier. This staining enables visualization of the macronucleus impregnated by the dye, both in cysts and in trophozoites. Biopsies of the colon and other infected organs, followed by staining of the biological material using hematoxylin-eosin, may be useful for observing the parasite and evaluating the damage caused by parasite invasion and the inflammatory response.

In cases of lung infection, erroneous diagnoses may be made, due to difficulty in differentiating between ciliate cells of the respiratory epithelium and trophozoites of *B. coli*. In these cases, direct examination of bronchoalveolar lavage should be performed. Infections of the common genitourinary tract can be detected by means of sediment microscopy techniques in the laboratory. Regarding samples of biological ocular material, including corneal scrapings, contact lenses and lens cleaning solutions, the diagnosis of *B. coli* can be made through direct sample examination, permanent staining or in vitro culturing with subsequent evaluation of the material under a microscope.

In Vitro Cultures

B. coli can be isolated and maintained in acellular culture media, generally in a *xenic* culturing system, or even in a *monoxenic* system when associated with the bacterium *Escherichia coli*. It is normally not done for routine diagnostic purpose.

Serodiagnosis

Immunological tests for the diagnosis of *B. coli* have already been developed by some authors.

However, no tests have yet been found to detect anti-*B. coli* immunoglobulins in serum or to detect coproantigens from fecal parasites. Few epidemiological studies have been conducted with the protozoan. Ratio titers $\leq$1:64 available in experimentally infected rabbits were able to cause immobilization reactions both in previous porcine strains and in human strains.

Molecular Diagnosis

Molecular biology is a fundamental tool for confirming *B. coli* infection, since the evolutionary form of the protozoan is of pleomorphic nature, which may lead to misunderstandings in making the diagnosis. For *B. coli*, few molecular markers have been described, and the main target used has been ribosomal RNA, especially the region ITS1-5.8s rRNA-ITS2, that amplifies a 500 bp gene fragment (Fig. 4c). Through analyzing the ITS regions, *B. coli* isolates can now be classified into A0, A1, A2, and B0 and B1 variant sequences and also into subvariants such as A0a, A0b, B0a, B0b, B1a, and B1b. In GenBank there are many sequences of *B. coli* isolated from pigs, nonhuman primates, ostriches, and humans.

Treatment

The drugs of choice for treating balantidiasis are tetracyclines, metronidazole, and iodoquinol. For tetracycline, the adult dosage is 500 mg four times a day and the pediatric dosage is 40 mg/kg of body weight in four doses per day over 10 days. For metronidazole, the treatment is over 5 days, with an adult dosage of 500–750 mg, three times a day, and a pediatric dosage of 35–50 mg/kg of body weight in three doses per day. For iodoquinol, the adult dosage is 650 mg three times a day and the pediatric dosage is 30–40 mg/kg of body weight in three doses per day, with treatment over 20 days. Tetracyclines are contraindicated during pregnancy and for children <8 years old.

Prevention and Control

The prophylactic measures for controlling balantidiasis include basic sanitation; careful washing of foods such as fruits and vegetables in running water; and diagnosis and treatment of parasitized hosts, especially those who are asymptomatic carriers.

People who work with animals, especially pigs and nonhuman primates, need to take extra care and should routinely include the use of personal protective equipment, such as boots, gloves, and even masks when handling animals. Health education measures are essential for raising community awareness, with the aim of promoting changes in people's behavior, so as to decrease the risk of infection.

Case Study

An 18-year-old woman living in a rural area in the state of Rio de Janeiro was seen at the Antônio Pedro University Hospital in Niterói, RJ, Brazil, with diarrhea symptoms comprising 15 bowel movements a day, along with muscle weakness, diffuse pain throughout the abdominal cavity, anorexia, pallor, and fever of 38 °C. Within 24 h of hospitalization, she started to have an acute condition, with severe cramps and intense sweating, with yellowish liquefied stools. Food poisoning was suspected. The patient's feces were collected and sent for analysis by parasitologists and professors at the Biomedical Institute of the Fluminense Federal University (Ottilio Machado, Antônio Luiz de Pinho, and Said Silva), who performed drug analysis to control for amebiasis. In the fecal material, the bacteria *Escherichia coli* and *Enterococcus* sp. were detected in in vitro cultures. In addition, the patient's feces were subjected to a series of coproparasitological tests, including direct examination, in which trophozoites and cysts similar to *Balantioides coli* and *Trichuris trichiura* eggs were identified. Moreover, the spontaneous sedimentation and floatation techniques were performed, and *B. coli* cysts, *T. trichiura* eggs, and *Blastocystis* sp. were detected.

The patient received a cocktail of drugs that included iodoquinoline, acetamide, and monodral. Twenty-four hours after taking the medication, the patient no longer complained about the infection and presented suppression of symptoms. In order to verify the effectiveness of the cocktail, two cure controls were performed, that is, on newly collected feces from the patient. The first control was performed 20 days after treatment and, the second, 38 days after treatment. No parasites were detected in either control. Even though the patient did not report having had any contact with pigs, these animals were being raised in the rural area where she came from. Thus, the parasitologists were led to believe that environmental contamination with feces from parasitized pigs was the source of the infection.

1. Which other parasitic diseases in humans are associated with pigs?
2. Describe the characteristics of *B. coli* in fresh wet mount specimen of stool.
3. Mention the methods by which it can be kept viable for experimental purposes.

Research Questions

1. How to design a molecular diagnostic method which can help to confirm balantidioides?
2. What is the immunological response and pathophysiology in balantidioides?
3. Is there involvement of other animal species in the zoonotic transmission of infection?

Further Readings

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Part III

Zoonotic Helminthic Infections: Trematode

Schistosomiasis

M. C. Agrawal and Suman Kumar

Learning Objectives

1. To be able to distinguish the three parasites on the basis of morphology of the worm and the egg.
2. To have a knowledge about hybrid species.
3. To have a knowledge about the newer serolgical tests for schistosomiasis.

Introduction

Schistosomiasis or Bilharziasis is a disease complex affecting both humans and animals. The genus *Schistosoma* contain at least 22 species, of which three species, mainly *Schistosoma haematobium, Schistosoma mansoni*, and *Schistosoma japonicum* have been affecting human beings for a long time. Interestingly, these species differ in their snail hosts and their geographical distributions according to the spread of the snail host. Schistosomiasis in humans remained a neglected waterborne disease complex as it affects mostly developing countries, but the scenario is rapidly changing. This is due to the launch of Special Programme for Research and Training in Tropical Diseases (TDR) by WHO, development of a simple faecal diagnostic technique (Kato's technique) for *Schistosoma* eggs, a single dosage of drug given orally, and nuclepore method for diagnosis of urinary schistosomiasis. However, such developments are lacking in animal schistosomiasis and hence difficult to control. This chapter describes human schistosomes including schistosomiasis in India.

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History

S. haematobium was first discovered by a German parasitologist Theodor Maximilian Bilharz in the portal veins of an Egyptian peasant in Cairo (Kasr El Aini Hospital) in 1851 and named it *Distoma haematobium* (*Distoma* = flukes with two suckers/mouths; *haematobium* = responsible for haematuria). A new genus, *Bilharzia*, was suggested by Cobbold in 1859 in honour of its discoverer, but 3 months earlier Weinland (1858) had named it *Schistosoma* (cleaved body); hence, the parasite was named as *Schistosoma haematobium*. Manson (1903) demonstrated lateral-spined eggs in the faeces of the patient who never had haematuria. He suggested the possibility of the two species one with the lateral-spined and the other with a terminal spine. Sambon (1907) first pointed out that these lateral spines eggs are associated with dysentery and belonged

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Table 1 Comparison of genome of three *Schistosoma* species

Genome character	*Schistosoma haematobium*	*Schistosoma mansoni*	*Schistosoma japonicum*
Genome size (Mb)	385	381	403
Chromosome number (2n)	8	8	8
No. of coding genes	13,073	13,184	13,469
Gene size (avg. bp)	11,952	13,397	10,003
Total GC content (%)	34.3	34.7	33.5
Coding regions (% of genome)	4.43	4.72	4.32

to a new separate species and named it as *Schistosoma mansoni* in honour of Manson. Majima (1888), for the first time, reported that hepatic cirrhosis in man in Japan is produced by the eggs of an unknown trematode. Katsurada (1904) found characteristic eggs (of *S. japonicum*) in faeces of five patients and suspected that the disease is caused by these eggs, and their adult worms may be present in the portal system and named it *S. japonicum*.

Taxonomy

The genus *Schistosoma* (Weinland, 1858) belongs to subfamily: Schistosomatinae (Stiles and Hassale, 1926); family: Schistosomatidae (Loss, 1899); superfamily: Schistosomatoidea (Stiles and Hassale, 1926); subclass: Digenea; class: Trematoda; and phylum: Platyhelminthes in the kingdom: Animalia.

The genus contains 226 species that are differentiated mainly on geographical distribution and snail compatibility. *S. haematobium, S. mansoni*, and *S. japonicum* are the main species that cause infection in humans. Other species which can infect humans are *Schistosoma mekongi, Schistosoma malayensis, Schistosoma intercalatum,* and *Schistosoma guineensis.*

Genomics and Proteomics

The nuclear genome of three *Schistosoma* species have been sequenced. *S. haematobium*, *S. japonicum*, and *S. mansoni* have genome sizes of 385, 397, and 363 Mb, respectively, which contain approximately, 13,073, 13,469, and 10,852 protein-coding genes. The comparison of the nuclear genomes of the three species is shown in Table 1. Apart from the nuclear genome, the mitochondrial genes have also been sequenced and used as molecular markers for species and strain identification. These genomes range in size from 13,503 to 16,901 bp and encode 36 genes comprising two ribosomal genes (large and small subunit rRNA genes) and 22 transfer RNA (tRNA) genes, as well as 12 protein-encoding genes. On the basis of mitochondrial gene analysis, 23 *Schistosoma* species have been proposed. Six clades were identified that correlate with the geographic distribution of the species analysed.

Proteomic studies have revealed many proteases (cathepsins and aminopeptidases), kinases, multidrug resistance transporters, and type V collagen in the various life stages of schistosoma. Potential vaccine targets including Dyp-type peroxidases, fucosyltransferases, G-protein-coupled receptors, leishmanolysins, tetraspanins, and the netrin/netrin receptor complex have been identified in different studies. SchistoDB integrates genome and proteome sequence data along with functional annotation of genes and gene products of the three important species. This resource also covers results from large-scale analysis including ESTs, metabolic pathways, and candidate drug targets.

Parasite Morphology

Adult

S. haematobium: The body of *S. haematobium* is dorso-ventrally flattened and elongated. They are dioecious; males are stouter (about 1–1.5 cm in length and 0.9 mm in breadth) and carry females (about 2 cm in length and 0.25 mm in the breadth) in gynaecophoric canal (Fig. 1), showing distinct sexual dimorphism. The integument, covering

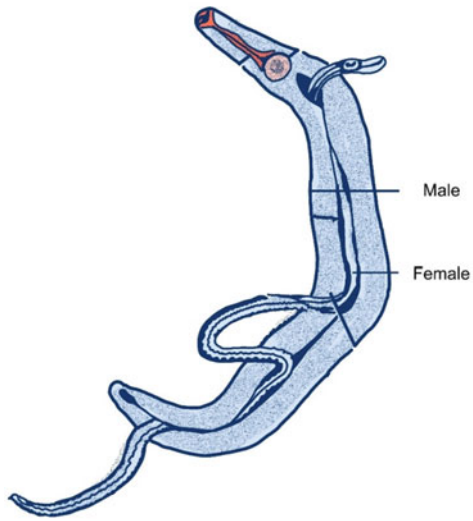

Fig. 1 Coupled male and female adult *Schistosoma mansoni* parasite in the blood vessel

body surface, is metabolically active, hence helps in exchanging nutrients in addition to protecting from the host immune responses. The oral sucker obliquely placed at the anterior end and posterior sucker is behind the oral sucker on the ventral aspect. The genital pore is situated a little behind the posterior sucker. The pharynx is absent, and oral sucker directly leads to the oesophagus which bifurcates into two intestinal caeca. The caeca reunite again before terminating in a blind end, giving it taxonomic importance.

The male reproductive system consists of four to six testes, a pair of vas deferens, and the seminal vesicle opens in the genital pore situated behind the posterior sucker. Adult female remains in the gynaecophoric canal of male fluke with free anterior and posterior ends though hybridization is common and reported from many parts of the globe including India. The female reproductive system consists of a single elongated ovary, oviduct, and uterus. The vitelline glands are situated in the posterior half of the body. The ova produced in the ovary are carried by the oviduct, and fertilization takes place in the ootype. The fluke releases 20–200 eggs per day with a ready-to-hatch miracidium and a terminal spine. The eggs laid in the small venules of the vesicle plexus tear the wall of vessel and bladder to get into the lumen and are passed out in the urine.

S. mansoni: Adult flukes reside in the mesenteric venule, draining the large intestine and posterior part of the ileum. Occasionally, they may also be found in branches of the portal vein, in the liver, upper branches of the super mesenteric vein, and vesicle plexus. The adult flukes are smaller than that of *S. haematobium*. The body is tuberculated behind the ventral sucker. In males, the integumentary tubercles are larger than that of *S. haematobium.* Male flukes are short, stout, and has six to nine testes that are arranged irregularly. In the female, the ovary is situated in the anterior half of the body. The uterus is conspicuously small and contains a few (one to four only) lateral-spined eggs. The female worms are characteristically held by the males in their gynaecophoric canal during the time of oviposition, in the small venules.

S. japonicum: Adults reside in the superior mesenteric vein draining the small intestine and occasionally the rectal plexus of the veins. The adult *S. japonicum* superficially resembles other human schistosomes except that the tegument of the body is non-tuberculated. Male flukes have seven testes that are arranged side by side in a single line. The female flukes have an ovary which is situated in the centre of the body. The well-developed uterus which is a long straight tube contains as many as 50–300 or even more eggs at one time.

Miracidium

It is the larval form of *Schistosoma* hatched out of the eggs. Miracidia are actively motile and swim in water due to the presence of cilia.

Cercaria

The furcocercous (fork-tailed) cercaria (Fig. 2) measure 175–250 μm × 50–100 μm in size. It has a long tail of about the same size as the body, and the furci are 60–100 μm in length. The oral sucker is absent, and a small spinous ventral sucker is present. It is a free-living aquatic form with a life span of 1–3 days. S. *japonicum* cercaria have four pairs of cephalic glands in contrast to two pairs in the other two species.

Fig. 2 A single, *Schistosoma mansoni* cercaria, which represented the larval stage of this parasitic fluke (Courtesy: PHIL, Dr. DS Martin, CDC)

Egg

Eggs are yellowish brown in colour and are non-operculated (Fig. 3).

S. haematobium eggs are 112–170 μm × 40–70 μm in size, elliptical, and have sharp prominent terminal spine.

S. mansoni eggs measure 114–175 μm × 45–70 μm in size. They are elliptical and have sharp prominent lateral spine.

S. japonicum eggs are 70–100 μm × 50–70 μm in size, and oval to almost spherical, with the presence of a rudimentary lateral spine.

Cultivation of Parasite

The cercaria with their tails separated by artificial means have been grown in a medium based on Basal Eagle's medium supplemented with lactalbumin hydrolysate, glucose, hormones, and human serum with added human blood cells (Group O). In this system, young schistosomules develop steadily to pairing, which can be first seen during the seventh week of culture. Egg hatching is also carried out using dechlorinated or spring water.

Laboratory Animals

Experimental studies have been carried out in baboon (*Papio* spp.), rhesus monkey (*Macana mulatta*), grivet monkey (*Cercopithecus aethiops*), mouse, and hamsters. African red monkey is the more efficient and less expensive animal model than baboons. Most of the experimental studies have been carried out with *S. mansoni* because it is relatively easier to maintain in the laboratory. *S. mansoni* in the snail (*Biomphalaria glabrata*) provides a source of cercariae for ready infection in laboratory animals.

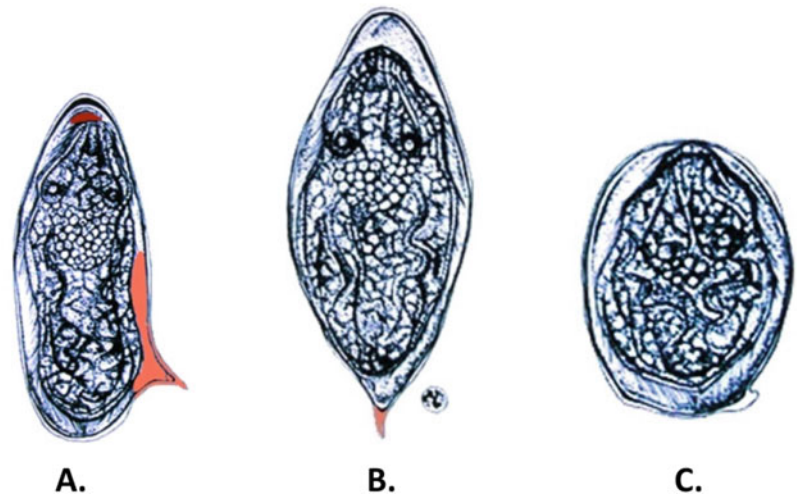

Fig. 3 Morphology of ova of A. *Schistosoma mansoni* B. *Schistosoma haematobium*, C. *Schistosoma japonicum*

Life Cycle of *Schistosoma* spp.

Hosts

Definitive Hosts

Humans, cattle, dogs, cats, rodents, pigs, horses, goats, and wild primates, depending on the species of the parasite.

Intermediate Hosts

Freshwater snails of genus *Bulinus, Physopsis, Biomphalaria,* and *Oncomelania.* The snail species is different for the various *Schistosoma* species.

Infective Stage

Cercaria is the infective stage of the parasite.

Transmission of Infection

Humans and other mammalian definitive hosts acquire the infection when they come in contact with cercaria present in the contaminated water (Fig. 4). The cercariae are attracted by skin secretions, following which they penetrate the intact epidermis by shedding up the tail. The glycocalyx of outer layer is shed, and the cercariae transform into schistosomules covered with a double-layered tegument. Within 24 h, the schistosomules enter the peripheral circulation and reach the heart. From the right side of heart, the small worms pass through the lung capillaries and enter the left side of heart and finally to the systemic circulation. A majority of the worms may be eliminated during passage through the lungs.

The schistosomules reach the liver by the hepatoportal system and continue to grow in the liver. After about 3 weeks in the liver sinusoids, the development of the adult worms becomes complete. The young male and female worms pair up and traverse together either to the intestine or to the urinary bladder depending on the species and start laying eggs. *S. haematobium* resides in the small venules of vesical and pelvic plexuses and sometimes in rectal venules; *S. mansoni* reaches the inferior mesenteric veins draining the large intestine, while for *S. japonicum*, the preferred site is the superior mesenteric veins that receive blood from small intestine. These two species can inhabit both the sites and may even move from one area to another. The inferior mesenteric plexus is also the habitat for *S. intercalatum* and *S. guineensis*, but at a lower region of the large intestine than *S. mansoni*. The deposited eggs reach the bladder or the intestinal lumen and are excreted with urine or stool.

The snail acts as an intermediate host, in which the parasite multiplies asexually to produce different larval stages. When eggs excreted by the definitive hosts come in contact with water, miracidium hatch out. The miracidium penetrates suitable snail host and undergoes asexual multiplication to give rise to first- and second-generation sporocysts and finally the fork-tailed cercaria. A single miracidium can produce up to 100,000 cercaria, and it takes about 4 weeks to complete the development. These cercaria enter the water and are the infective forms for the definitive hosts.

Pathogenesis and Pathology

The major pathogenic changes in the infected hosts are caused by *Schistosoma* eggs and in untreated cases may lead to chronic infections with the development of granuloma which may be fatal.

After penetration of the skin, the larvae are transported by the blood, but the dying or dead larvae may give rise to hypersensitivity reactions. This condition is known as cercarial dermatitis or *swimmer's itch*. The schistosomules or the eggs may trigger systemic hypersensitivity reaction and initiate immune complex formation particularly if the individual is exposed for the first time. In endemic areas, the patient remains asymptomatic, in certain species, till the development of the adult worms and excretion of eggs. The adults do not elicit any host reaction. The main pathology of patent infections is caused by inflammatory responses against parasite egg. The eggs secrete a glycoprotein which is antigenic. This egg substance produces inflammatory reactions as well as promotes granuloma formation around the eggs (Fig. 5).

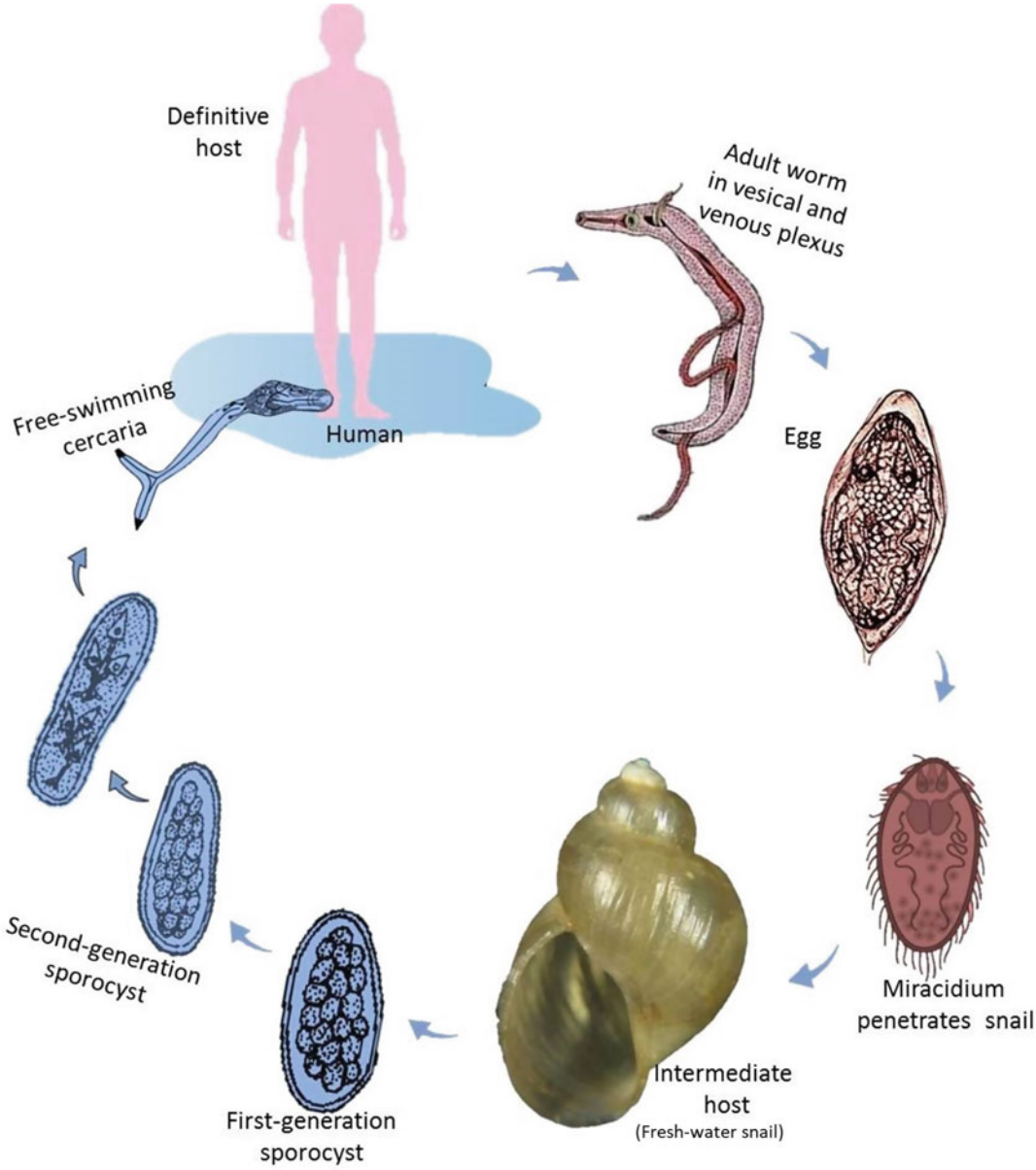

Fig. 4 Life cycle of *Schistosoma* spp.

Immunology

Host's immune response against *Schistosoma* spp., like other helminth infections, is complex. The host immune system has to encounter four different life stages of the parasite: cercaria, schistosomule, adult worm, and the eggs. This causes a myriad of alterations and modifications of the immune response due to the galaxy of antigens elaborated by different stages of the parasite.

Parasite immunity is largely the response of the host to the cercarial and possibly other developmental stages of the parasite. On the other hand, pathological consequences are almost always due to the immune responses against the

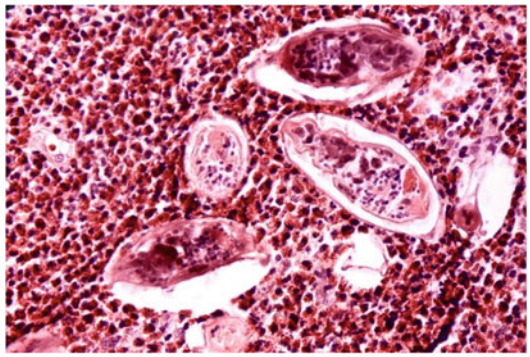

Fig. 5 Histopathological details seen in a bladder tissue specimen, in the case of *Schistosoma haematobium*. Presence of clusters of *S. haematobium* ova, surrounded by intense eosinophilic infiltrates, and other inflammatory cells can be seen. [Courtesy: PHIL, Dr. Edwin P. Ewing, Jr., CDC]

egg antigens. The host slowly develops immunity to the parasite in about 10–15 years of time. This explains why the paediatric population in endemic areas are susceptible to re-infection but adults are resistant. Host immunity is primarily a function of TH2 immune response with eosinophilia, production of specific IgE, IL-4, and IL-5. On the other hand, specific IgG4 has been associated with susceptibility to infection. Dead worms also evoke a protective response due to release of antigens which stimulate the release of IgE.

Infection in Humans

Urinary Schistosomiasis

Once *S. haematobium* infection is established in the ureter and bladder, painless haematuria is the first clinical sign in chronic schistosomiasis. The haematuria is frequently accompanied by dysuria, proteinuria, and frequent micturition. The disease usually progresses through the phases of the hydro ureter, hydronephrosis, secondary infections, and finely uraemia to reach the terminal stage. At times *S. haematobium* infection may also affect the seminal vesicles, prostate and testes in the males, and the cervix and vagina in the females leading to sterility and infertility. Inaction is closely linked with bladder carcinoma in endemic areas. Chronic urinary schistosomiasis has been associated with the development of bladder carcinoma. Pulmonary schistosomiasis due to migration of egg to the lungs may be seen occasionally.

Intestinal Schistosomiasis

The clinical manifestation in intestinal schistosomiasis is attributed to three distinct clinical phases: incubation, deposition of egg and extrusion, and tissue proliferation and repair.

During the phase of incubation, a pruritic papular skin rash known as *swimmer's itch* appears after 24 h of penetration of the skin by cercariae. This cercarial skin reaction is a sensitization phenomenon that occurs mostly in the cases of re-infection by the cercariae. This occurs rarely in the primary infection. The next clinical phase (5–7 weeks after the infection) corresponds to the beginning of oviposition in the intestine, liver, and spleen. Dysentery, with the passage of blood and mucus in the stool, is a distinguishing feature of this phase of infection. The patient complains of abdominal pain with frequent diarrhoea/dysentery and passing of egg mixed with exudate, mucus, and blood in the stool. As the disease progresses, the wall of the bowel becomes inflamed, thickened, and fibrosed with the formation of abscess and ulcers, accompanied by the loss of blood from the ulcers. Prolapse of the rectum is frequently present in the advanced stages of the disease. The liver and spleen are grossly enlarged due to infiltration by the eggs. The mesenteric lymph nodes are markedly enlarged due to infiltration by the eggs producing the cellular reaction. Occasionally, the eggs escape producing pathological changes in the kidney, spleen, lungs, pancreas, and other unusual sites.

The liver is grossly enlarged but characteristically firm. The *pipe-stem periportal fibrosis* of the liver, ascites, and oesophageal varices are frequent complications in the advanced stage of the infection. This may be accompanied by sudden episodes of hematemesis. The terminal stage of the disease is marked by decompensated liver diseases, ascites, jaundice, and liver failure. Schistosomiasis affecting the pulmonary and

cerebral nervous system, though rare, may occur as a late complication of the hepatosplenic schistosomiasis. *S. mansoni* infection has been increasingly associated with gram-negative bacteraemia.

Infection in Animals

Natural *Schistosoma* infections in chimpanzees in West Africa, rodents in Kenya and South Africa, and pigs in Nigeria have been reported. The eggs have been demonstrated in the faeces of these infected animals.

Epidemiology and Public Health

S. haematobium infection is endemic in Congo, Morocco, Tunisia, and Tanzania, and is widely distributed in the countries of Africa and the eastern Mediterranean region, and a few countries of Europe (Fig. 6 and Table 2). In sub-Saharan Africa, an estimated 112 million people are infected and 436 million people are at risk. Except occasional foci, the infection is absent in other countries of Southeast Asia and Western Pacific regions. Urinary schistosomiasis is common in children (10–14 years of age), and heavy infection is almost always accompanied by haematuria and proteinuria. Baboons and monkeys in East Africa, chimpanzee in West Africa, pigs in Nigeria, and rodents in Kenya and South Africa have been identified as the reservoirs of the infection. The possibility of inbreeding among schistosome species in nature has also been documented. Thus the epidemiology of *S. haematobium* is complex due to the interplay of multiple factors.

S. mansoni infection is widely distributed in and around the countries of South America, Africa, and certain Caribbean Islands. An estimated 54 million people are infected and 393 million are at risk in sub-Saharan Africa. The infection is more common in the individuals of 10–24 years of age. Heavy infection is noted in children between 10 and 14 years of age. The involvement of the spinal cord rather than the brain has been reported in the central nervous

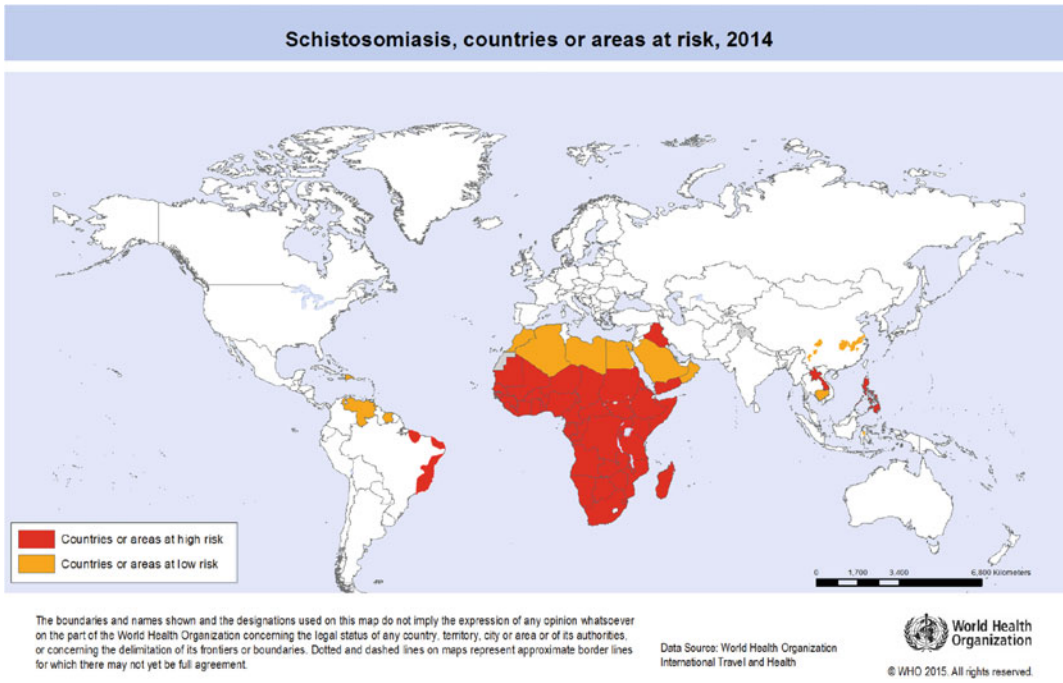

Fig. 6 Geographical distribution of human schistosomiasis (Courtesy: WHO)

Table 2 Geographic distribution and definitive hosts of *Schistosoma* species affecting humans

Schistosoma species	Distribution	Natural definitive host species (excluding humans)	Human public health importance
Schistosoma mansoni	Africa, Middle East, South America, Caribbean	Non-human primates (including apes), rodents, insectivores, artiodactylids (waterbuck), procyonids (raccoon)	High
Schistosoma haematobium	Africa, Middle East	Non-human primates (not apes), artiodactylids (pigs, buffalo)	High
Schistosoma intercalatum	Central Africa (D Congo only)	Possibly rodents	Low
Schistosoma guineensis	West Africa (Lower Guinea)	Possibly rodents	Low
Schistosoma mattheei	Southern Africa	Non-human primates (not apes), artiodactylids (cattle, antelope)	Low
Schistosoma japonicum	East Asia (China, Philippines, Indonesia)	Non-human primates, artiodactylids (water buffalos in particular), carnivores, rodents, perissodactylids (horses)	High
Schistosoma mekongi	SE Asia (Vietnam, Cambodia, Laos, Thailand)	Carnivores (dogs), artiodactylids (Pigs)	Moderate
Schistosoma malayensis	Peninsular Malaysia	Rodents (van Mueller's rat)	Low

schistosomiasis from South America and Caribbean countries. Natural infection has been documented in baboon, rodents, and dog in East Africa, rat in Egypt, gerbils in the Nile, rodent, cattle, and wild animals in Brazil, and rodents in South Africa and Zaire. Therefore, these animals are suggested to be responsible for maintaining the source of infection for man.

S. japonicum infection is an important zoonotic disease endemic in China and Philippines. The high prevalence of infection is observed in children of 10–14 years of age and adults of 35–44 years of age. Man gets infection by coming in contact of water having infective cercariae. Cat, dog, pig, cattle, rat, field mouse, water buffalo, sheep, goat, and other mammals serve as a reservoir of infection. The snails of *Oncomelania* spp. serve as an intermediate host.

Diagnosis

The clinical signs exhibited by the patients and history of occurrence of the disease in the particular geographical area give a clue of the infection; however, the confirmatory diagnosis is made based on the demonstration of eggs in the urine or stool and other indirect or direct methods (Table 3).

Microscopy

Demonstration of eggs in the urine sample by microscopy is the parasitological method for the detection of patent *S. haematobium* infection. The number of eggs excreted in the urine depends upon the intensity and duration of infection. Excretion of *S. haematobium* eggs in the urine of infected individuals follows a circadian rhythm; the eggs are discharged in the maximum number during the afternoon. Therefore, quantitative methods of urine sample examination are essential to assess the intensity of infection and also to assess the efficacy/response of the treatment. Nuclepore membrane filtration technique is a sensitive procedure for the diagnosis of urinary schistosomiasis. In this method, urine is filtered through a nuclepore membrane filtration system, then the filter membrane is examined for the presence of eggs by microscopy.

The specific diagnosis of intestinal schistosomiasis is made by the demonstration of the characteristic *S. mansoni* and *S. japonicum* eggs in the faeces. In chronic cases, the eggs may be scanty

Table 3 Laboratory diagnosis of schistosomiasis

Diagnostic approach	Methods	Target	Remarks
Microscopy	Urine: Concentration by nuclepore filtration, stool: Concentration by Kato-Katz technique, Bladder: Mucosal or rectal biopsy	Eggs	Quantitative methods are required to assess the intensity of infection and also to assess the efficacy/response of the treatment
Immunodiagnostics	Antibody detection by ELISA, Western blot, indirect haemagglutination tests	IgG	The detection of antibodies cannot differentiate the present and the previous exposure
	Antigen detection by ELISA	Circulating anodic antigen (CAA) and circulating cathodic antigen (CCA)	More sensitive for *Schistosoma mansoni*
Molecular assays	PCR, real-time PCR	Sm1-7 tandem repeat sequence for *Schistosoma mansoni*. Repetitive *Dra1* sequence of *Schistosoma haematobium*	Useful for early diagnosis during acute invasion phase, before eggs could be detected

and not uniformly excreted in the faeces. Hence, the direct smear examination of the faeces may not always demonstrate the eggs. The concentrations of stool by 0.5% glycerinated saline and by acid-ether technique are helpful in the diagnosis of chronic cases. Kato-Katz faecal thick smear technique is a frequently used method for the diagnosis. The egg count is obtained within 30 min after the preparation of slides. The stool containing 1–4 eggs (corresponding to 24–96 eggs/g of faeces) in the Kato-Katz slide is considered as light infection while the faeces showing 5–33 eggs (corresponds to 120–792 eggs/g of faeces) and more than 34 eggs (corresponds to more than 816 eggs/g of faeces) are regarded as moderate and heavy infections, respectively. In the pulmonary complications, the eggs may also be demonstrated in the sputum of patients.

Serodiagnosis

The detection of serum antibody by cercarian hullen reaction (CHR), miracidia immobilization test (MIT), circum oval precipitation (COP), using living cercaria, miracidium, and viable eggs, respectively, as the antigens have largely been abandoned in schistosomiasis serology. Indirect haemagglutination (IHA), indirect fluorescent antibody test (IFAT) and immune precipitation, ELISA, and modifications such as Dot ELISA, Falcon, and Fast ELISA are used to detect circulating antibodies. The detection of antibodies cannot differentiate between the present and the past infection as the antibodies may be present in the circulation, even after the elimination of the fluke. Detection of antibodies is useful in travellers who present with clinical symptoms and in those with very low or no egg excretion. Antibodies start developing against the adult parasites about 1–2 months post-exposure, and by 3 months complete seroconversion occurs.

The focus has now been shifted to the detection of two circulating antigens, circulating anodic antigen (CAA) and circulating cathodic antigen (CCA). Either CAAs or CCAs are positive in active infections with viable parasites, and they actually become positive even before the excretion of eggs. Several monoclonal antibody-based methods including up-converting phosphor lateral flow (UCP-LF CAA) technology,

commercially available as urine point-of-care (POC)-CCA test, have been developed and evaluated for the detection of these antigens in serum and urine for diagnosis. This point-of-care test (POC-CCA) for detecting urine cathodic antigen has an excellent diagnostic capability to diagnose *S. mansoni* infection but to a lesser extent for *S. haematobium* detection. It is commercially available and economical. This assay is now currently used in *S. mansoni* endemic areas for screening in relation to mass drug administration programmes.

Molecular Diagnosis

Several molecular techniques and a range of DNA targets have been described for the detection and quantification of *Schistosoma*-specific DNA in clinical samples. Even though PCR-based technology has high specificity and sensitivity, it is used less frequently for clinical diagnosis of schistosomiasis in endemic countries. This is because they require expensive laboratory equipment and highly skilled personnel. Detection of parasite DNA in stool, urine, vaginal lavage, and CSF is more sensitive than other methods and being used in high resource settings. Loop-mediated isothermal amplification (LAMP) technology has been mainly used to study *Schistosoma* infection in animal models or for monitoring of *Schistosoma*-infected snails.

Treatment

Praziquantel is the drug of choice for the treatment of all varieties of schistosomiasis. The exact mechanism of its action is not known. It may act by damaging the tegument of the parasite possibly by increasing the calcium uptake by the parasite through calcium channels. The partial erosion of the body surface causes the fluke to lose its immunological disguise and is, therefore, recognized by the host immune system as foreign and consequently destroyed by the host immune system. It also causes tetanic contraction of the parasite. A single dose of 40 mg/kg body weight is recommended for *S. haematobium and S. mansoni*. It is well tolerated at the recommended dose but diarrhoea, abdominal discomfort, dizziness, etc. may be encountered in some cases.

Oxamniquine is an alternate drug for the treatment of *S. mansoni* infection, including patients with advanced disease and hepatosplenomegaly. The drug is found to be more effective against adult male than the female. The early developmental stages are also susceptible to oxamniquine therapy. However, resistance to oxamniquine is a serious drawback for this drug for which it is not preferred. *S. japonicum* is considered the most resistant among human schistosomes for which Praziquantel has to be given orally in three doses, each 20 mg/kg body weight (total dose 60 mg/kg) at an interval of 4 h. Praziquantel is also the only drug for mass drug administration programmes worldwide for schistosomiasis control programmes.

Prevention and Control

The primary objective of surveillance and prevention is to reduce transmission, morbidity, and prevent mortality. Reduction in the contamination of natural water by human faeces and stool can be achieved by educating people about the health hazards of the infection and providing proper sanitary facilities. Since these are long-term measures and also involve large financial outlays, the chemotherapy of cases provides the basis for an immediate and feasible approach to control the morbidity due to the disease. Schistosomiasis is a disease of poverty, and hence good sanitation and safe water are often lacking in the endemic areas. Provision of these measures and prevention of occupational exposure to ponds or rivers should be avoided. Preventive measures directed at controlling animal infections should also be undertaken.

The WHO has marked the year 2025 by which total elimination of schistosomiasis has to be achieved worldwide. The mainstay of disease reduction is periodic mass drug administration of praziquantel. Other measures like provision of safe drinking water, proper sanitation, and control of snail population can be of help in

breaking the chain of transmission. As per WHO data, 97.2 million population was treated for schistosomiasis in 2018 of the total 290.8 million who required preventive treatment. Children and adults at risk in the endemic areas, particularly the population which have occupational exposure to water (like fishermen and farmers), and entire communities living in highly endemic areas are the groups targeted for the treatment of schistosomiasis. WHO is also reinforcing snail control as part of its strategic approach to achieve the target of eliminating schistosomiasis based on guideline with specific, standardized procedures and criteria for efficacy testing and evaluation on the use of molluscicides in the field.

Other *Schistosoma* Species

Schistosoma intercalatum

S. intercalatum is a blood fluke, causing intestinal/rectal schistosomiasis in man and is endemic in Cameroon, Gabon, Central and West Africa, and the Democratic Republic of the Congo. The parasite exists in two geographically isolated strains: one is the Lower Guinea strain and the other is the Zaire strain; Lower Guinea strain uses *Bulinus forskalii*, whereas, the Zaire strain uses *Bulinus globosus* as the intermediate host. The electron microscopic studies of the male worms revealed that they have tuberculated tegument with spines. Natural hybridization occurs between *S. intercalatum* and *S. haematobium*. Female flukes lay eggs with terminal spined eggs. Since the size of eggs is of an intermediate range (smaller than *S. haematobium* and larger than *S. bovis*), so the name "intercalatum" has originated.

Mice, baboons, hamsters, goats, gibbons, chimpanzees, etc., are used as laboratory animals. Many animals have been infected experimentally in the laboratory, and they pass viable eggs in the stool; however, the natural infections with *S. intercalatum* have been reported in only one species of wild rodent (*Hybomys univittatus)*. The zoonotic infection has not yet been proved to play any major role in the epidemiology of human disease. Bloody diarrhoea with abdominal pain is the main clinical symptom. The pathological changes are milder than that of *S. mansoni,* and splenomegaly is rare. Interestingly, most of the people in the endemic areas are asymptomatic. The diagnosis is performed by stool concentration technique.

Schistosoma mekongi

S. mekongi was first diagnosed in a human patient (from Mekong River Basin) in 1957 causing intestinal schistosomiasis and later from Cambodia in 1968. Presently, the infection is endemic in the Mekong River Basin in Lao People's Democratic Republic (Lao PDR) and Cambodia and has been reviewed extensively by Attwood in 2001. The parasite was identified as separate species based on the susceptibility of a different snail species, that is, *Tricula aperta* or *Neotricula aperta.*

The electron microscopic studies of the male worms revealed that they have non-tuberculated tegument. Adult flukes reside in the mesenteric vein, but eggs are relatively smaller and more spherical than that of *S. japonicum*. In man, it causes intestinal schistosomiasis, and the main pathological changes observed are in the liver and spleen; however, brain involvement can also occur. The infection is common in children, due to their high level of water contact. Though the infection is restricted in Lao People's Democratic Republic and Cambodia, the parasite has so far not been reported from India, yet its existence in the country cannot be ruled out as the snail, *Tricula* (intermediate host of *S. mekongi*) is prevalent in India. The infection has been reported in animals (dogs and pigs); however, the role of these reservoir hosts in the epidemiology of *S. mekongi* has not been established. The diagnosis of the disease and treatment is similar to *S. japonicum*.

Schistosoma malayensis

S. malayensis and *Schistosoma sinensisum* are considered as strains of *S. japonicum*, while *S. mekongi* has emerged as a subspecies or a

separate species than *S. japonicum. S. malayensis* spp.nov. was first recovered in histological sections of the liver, pancreas, and mesentery of a 38-year-old female from the Pahang state of Malaysia, in 1973. The adult stage of *S. malayensis* is smaller than that of *S. mekongi* and *S. japonicum*. The rodent *Rattus muelleri* is the primary vertebrate/definitive host in Peninsular Malaysia. *Robertsiella kaporensis* and *Robertsiella gismanni* are identified as intermediate hosts for *S. malayensis*. Symptom in man is not very clear, and further study needs to be done.

Case Study

A 55-year-old male presented with chief complaints of haematuria and dysuria of 7-month duration. After the initial workup when no abnormalities were detected, an ultrasound abdomen was done, which revealed a mass on the left wall of bladder. On cystoscopy papillary tumour-like lesions were seen, and transurethral resection of the lesion was performed. Histopathology revealed high-grade papillary carcinoma and eosinophilic spherical structures resembling parasite eggs. The patient was again interviewed, and a detailed history was obtained. It was revealed that the patient had worked in Libya for about 10 years, and during that time he had travelled to neighbouring countries in North Africa like Tunisia, Egypt, Morocco, etc. About 4 years back, he had developed an itchy lesion on his leg and a rash which had disappeared after a few days. The patient was tested for antibodies against *S. haematobium* and *S. mansoni*, and the *S. haematobium* IgG was found to be elevated. A diagnosis of bladder carcinoma due to *S. haematobium* was made, and apart from adjunctive therapy for bladder carcinoma, Praziquantel was prescribed for the patient.

1. What is the mechanism of bladder carcinoma in chronic urinary bilharziasis?
2. What is the reason that children and young adults commonly suffer from the disease and not the adults?
3. What are the commercial diagnostic kits available for the diagnosis of schistosomiasis?

Research Questions

1. What are the protective antigens that can be used to develop effective vaccines against various *Schistosoma* species, and what should be the strategies for testing the vaccine efficacy in field conditions?
2. What inexpensive, rapid point-of-care tests can be used for the diagnosis of schistosomiasis?
3. How to understand the transmission patterns using remote sensing and geographical information system techniques?
4. What measures can be effective in the control of schistosomiasis?

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Fasciolosis

V. C. Rayulu and S. Sivajothi

Learning Objectives

1. To be able to recognize the differentiating features of the morphologies of trematode eggs based partially on geographic distribution of these parasites.
2. To understand the importance of Falcon assay screening test-enzyme-linked immunosorbent assay (FAST-ELISA) in the diagnosis of fasciolosis.
3. To know the preventive measures that need to be adopted for the use of vegetable products.

Introduction

Fasciolosis caused by *Fasciola* spp. is one of the neglected food-borne zoonotic parasitic diseases of humans. *Fasciola hepatica* and *Fasciola gigantica* as well as hybrids between these two species cause infection in humans and animals worldwide. These flukes are commonly called as liver flukes. Various snail species of the family *Lymnaeidae* act as intermediate host for these flukes. Humans and animals become infected by ingesting contaminated freshwater plants, especially watercress. The adult flukes live in the large biliary ducts of the mammalian host. Humans are accidental hosts for these parasites. The public health importance of human fasciolosis has increased in the recent past, as reported by a greater number of human cases. According to WHO, 17 million people are infected with *Fasciola* species and 180 million are at risk all over the world. There is an increased prevalence of fasciolosis among the animal-rearing communities in low-income countries because of their constant close association with livestock. No vaccine is available for prevention of fasciolosis.

History

Evidence of fasciolosis in humans exists dating back to Egyptian mummies that have been found with *Fasciola* eggs. *Cercariae* of *F. hepatica* in a snail and flukes infecting sheep were first observed in 1379 by Jehan De Brie. The life cycle and hatching of eggs were first described in 1803 by Zeder. Later, Steenstrup (1842) elucidated the idea of an alternating generation in the development of the parasite. Weinland (1875) described the role of *Luteola truncatula* as the intermediate host for the larval stage. Mode of transmission of *Fasciola* parasites to

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herbivores was identified by Lutz (1892), whereas the route of transmission to humans and organ damage caused by the parasite were described by Sinitsin (1914).

Taxonomy

The genus *Fasciola* belongs to family: Fasciolidae; subclass: *Digenea;* class Trematoda; Phylum: Platyhelminthes and order: Echinostomiformes. *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855.

Genomics and Proteomics

Among the trematodes, the genome of the *F. hepatica* is the largest with about 1.3 Gb size. Next to *F. hepatica, Paragonimus westermani, Ophistorchis viverrini* and *Clonerchis sinensis* possess genome sizes in the decreasing order of 0.9 Gb, 0.6 Gb and 0.5 Gb, respectively. The genome of *F. hepatica* is organized into 10 pairs of chromosomes with 15,740 protein coding genes and 57.1% repetitive DNA. The total mitochondria genome of the organism is similar to most other eukaryotic mitochondrial genome 14.5 kb. This mitogenome includes 22 t-RNA genes and 12 protein-coding genes. The larger genome size of *F. hepatica* is attributed to gene duplication and polymorphism. Generally, the larger genomes tend to evolve rapidly to adapt to changes in that environment and increasing the fitness of the organism. Similarly, in *F. hepatica*, the larger size may gain quick adaptation to new hosts, drug intervention and escaping vaccines.

The differential expression of *F. hepatica* somatic proteins in different growth phases has been reported. The high-performance liquid chromatography-tandem mass spectroscopy has identified 629, 2286, 2254 and 2192 proteins in metacercariae, juvenile flukes, immature flukes and adult phases, respectively. Gene ontology analysis revealed that differentially expressed proteins are involved in the transport, localization, metabolism, enzyme regulation, protein folding and binding, and nucleoside and nucleotide binding. In addition, the excretory-secretory proteins of adult worm have been studied to find their interaction with various cytokines and immune cells. Such studies would help in understanding the molecular mechanism of host parasite interaction. Further those studies also would help in understanding new drug and vaccine targets.

The Parasite Morphology

F. hepatica and *F. gigantica* have following life forms seen in the definitive hosts, snail, water and vegetation.

Adult

Both *F. hepatica* and *F. gigantica* have been traditionally classified based on their morphological features and size of body length and width. The adult of *F. hepatica* is leaf-shaped with a broad and cone-shaped anterior projection (Fig. 1). It is greyish brown in colour changing to grey when preserved. The tegument is armed with sharp spines. The young fluke at the time of entry into the liver is 1–2 mm in length and is lancet-shaped. Flukes become fully mature in the bile ducts and measure 3.5 cm in length and 1 cm in width. *F. gigantica* is larger than *F. hepatica* and can reach even up to 7.5 cm in length. The leaf-shaped *F. gigantica* has a short conical anterior end and an inconspicuous shoulder. The fluke possesses a prominent oral sucker at the end of the anterior cone and a ventral sucker at the base of the cone which allow it to attach to the lining of the biliary ducts. Each worm possesses ovaries and testes which are highly branched.

Eggs

The eggs are yellowish brown, oval, operculated and measure 130–145 μm in length and 70–90 μm in width. They consist of a fertilized ovum surrounded by many yolk granules. Undeveloped eggs are excreted in faeces, which contaminate the pasture and undergo embryonation outside the hosts. Several factors such as temperature, humidity and oxygen tension influence the

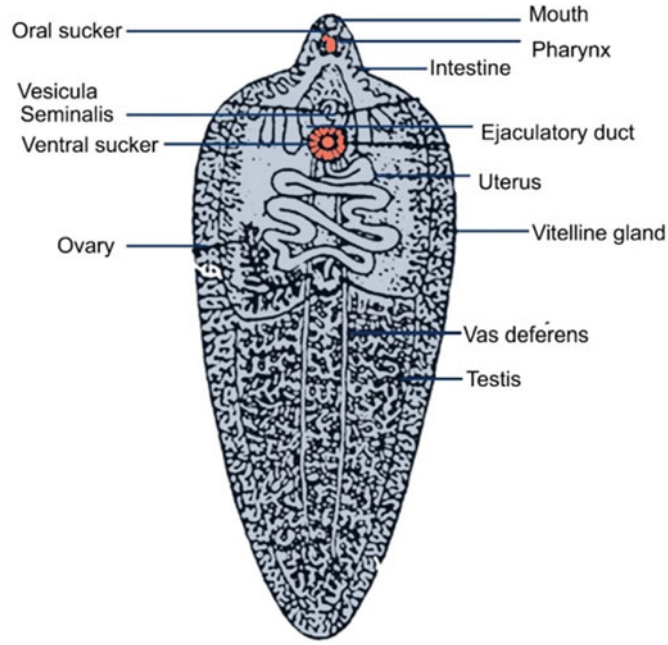

Fig. 1 Schematic diagram of adult of *Fasciola hepatica*

development of eggs in the environment. *Miracidia*, the first larval stage hatches out eggs in the aquatic medium.

Larval Stages

Miracidia are short-lived pyriform motile larval stages covered with cilia. Inside the snail, miracidia transform into sporocysts that are pleomorphic sac-like bodies containing germinal cells which further give rise to small rediae. Mature cercariae are free-swimming stages with simple elongate club-shaped tails, which are subsequently shed when they encyst on vegetation to form membrane-bound metacercariae.

Metacercariae

Metacercariae are the infective stage of the parasite. They measure nearly 0.2 mm in diameter. These larvae under natural conditions remain submerged in water and may survive for 6 months.

Cultivation of Parasite

Fasciola is maintained in tissue culture medium for harvesting excretory/secretory and metabolic products of the parasite for various vaccination and diagnostic studies. The culture medium NCTC 135, supplemented with 50% heat inactivated chick serum and sheep red blood cells, is frequently used for the growth and development of excysted *F. hepatica* metacercariae. The freshly excysted juveniles incubated at 37–38 °C, undergo somatic development similar to those of flukes recovered from mouse liver at 11 days after infection. In vitro culture of immature flukes recovered from abdomen and liver of mice continue their somatic growth in the medium but fail to develop the reproductive system.

Laboratory Animals

Fasciola is adapted to complete its sexual phase of cycle in a wide range of hosts including rabbits and rodents besides ruminants and wild herbivore animals. However, these two species differ in

their development in the laboratory animals with respect to their growth, development and maturity. *F. hepatica* develops sexually in rats and mice. Therefore, extensive work related to immunology, immunoprophylaxis and chemotherapeutic trials has been carried out in rats. *F. gigantica*, on the other hand, develops in guinea pigs and rabbits but not in rodents such as rats and mice. Owing to its larger size and prolonged prepatent period, *F. gigantica* in guinea pigs and rabbits develops to a limited period, and before attaining maturity, the infected laboratory animals die of infection in 11 to 12 weeks. However, if a rabbit survives, the parasite matures in about 22 weeks. In such instances, the parasite as well as the eggs remain entrapped in the liver parenchyma. Neither the parasite nor the eggs gain access into the minute bile ducts of the rabbits.

Life Cycle of *Fasciola* spp.

Hosts

Definitive Hosts

F. hepatica and *F. gigantica* are primarily the parasites of domestic and wild ruminants, including sheep, cattle, buffaloes, goats, camelids and cervids. Infections occasionally occur in aberrant, non-ruminant herbivore hosts such as equids, swine, lagomorphs, macropods and rodents. Detection of *Fasciola* spp. eggs in the faeces of carnivores probably represents spurious passage following consumption of contaminated liver. Humans are accidental hosts for these parasites.

Reservoir Hosts

These include: (1) the main domestic animals such as cattle, buffaloes, sheep, goats, pigs and donkeys, (2) sporadic domestic animals such as horses, dromedaries, camels and (3) sylvatic reservoir such as hares and rabbits for both *Fasciola* species and rodents for *F. hepatica* only.

Intermediate Hosts

Air-breathing freshwater snails of the family Lymnaeidae act as intermediate hosts for *Fasciola* spp. About 20 snail species under the genera *Lymnaea, Fossaria, Galba* and *Pseudosuccinea* act as intermediate hosts for one or more *Fasciola* spp. Snail species as intermediate hosts for *F. hepatica* and *F. gigantica* may differ according to the geographical region.

Infective Stage

Metacercaria is the infective stage

Transmission of Infection

Fasciolosis is transmitted through both water and food. Humans and other mammals acquire infection by: (i) ingesting encysted metacercariae attached to aquatic or semi-aquatic plants, (ii)-drinking water contaminated with floating metacercariae and (iii) ingesting metacercariae attached to the surface of food or kitchen utensils washed using water contaminated with floating metacercariae. Transmission to humans is frequently attributable to environmental contamination by infected animals (Fig. 2).

Ingested metacercariae excyst and release juvenile flukes in the duodenum. The juvenile flukes migrate through the intestinal wall, peritoneal cavity, liver parenchyma and finally reach into the biliary ducts, where they mature into adults in about 3–4 months. Adult flukes present in the bile duct shed eggs that are released through the common bile duct into the intestine, and subsequently are excreted in the faeces. The eggs are not fully developed when passed in the faeces and require minimum of 10 days to reach miracidial stage. The miracidia are pyriform motile larva measuring 150–200 μm in length and are covered with cilia. The eggs release miracidia, which swim freely in the water and subsequently invade a suitable snail intermediate host present in water. In the lymph spaces of the snail, the miracidia develop into *sporocysts*. Sporocysts are pleomorphic sac-like bodies (0.3–1.5 mm in diameter) containing germinal cells which give rise to small rediae (embryos). Two generations of rediae and finally the cercriae are released from the snail in the aquatic environment. The whole cycle takes a period of 30–60 days in the snail.

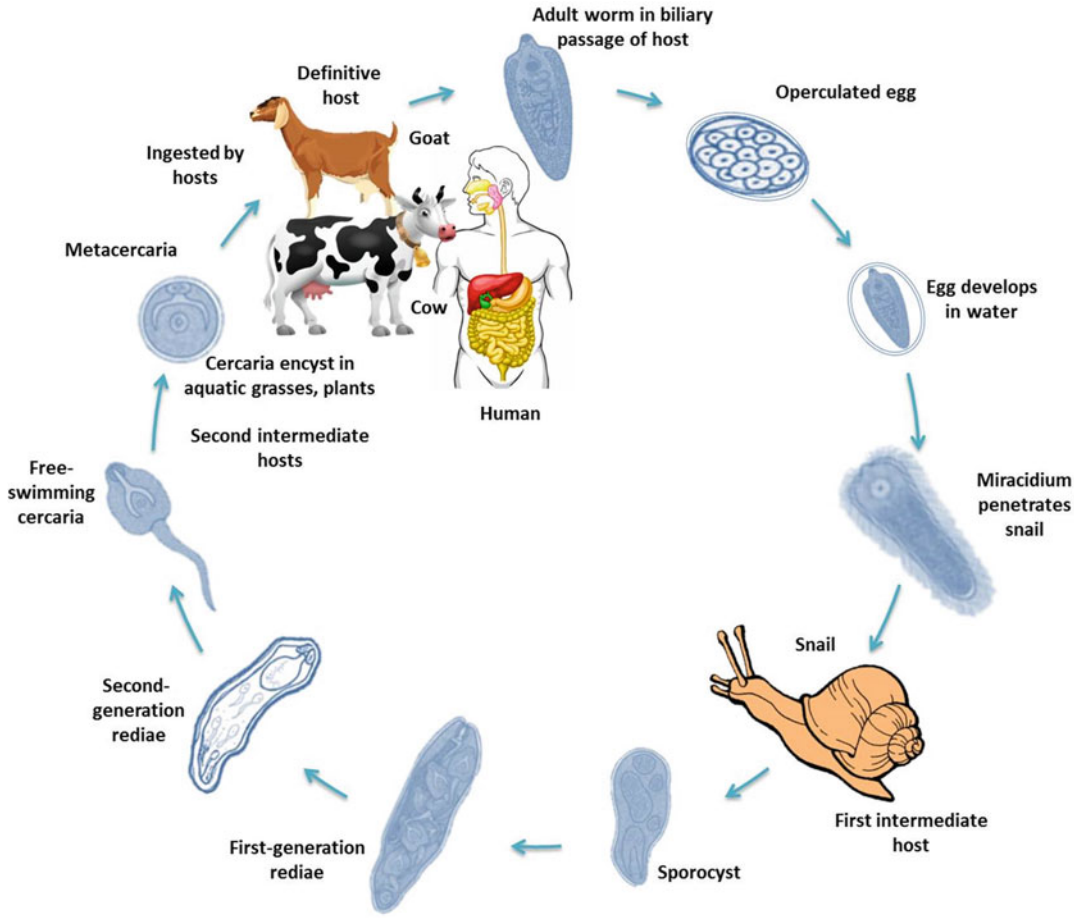

Fig. 2 Life cycle of *Fasciola hepatica*

The cercariae are nearly 0.5 mm long, free-swimming gymnocephalous stages with simple elongated club-shaped tails. The mature cercariae attach themselves to blades of grass or aquatic vegetations and encyst resulting in the metacercaria stage which is infective for humans and other definitive hosts.

Pathogenesis and Pathology

The disease process in fasciolosis begins with the entry of juvenile fluke in the hepatic tissues. The pathogenesis of the disease in different hosts also may vary in severity depending on the number of metacercariae ingested, *Fasciola* species and the stage of the parasitic development in the infected hosts. The juvenile flukes migrate in the liver parenchyma and cause extensive traumatic lesions with haemorrhage and inflammation. Lesions produced by *F. gigantica* are more severe with even fewer flukes, possibly due to their longer duration of migration in hepatic parenchyma, larger size and spines present all over the tegument of the parasite.

In sheep, ingestion of nearly 2000 metacercariae of *F. hepatica* and 300 metacercariae *F. gigantica* can produce acute condition. In large ruminants, buffalo and cattle, ingestion of 1000 metacercariae of *F. gigantica* produces acute disease. The acute phase is characterized by hepatomegaly, splenomegaly, and/or peri-portal lymphadenopathy, especially severe haemorrhage caused by the migrating juvenile flukes. The liver

parenchyma, particularly the ventral lobe associated with gall bladder, is severely damaged assuming an uneven surface covered with blood clots. The liver is enlarged, and haemorrhagic covered with fibrous clots and necrotic tunnels with migrating flukes. Liver damage in sheep and sometimes in cattle, characterized by hepatic fibrosis and hyperplastic cholangitis, correlates well with the parasite load. Adult flukes may partially or completely obstruct the bile ducts, over time causing fibrosis, hypertrophy and later dilation of the proximal biliary tree. There may be subcapsular lesions in the parenchyma of the liver. Bile ducts are often calcified resulting in a 'clay-pipe' or '*pipe-stem*' liver. Tortuous tracks are left behind by the migrating parasites. Subcapsular hematoma, capsular thickening or parenchymal calcifications can also be seen.

Immunology

Several studies in fasciolosis have shown that the resistance at the gut wall is thymus-independent and that non-specific and hypersensitivity reactions may play a role. The juvenile flukes migrating through intestinal wall and peritoneum induce infiltration of eosinophils, IgG1and IgG2 antibodies. Antibody-dependent cell-mediated cytotoxicity (ADCC) plays an important role in *Fasciola* infection, like other metazoan parasites. The isoenzyme glutathione S-transferase (GST) in both *F. hepatica* and *F. gigantica* suppress the release of toxic oxygen resulting in neutralization of immediate hypersensitive type-I mediated immune response involving both neutrophils and eosinophils.

Experimental studies in animal models suggest that the immature parasite is the target of protective host immune responses but the effector mechanisms employed vary among the hosts. In the rat model, in vitro killing of immature *F. hepatica* involves an antibody-dependent cell cytotoxicity mediated by nitric oxide produced by activated monocytes and macrophages. *Fasciola* spp. actively modulate the host immune response, downregulating type 1 responses during infection. Analysis of IL-4 and interferon-γ cytokines suggested a predominant type 2 immune response in BALB/c mice infected with metacercaria of *F hepatica*. Level of IL-4 mRNA assessed by reverse transcriptase-polymerase chain reaction has demonstrated that the immune response becomes polarized within 24 h of infection.

Infection in Humans

Incubation period varies from few days to 3 months based on the number of ingested metacercariae and immune status of the host. Nearly, half of *Fasciola* infections in humans are subclinical.

Acute fasciolosis, otherwise known as invasive fasciolosis, usually occurs in sheep but rarely in humans because it requires a large number (>10,000) of metacercariae to be ingested. Usually, the invasive phase lasts many weeks, with the most common symptoms being intermittent fever, hepatomegaly and abdominal pain. Abdominal pain is confined to the epigastrium or right hypochondrium. Malaise, wasting, urticaria and eosinophilia are the other symptoms.

Chronic or obstructive fasciolosis is a latent phase that lasts for months or even years when infection is asymptomatic. The condition is often diagnosed during screening for other infections. Nevertheless, the maturation of the parasite to an adult stage may lead to an obstructive manifestation leading to hepatitis, cholangitis and or pancreatitis. Anaemia, pancreatitis, biliary fibrosis, cholelithiasis and obstructive jaundice can occur. Large numbers of migrating larvae invade the liver and cause a traumatic hepatitis which is frequently fatal. Sometimes, the liver capsule may rupture into the peritoneal cavity, causing death from peritonitis.

Fasciola may also cause ectopic infections, especially in the lungs and subcutaneous tissues, where they may form cysts. *Halzoun* is an example of such condition, which occurs following the consumption of raw liver dishes prepared from fresh livers of sheep, goats, etc. infected with

immature *Fasciola* spp. The condition is characterized by severe pharyngitis and oedematous congestion, dysphagia, sensation of a foreign body in the throat and bleeding from the pharynx. This condition has been described in Lebanon, Syria, and parts of Middle East and North Africa.

False fasciolosis (pseudo fasciolosis) refers to the presence of eggs in the stool not because of an actual infection but rather because of recent ingestion of liver contaminated with eggs, which are not infective for humans.

Infection in Animals

Clinical manifestations of fasciolosis in sheep and cattle depend on the number of metacercariae ingested. The clinical presentation is divided into four types: Acute type I fasciolosis, acute type II fasciolosis, subacute fasciolosis and chronic fasciolosis.

Acute type I fasciolosis usually occurs when more than 5000 metacercariae are ingested. Ascites, abdominal haemorrhage, icterus, pallor of membranes and weakness are observed. Animals may suddenly die without any previous clinical signs. Acute type II fasciolosis is caused by a high number of (1000–5000) metacercariae ingested by sheep. Sheep usually die of infection but may briefly show pallor, loss of condition and ascites. Subacute fasciolosis is caused by a moderate number of (800–1000) metacercariae ingested by sheep. Infected sheep are lethargic, anaemic and show weight loss, characteristic of the infection. Chronic fasciolosis is caused by 800 or less number of ingested metacercariae. The condition is characterized by asymptomatic or gradual development of bottle jaw, ascites, emaciation and weight loss in infected sheep or cattle. Anaemia, hypoalbuminemia and eosinophilia may be noticed in all types of fasciolosis in animals.

Mostly in sheep and sometimes in cattle, the damaged liver tissue may be secondarily infected by *Clostridium* spp. The bacteria release toxins into the bloodstream, resulting in black disease which is usually fatal.

Epidemiology and Public Health

Fasciolosis in humans caused by *F. hepatica* is a global disease, being documented from more than 75 countries worldwide (Table 1 and Fig. 3). The disease is found in Papua New Guinea, the Caribbeans, Columbia, Venezuela, Bolivia, Peru, Cuba, Ecuador, Egypt, England, Portugal France and Iran. *F. gigantica* on the other hand, is found mainly in tropical and subtropical countries such as Africa, Asia Minor, Southeast Asia, Southern Europe, State of Hawaii (USA), USSR and Southern USA.

Both humans and animals acquire fasciolosis in the same way, that is, by ingesting metacercariae encysted on leaves or vegetables. Transmission of the infection in the environment is usually perpetuated by animals. Humans usually do not contribute to the parasite's life cycle but are occasionally infected after failure to observe basic hygiene measures. Further, *Fasciola* spp. are not well adapted to humans and, in some cases, fail to develop into mature adult worms and do not produce any eggs. In some areas, transmission to humans is constant and intense, and a geographical aggregation of cases has been demonstrated. This pattern is possibly explained by a human-to-snail-to-plant-to-human transmission cycle, without the involvement of any animal.

The epidemiological pattern of fasciolosis is quite varied across the globe. However, the infection usually has a hypo-endemic pattern, with low and stable levels of prevalence among a defined population. Fasciolosis affects people from all age groups. The prevalence and intensity of infection tend to be high in school-age children in highly endemic areas of fasciolosis. People in rural areas are more likely to become infected. In Africa and Asia, where both *F. hepatica* and *F. gigantica* are present, mixed infections are possible. In Asia, hybridization among the two species occurring in co-infected humans or animals has been described.

Table 1 Global distribution of *Fasciola* spp.

Species	Distribution	Intermediate host
Fasciola hepatica	East of Iran	*Lymnaea truncatula*
	Egypt	*Lymnaea truncatula*
	Europe, Asia, Africa and North America	*Lymnaea truncatula*
	China	*Lymnaea truncatula*
Fasciola gigantica	East of Iran	*Lymnaea truncatula*
	Eastern India	*Lymnaeaa uricularia,* *Lymnaea rufescens* *Lymnaea acuminata*
	Nepal and Bangladesh	*Lymnaea auricularia*
	Malaysia	*Lymnaea rubiginosa*
	Africa	*Lymnaea natalensis*
	East Africa	*Lymnaea cailliaudi*

Diagnosis

The mainstay of diagnosis is the detection of eggs in the stool specimen, but in recent years, antibody detection and nucleic acid detection have also been increasingly used (Table 2).

Microscopy

Microscopy of stool or duodenal bile aspirates for *Fasciola* eggs (Fig. 4) is useful for detecting chronic infections. A single adult fluke can release over 20,000 eggs per day, but excretion of eggs is intermittent. Therefore, microscopy of multiple stool or duodenal aspirate specimens may be needed as a negative microscopy of a single specimen does not necessarily rule out

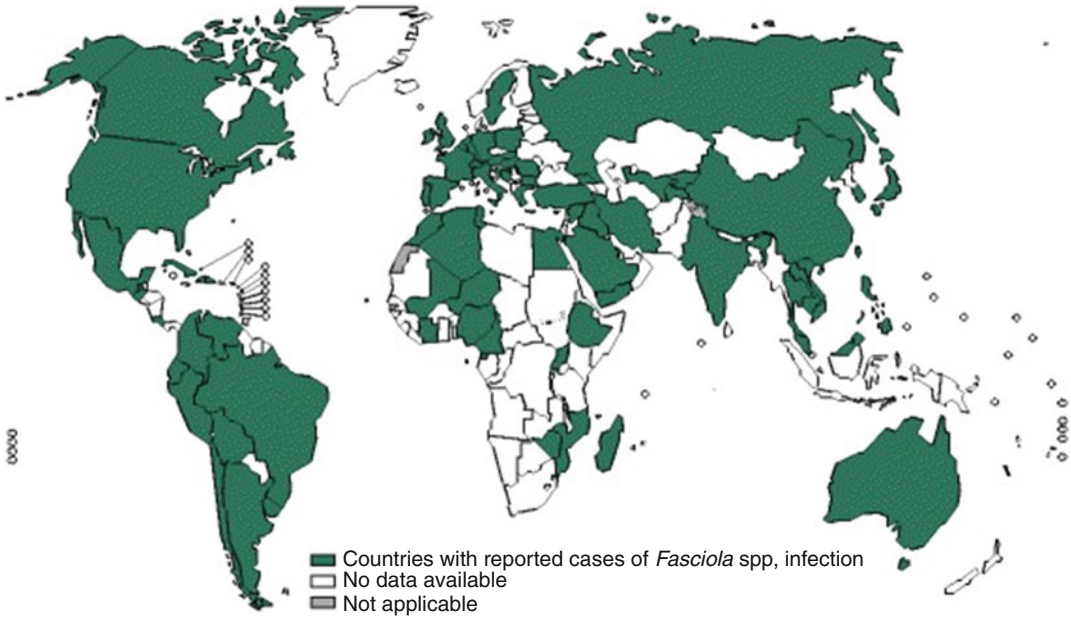

Fig 3 Global distribution of fasciolosis (Source: Lu XT, Gu QY, Limpanont Y. et al. Snail-borne parasitic diseases: an update on global epidemiological distribution, transmission interruption and control methods. *Infect Dis Poverty* **7,** 28 (2018). https://doi.org/10.1186/s40249-018-0414-7)

Table 2 Diagnostic methods for fasciolosis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Faecal examination	Eggs of *Fasciola* spp.	• Gold standard test • Sensitivity is not always optimal • Unable to identify intra-specific differences for *Fasciola* species
Immunodiagnostics	Copro Ag-ELISA	MabMM3	• High sensitivity • Detection of active infections • Efficient testing of many samples • False negatives due to inhibitory host antibodies
	FAST-ELISA		• High sensitivity • Limitations in specificity (due to crude antigen preparations)
	Dot-ELISA or Dipstick		• High sensitivity • Limitations in specificity (due to crude antigen preparations)
	Ab-ELISA	Ig-G	• High sensitivity • Limitations in specificity (due to crude antigen preparations) • Limitations in sensitivity (when recombinant antigens or synthetic peptides are used) • Persistence of host antibodies after cure
Molecular assays	PCR, RT-PCR, LAMP, Luminex and PCR-ELISA	ITS-1, ITS-2	• More specificity and sensitivity • Require sophisticated equipment

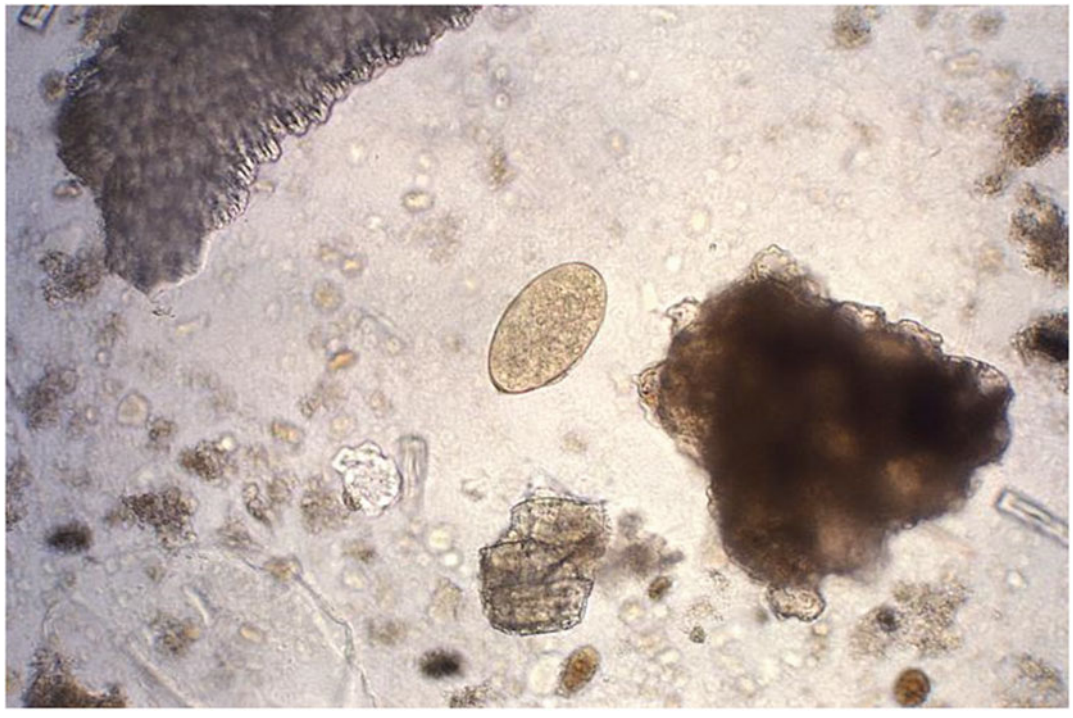

Fig. 4 The egg of *Fasciola hepatica*, which is described as broadly ellipsoidal, operculated, and measures 130 μm to 150 μm long by 60 μm to 90 μm wide. (Courtesy: PHIL, CDC/ Dr. Mae Melvin)

the diagnosis of fasciolosis. *F. hepatica* and *F. gigantica* eggs are morphologically indistinguishable and also difficult to distinguish from eggs of *Fasciolopsis buski* and eggs of some *Echinostoma* spp.

Serodiagnosis

Antibody-based tests such as ELISA, lateral flow immunoassay, Western blot and other serological tests have been evaluated for diagnosis of fasciolosis by using excretory-secretory or recombinant *Fasciola* antigens. Falcon assay screening test (FAST-ELISA) shows a sensitivity of 95% and can detect specific *F. hepatica* antibodies as early as 2 weeks after infection. The FAST-ELISA is also used as a prognostic test for effective cure as antibody levels return to normal in 6–12 months after the cessation of infection. A new immunoblot assay for the diagnosis of *Fasciola* infection is based on a recombinant *F. hepatica* antigen (FhSAP2). The presence of a band at ~38 kDa indicates a sensitive reaction. The assay is $\geq$94% sensitivity and $\geq$98% in diagnosis of chronic *Fasciola* infection in humans. However, persistence of circulatory antibodies at latent phase and lack of defined antigens are the major limitations for the antibody-based serological assays.

Coproantigen ELISA to detect specific *Fasciola* antigen in stool of infected humans and other mammals is a useful test to detect acute infection.

Molecular Diagnosis

Because of variations in size of *F. hepatica* and *F. gigantica*, the discrepancy of morphological features, and the presence of intermediate forms, it might be difficult to distinguish between two species, solely based on morphological features of *Fasciola.* PCR, real-time PCR, PCR-RFLP and nested PCR have been developed for specific detection and identification of *Fasciola*. Molecular studies have demonstrated that these two species can be distinguished by DNA sequencing of ITS1 and ITS2 and mitochondrial genes of NDI and COI.

Other Tests

Ultrasound, computerized tomography (CT) scan, cholangiogram, and endoscopic retrograde cholangiopancreatography (ERCP) are frequently helpful for supplementing diagnosis of fasciolosis. These may demonstrate mobile, leaf-like flukes in biliary ducts or gallbladder during the biliary stage of the disease. CT may show multiple, nodular, small (approximately 25 mm in diameter), branching, sub-capsular lesions in parenchyma of the liver, including tortuous tracks left behind by the migrating parasites.

Treatment

Triclabendazole is the drug of choice for the treatment of fasciolosis in humans and animals. It is an imidazole derivative that acts by preventing the polymerization of tubulin into microtubules rendering cells incapable of producing their cytoskeletal structures. It is effective against all stages of fasciolosis with a cure rate of over 90% with a two-dose regimen of 10 mg/kg/dose, at an interval of 12 h. Nitazoxanide is a good alternative to triclabendazole, especially for use in treatment of chronic fasciolosis. It is administered as 500 mg twice a day for 7 days in adults. Rafoxanide, oxyclozanide and closantel are also used for the treatment of fasciolosis but resistance has been reported to these anthelminthic in some countries. Parasite removal at endoscopic retrograde cholangiopancreatography is effective in the biliary stage. Ascending cholangitis may require surgery.

Prevention and Control

Currently, no vaccine is available for prevention of fasciolosis either in humans or animals. Timely treatment with triclabendazole is the quickest way to control morbidity associated with fasciolosis. Public health education on fasciolosis by promoting cultivation of vegetables in water free from

faecal pollution and thorough cooking of vegetables before consumption are important to prevent fasciolosis. The practice of washing water-grown vegetables with 6% vinegar or potassium permanganate for 5–10 min, which kills the encysted metacercariae, is effective. Cooking of water-grown vegetables thoroughly before eating and avoiding sewage contamination of growing areas are the best practices to be adapted for preventing fasciolosis.

Veterinary public health measures include treating domestic animals and enforcing separation between husbandry and humans. Environmental measures include containment of the snail intermediate hosts and drainage of grazing lands and the use of molluscicides for control of snail intermediate hosts will prevent transmission of not only *Fasciola* spp. but also many other trematodes.

Case Study

A 40-year-old woman from the north-eastern part of India was admitted with the chief complaints of pain in the right hypochondriac region and irregular fever for the last 2 years. Physical examination revealed hepatomegaly, and haematological tests revealed moderate leucocytosis (12,000 cells/cu mm) but with 50% eosinophils. Bilirubin levels and liver enzymes were within normal limits. Ultrasonogram showed hypoechoic regions in the liver with possibility of necrosis. Ultrasound-directed biopsy of the area showed necrotic granuloma formation with eosinophilia. Bile specimen aspirated by endoscopy revealed many brown, ellipsoidal, unembryonated ova with a small operculum resembling *F. hepatica* eggs. The patient was treated with a single dose of triclabendazole and on follow-up the patient's pain and fever had resolved and repeat bile examination showed absence of any ova (adapted from Ramachandran et al. 2012).

1. What are the different anti-trematodal drugs recommended for the treatment of fasciolosis?
2. What dietary history is important in the above case, which has not been mentioned?
3. What alternative non-invasive test could have been done in the above case without taking resort to liver biopsy?

Research Questions

1. Which antigenic target of *F. hepatica* should be utilized for development of sensitive immunodiagnostic test?
2. How to proceed for vaccine development using proteomic studies and reverse vaccinology for fasciolosis?

Further Readings

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Fasciolopsiasis

Sumeeta Khurana and Priya Datta

Learning Objectives

1. To know the importance of snail as intermediate host in various trematode infections.
2. To understand the necessity for identification of the egg and the adult worm for diagnostic purpose.
3. To have a knowledge about the control measures that should be taken in the aquatic ecosystem of the endemic areas.

Introduction

Fasciolopsiasis is an infection caused by *Fasciolopsis buski* or 'Giant intestinal fluke'. The adult flukes of these food-borne parasites reside in the small intestine of definitive host, that is, man and pigs. However, the parasites require one or more intermediate hosts to complete their life cycle. These infections are predominantly seen in Southeast Asian countries, including those in Central and Southern China and India. WHO recently included fasciolopsiasis along with other food-borne trematodioses to the list of neglected tropical diseases (NTDs). The 'One World: One Medicine: One Health' approach of WHO offers the most hopeful and comprehensive solution for control of various NTDs. However, fasciolopsiasis still continues to remain a major public health problem in endemic countries.

History

F. buski was first described by English surgeon George Busk in 1843 in London. It was discovered in the duodenal lumen during post-mortem examination of an East Indian sailor. This parasite was subsequently named *Distoma Buskii* by ER Lankester and studied in detail by T Spencer Cobbold in 1859. Nakagawa was credited for interpreting the life cycle of *F. buski* in 1920 in pig, and it was confirmed by Barlow in 1925 in man.

Taxonomy

The genus *Fasciolopsis* belongs to family: Fasciolidae; superfamily: Echinostomatoidea; order: Echinostomida; subclass: Digenea and class: trematoda, in the phylum: Platyheminthes

F. buski is the species that causes infection in humans and animals.

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Genomics and Proteomics

Although *F. buski* is the only reported species in the genus, morphological variations have been observed in the parasites from different geographical regions suggesting genetic polymorphism. The circular genome consists of 14,118 ntbp and is almost similar to that of *Fasciola hepatica*. There are 12 protein-coding genes, 2 genes encoding ribosomal RNA subunits, that is, the large subunit (rrnL or 16S) and small subunit (rrnS or 12S). Using next-generation sequencing techniques, the complete mt genome sequences of the intestinal fluke comprising 14,118 bp were identified. The gene content is identical to that of *F. hepatica*. *F. buski* mitochondrial DNA genome has a close resemblance with *F. hepatica* and has a similar gene order. In a study from India, 12,380 key genes that encode cytoskeletal proteins were reported to be highly expressed. Moreover, genes encoding fatty-acid-binding proteins that capture, store and transport lipid molecules were identified.

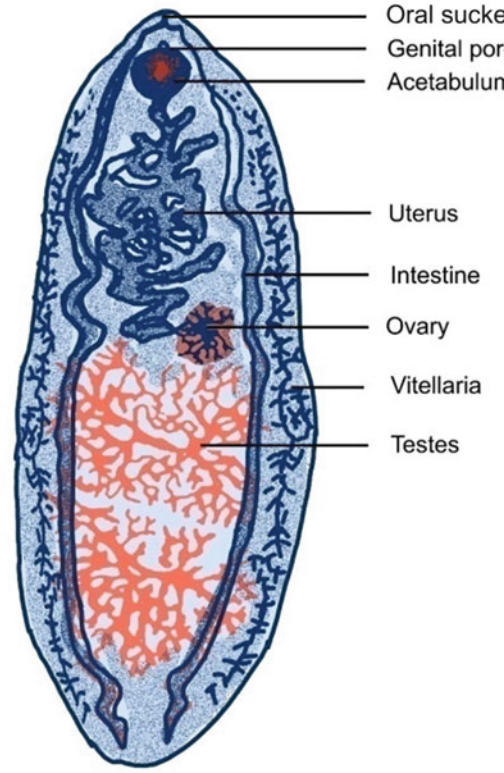

Fig. 1 Schematic diagram of an adult *Fasciolopsis buski*

The Parasite Morphology

Three distinct stages are seen in its life cycle of *F. buski*—egg, larval stage adult worm.

Adult Worm

F. buski is the largest trematode infecting humans. The elongated, leaf-like trematode inhabits the small intestine of man or pig. It grows up to 2–7.5 cm in length, 0.8–2 cm in width and 0.5–3 mm in thickness. When freshly passed, it is flesh-coloured and is covered with transverse spinous projections on the ventral surface (Fig. 1). A syncytial epithelium called tegument covers the body surface of the adult worm. The main function of the tegument is to aid in the process of osmoregulation, nutrient absorption, secretion and sensory function, and also protection from host defences. Its anterior end is narrow as compared to posterior end.

The anterior end has the mouth located sub-terminally and has two suckers. The ventral sucker or acetabulum is 3 mm in diameter and larger in size than oral sucker (0.5 mm). The genital pore lies near the acetabulum. The alimentary canal, like all trematodes, is incomplete having no anal opening. It consists of an oval pharynx, and short oesophagus which bifurcates into a pair of blind intestinal pouches called caeca. This is situated in the posterior end of the body.

F. buski is *monoecious*, that is, both male and female reproductive organs are present in the same worm. The male reproductive organ consists of highly branched testes which occupy nearly half of the posterior part of the body near caeca. The vas deferens enters into the cirrus sac and finally opens at genital pore located near the ventral sucker. The cirrus is the copulating organ. The female reproductive system consists of a

single ovary (located anterior to testis), vitelline glands, oviduct and a uterus which contains eggs and opens behind the ventral sucker. The bilaterally symmetrical excretory system comprises of flame cells and collecting tubules which open as excretory pore on the dorsal surface at the posterior end of the body. The adult worm lacks the circulatory and respiratory system.

The life span of adult worm is approximately 6 months. It produces 15,000–16,000 eggs/day.

Eggs

Eggs of *F. buski* are operculated, 130–-140 μm × 80–90 μm in size and oval in shape (Fig. 2). They contain fertilized, unsegmented ovum surrounded by refractile yolk globules. The freshly passed eggs are dark brown in colour and characteristically the operculum is not noticeable. When the eggs are kept for 8–10 days in moist environment at room temperature, they gradually turn light brownish to yellow in colour with a well-distinguished operculum. On further incubation after 10 days, open operculum and transverse grooves are observed on the egg shell. This signals the maturation and subsequent escape of the miracidium from the eggs.

Infective Larva

Cercariae are the last stages of larval development, becoming the infective form to human and pigs. They measure from 0.21 × 0.23 mm in length and 0.12 × 0.15 mm in breadth. The tail is long, measuring two or three times the body land tail (0.4 to 0.5 mm). Cercariae are released from snails into water where these encyst on the surfaces of freshwater aquatic plants to give rise to metacercariae. Metacercariae are infective to humans and pigs.

Cultivation of Parasites

F. buski has not been cultivated in any cell-free culture media so far.

Laboratory Animals

In laboratory setting, young rabbits, guinea pigs and squirrel monkeys (*Saimiri sciureus*) have been found to be susceptible to infection with *F. buski.* Pigs and young rabbits are good models for various experiments.

Life Cycle of *Fasciolopsis buski*

F. buski complete their life cycle in two or more different hosts (Fig. 2).

Hosts

Definitive Host

Generally, pigs or humans harbour the adult worm and excrete the eggs in faeces, urine or sputum, which develop into free-swimming ciliated embryo called *miracidium* in the water. The miracidium infects the intermediate host.

Intermediate Host

Different species of freshwater snails are the first intermediate hosts. Inside the snail, the miracidium develops into a *sporocyst,* then *redia*, and then into *cerceriae* which are released in water (Fig. 2).

Infective Stage

The cercariae released from the snails into water develop into metacercariae in about 4 weeks and encyst on the aquatic plants like water caltrops, water chestnuts, lotus, etc.

Transmission of Infection

Humans and pigs acquire infection by consumption of raw, undercooked aquatic plants having metacercaria larva during peeling off the skin of aquatic plants or contaminated water. The percentage of infections occurring due to contaminated drinking water is 10–12% in humans and 35–40% in pigs.

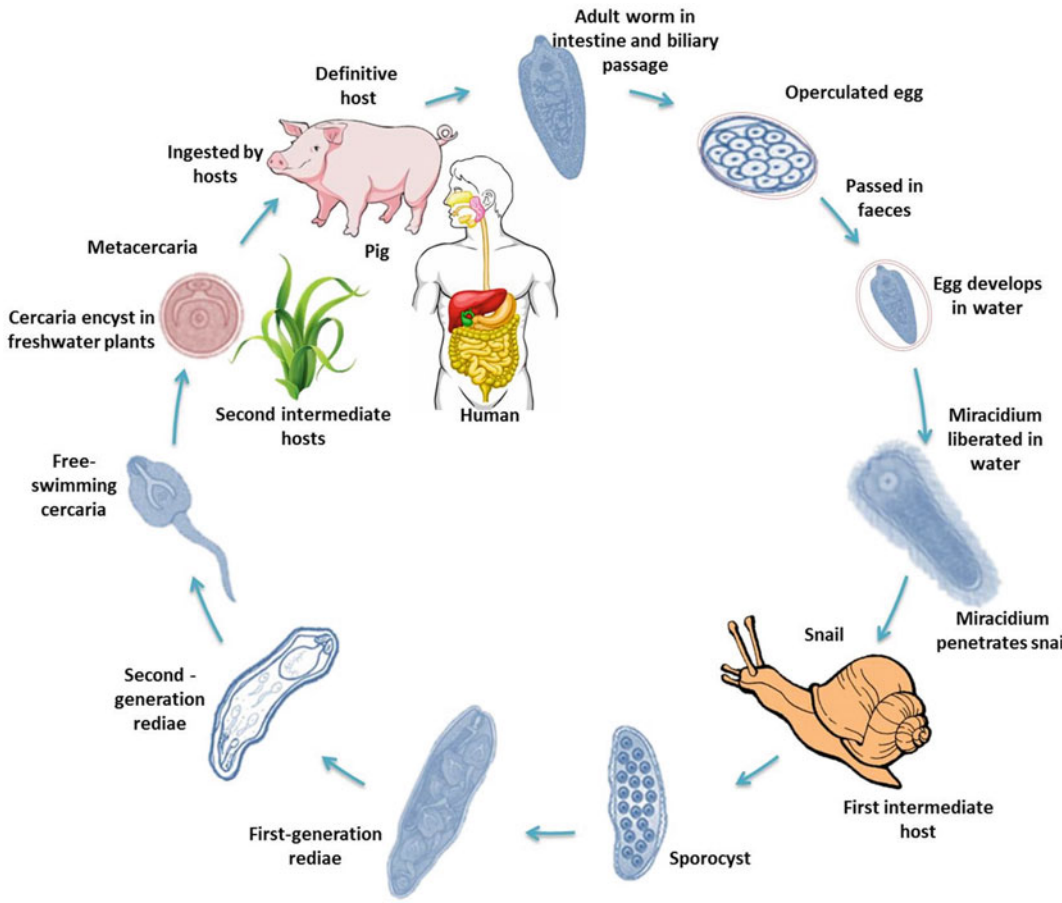

Fig. 2 Life cycle of *Fasciolopsis buski*

The encysted metacercariae pass through the stomach and undergo excystation in the duodenum. These attach themselves to the mucosa of small intestine, mainly duodenum or jejunum and grow to form adult flukes. The time taken for development from metacercaria into adult flukes is about 3 months. The adult flukes become sexually mature and lay eggs which are excreted in faeces. The adult produces an average of 15,000–16,000 eggs/day. Further maturation of eggs takes place in water, at optimum temperature of 27–30 °C in which ciliated miracidia are formed. On attaining maturity in 3–7 weeks, miracidium escapes from the egg into the water through the operculum. This freely swimming miracidium remains in water until it reaches a suitable host, that is, snail. Then miracidium penetrates the snail, with the help of secretions of the cephalic glands and reaches the lymph space. Inside the lymph space of snail, miracidium takes around 30 days to pass through the various stages of sporocyst, first-generation redia, second-generation redia and finally cercaria. This development in snail tissues can take place between 6 and 8 weeks. Cercariae that emerge from snails are short-lived, swim in freshwater until appropriate substrate, that is, plant and debris are available for encystment. They undergo encystment on the surface of aquatic plants like water hyacinth, lotus, water chestnut and water bamboo etc. and develop into metacercariae. These water plants grow in shallow ponds and water bodies, which are fertilized by night soil; thereby snails are plentiful in these ponds. The metacercaria survives for 64–72 days in water. The metacercaria present on aquatic plants is eaten by man or pig, and the life cycle is repeated.

Pathogenesis and Pathology

In fasciolopsiasis, pathological effects may be traumatic, toxic or obstructive. The adult worm, at the site of attachment in duodenum and jejunum, causes localized inflammation leading to ulceration. In severe infestation, *F. buski* may be found in the pylorus, ileum and colon. In complicated cases, deep erosions can result in intestinal haemorrhage from capillaries in the intestinal walls. Occasionally, damage to the intestinal mucosa by adult worms can lead to abscess and catarrhal inflammation. Due to large size of the worm, intestinal obstruction may be rarely seen. Additionally, in heavy infestation, allergic and toxic effects of the worms and their metabolites lead to various pathological effects like ascites, oedema, etc.

Immunology

Most of the trematodes inhabit in a state of equilibrium with their host as evidenced by asymptomatic or mild disease in majority of infected individuals. This is because of the long-term co-evolution of the parasite and the host. The immune response of host to most of helminthic infections is characterized by a predominant Th-2 response and is typically associated with eosinophilia and significant IgE production, mastocytosis and goblet cell hyperplasia. IL-4 and IL-13 are required for IgE isotype switch and IL- 5 is important for eosinophil production. Antibody-dependent cell-mediated toxicity is mediated by eosinophils, mast cells, neutrophils and macrophages as effector cells and IgE, IgG and IgA antibodies. The worms coated with antibodies are destroyed by immune cells, carrying receptors for Fc fragment by release of toxic products. Some of these toxic products include major basic protein, eosinophilic cationic protein, reactive nitrogen intermediates etc. However, parasites have developed certain mechanisms to evade the effector response of the host, for example, by production of superoxide dismutase that neutralizes superoxide radicals, cathepsin L protease that cleaves IgE and IgG.

Infection in Humans

The symptoms of fasciolopsiasis manifest after an incubation period of 1–3 months. The clinical features of fasciolopsiasis vary according to the parasite burden, disease severity and physiological state of the person.

In humans, light infections are mostly asymptomatic or are associated with mild symptoms such as abdominal pain, headache, dizziness, diarrhoea etc.

Moderate infection presents with severe epigastric pain, poor appetite, headache, abdominal colic, fever and chronic diarrhoea. Heavy or severe infections can be fatal especially in endemic countries. It is associated with severe intestinal inflammation causing severe epigastric pain, ascites, abdominal distension, fever, dysentery, obstructive jaundice (if worms ectopically lodge in the biliary system), acute kidney injury, small bowel stricture, acute ileus, intestinal perforation with malabsorption syndrome causing malnutrition and lowering of Vitamin B_{12} content. Generalized toxic and allergic symptoms are commonly seen, usually in the form of oedema of face, abdominal wall and lower extremities.

Infections in Animals

Pigs are the main host and reservoir of *F. buski*. Cattle, horses and dogs are resistant to infection by *F. buski*. Pigs naturally harbour only 3–12 flukes/pig and the infection is asymptomatic.

Epidemiology and Public Health

Fasciolopsiasis is prevalent in Southeast Asian countries especially in China, Taiwan, Thailand, Vietnam, Laos, Cambodia, Bangladesh, India, Indonesia, Myanmar, Philippines, Singapore and Malaysia (Fig. 3). It is estimated that in the

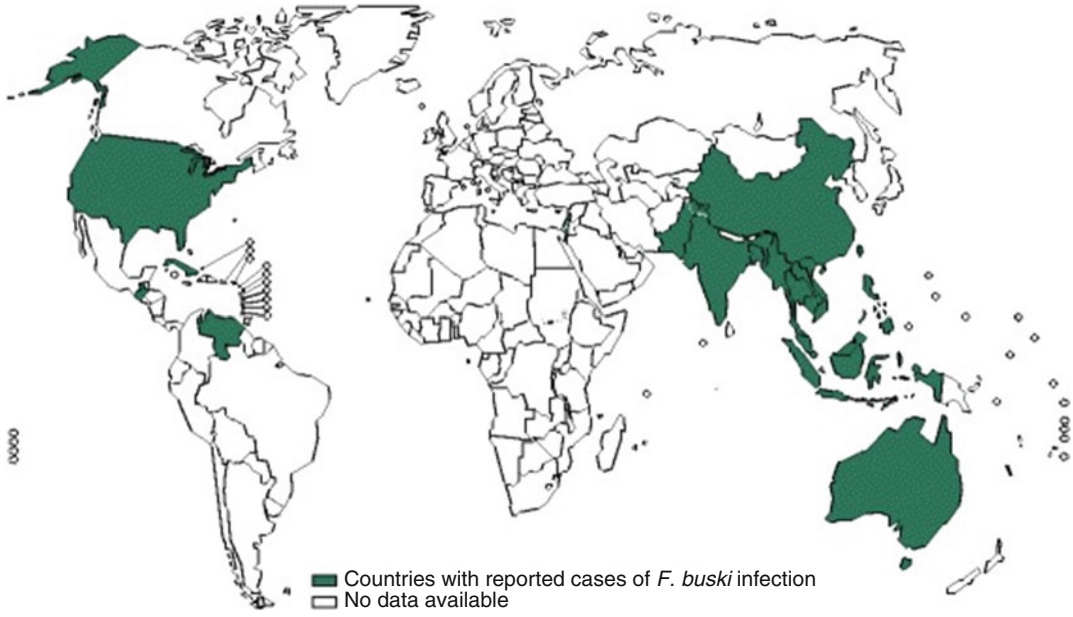

Fig. 3 Global prevalence of *Fasciolopsis buski* (Source: Lu, XT, Gu, QY, Limpanont, Y. et al. Snail-borne parasitic diseases: an update on global epidemiological distribution, transmission interruption and control methods. *Infect Dis Poverty* **7,** 28 (2018). https://doi.org/10.1186/s40249-018-0414-7)

beginning of the twenty-first century, at least ten million people were infected with this food-borne trematode infection. Disease is underreported in endemic areas and is mainly prevalent in semi-urban and rural areas of the world (Table 1).

The highest prevalence of fasciolopsiasis is observed in age group of 10–14 years worldwide. The prevalence of infection in children ranges from 10% in Thailand, 25% in Taiwan, 57% in China to 60% in India. The reason for high prevalence in this age group could be attributed to the habit of picking up and eating contaminated water plants, while going and coming back from school.

In India, the prevalence of this food-borne trematodiases is estimated to be 63% in Maharashtra, 60% in Assam, 22.4% in Uttar Pradesh and 45–80% in Bihar. Sporadic cases of fasciolopsiasis have been reported from West Bengal, Odisha, Tamil Nadu, Manipur and Karnataka. The key reservoirs of infections are the pigs and harbour only 3–12 flukes/ pig. Studies have detected prevalence of infection in pigs in different countries: 10% of pigs from Kwangtung, China, 30% from pigs in Uttar Pradesh in India and 52% of pigs in Taiwan.

In endemic areas, disease is closely linked to various common social practices practised in areas near close proximity to stagnant or slow moving water. Pollution of water with human excreta (night soil) and pig excreta are important contributory factors in transmission of fasciolopsiasis in a community. These are fed to fish and added to pond as fertilizer which facilitates transmission of the parasite. Poor sanitation, poverty, malnutrition and low standard of living are the other socioeconomic factors that contribute to the spread of fasciolopsiasis.

The water chestnut (*Eleocharis tuberosa*), water caltrop (*Trapa natans* in China, *Trapa bicornis* in Thailand and Bangladesh, *Trapa bispinosa* in Taiwan), water bamboo (*Zizania* sp.), water hyacinth (*Eichhornia* sp.), water lotus (*Nymphaea lotus*), water cress, water lily (*Nymphae* sp.), water spinach (*Ipomoea*

Table 1 Distribution of *Fasciolopsis buski* in humans

Species	Distribution	Definitive host	First intermediate host
Fasciolopsis buski	Southeast Asian countries	Humans, pigs	Freshwater snails

aquatica) and water morning glory are various aquatic plants implicated in transmission of *F. buski*. Small planorbid snails of the genera *Segmentina* and *Hippeutis* and *Gyraulus* are mostly involved in life cycle of *F. buski*. Important species are *Segmentina hemisphaerula, Segmentina trochoideus, Hippeutis cantori, Hippeutis umbilicalis, Gyraulus convexiusculus*, etc. Snails especially those of species *S. hemisphaerula* and *H. scantori* are particularly susceptible to desiccation. *F. buski* causes 100% mortality in snails, due to damage to the ovotestis during the life cycle.

Diagnosis

The diagnosis of the condition is made by detection of a large number of operculated eggs in faeces of infected hosts. Adult worms may also be occasionally seen in faeces in heavy infections.

Microscopy

Eggs may be demonstrated in the faecal specimens by microscopy. The eggs are large (130–140 μm × 80–90 μm), oval, operculated and bile stained (Fig. 4). The sedimentation technique is recommended for concentrating the trematode eggs. The zinc sulphate flotation concentration is not used because the high specific gravity of zinc sulphate tends to open the operculum of egg, and thereby filling the eggs with the solution, resulting in the eggs sinking to the bottom of the container. The concentration techniques recommended and used are Kato-Katz, Stoll's dilution, formalin-ethyl acetate centrifugation techniques and FLOTAC techniques. These methods have high sensitivity and help in quantification of infection. *F. buski* eggs appear to be morphologically similar to those of other trematodes such as *F. hepatica, Echinostoma* spp. and *Gastrodiscoides* spp. Due to the geographical overlap of areas endemic for these trematodes, a misclassification of eggs may occur.

In heavy infection, the adult worms lose the ability to remain attached to the intestinal wall, and thus are expelled and demonstrated in the faeces.

Serodiagnosis

Serology plays no role in diagnosis of fasciolopsiasis because of extensive cross reaction with antigens of other trematodes. Therefore, no serological tests are available commercially.

Molecular Diagnosis

Molecular techniques using sequences corresponding to 18srRN, ITS-1 and ITS-2 of ribosomal DNA are used for specific diagnosis of fasciolopsiasis. PCR using ITS2 is a promising epidemiological tool for accurate identification of different various food-borne trematodes. Additionally, rDNA (ribosomal DNA) and mtDNA (mitochondrial DNA) sequences have been used to find the phylogenetic relationship of *F. buski* isolates from China, India and Vietnam (Table 2).

Other Diagnostic Parameters

Low-grade eosinophilia is constantly present. Anaemia, especially macrocytic anaemia, may be seen.

Treatment

Praziquantel is the drug of choice for treatment. It causes severe spasm and paralysis of the worm's muscle. This isoquinoline derivative is well-tolerated and given orally either as a single dose of 75 g/kg/day or in three divided doses for 1–2 days. The use of praziquantel is

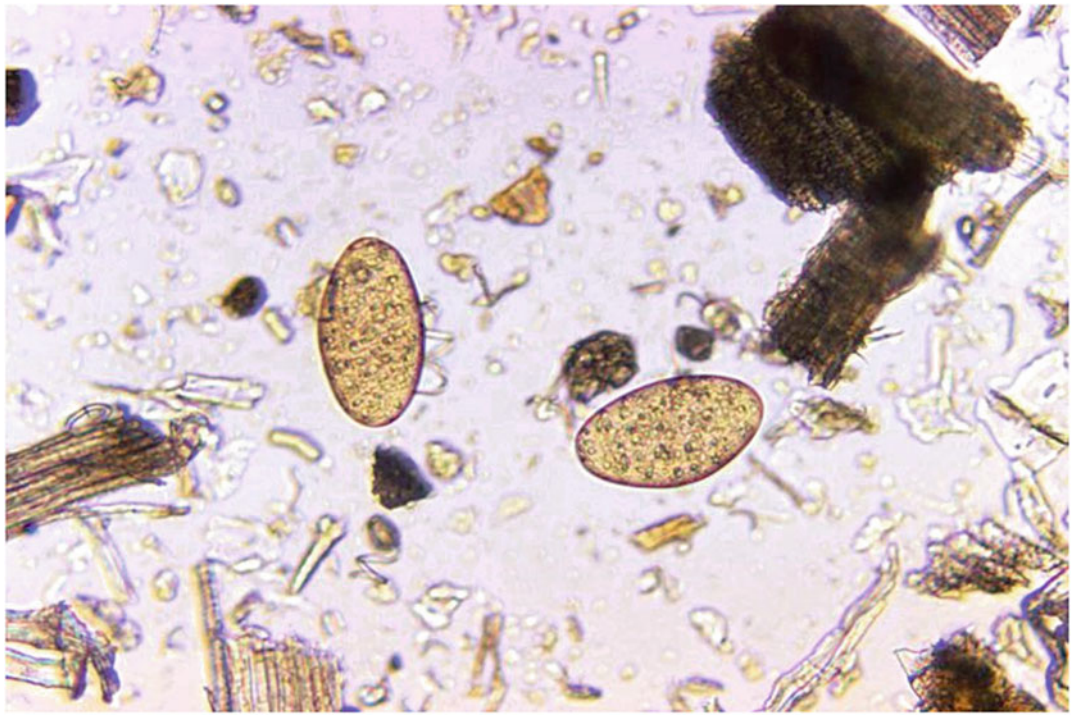

Fig. 4 *Fasciolopsis buski* eggs in an unstained, formalin-preserved stool sample (125×). (Courtesy: CDC/Dr. Mae Melvin)

Table 2 Diagnostic methods in human fasciolopsiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Stool examination	Large (130–140 μm × 80–90 μm), oval, operculated and bile stained	Kato-Katz, Stoll's dilution, formalin-ethyl acetate centrifugation techniques and FLOTAC techniques can be used
Molecular assays	PCR, qPCR	18s rRNA, ITS-1, ITS-2	High sensitivity and specificity *Limitations:* Require skilled personnel

contraindicated in pregnancy, cerebral paragonimiasis and ocular cysticercosis. In endemic areas, a single dose of praziquantel is given to children with the objective to prevent re-infection and having broad-spectrum antihelmintic effect, improves the children's health and development.

Niclosamide, a salicylamide derivative is an alternative drug available for treatment of *F. buski*. It is recommended for 1–2 days in a dosage of 40 mg/kg/day. The drug acts by diminishing the potential of inner mitochondrial membrane to inhibit oxidative phosphorylation.

Recently, triclabendazole, oxyclozanide and rafoxanide have been evaluated in naturally infected pigs for their efficacy based on the basis of egg reduction and clinical improvement. After treatment for 28 days, maximum efficacy was seen for triclabendazole (97.1%), followed by oxyclozanide (93.2%) and rafoxanide (83.1%).

Prevention and Control

Avoidance of eating raw aquatic food is the simplest and most practical method to prevent transmission of fasciolopsiasis. Nevertheless, changes in age-old food habits, food cooking practices, customs and traditions are difficult. Blocking and/or interrupting disease transmission is achieved by improved pig farming methods, elimination of intermediate host snails, early diagnosis and treatment of humans and pigs. Use of modern pig farming techniques, use of alternative pig feed instead of aquatic plant, prevention of faeces polluting water bodies, discontinuing use of unsterilized night soil as fertilizer and total ban on open defecation are the suggested ways to control fasciolopsiasis.

Various preventive measures like proper examination of aquatic plant before consumption can prevent fasciolopsiasis since metacercaria cysts are large enough to be seen by the naked eye (2–4 mm) and on average 15–20 cysts are found on one water caltrop. Eating dried aquatic plant is safe because drying at 27 °C for nearly 19 h or direct sunlight for 30 min kills the metacercaria. Additionally, immersing the plants in boiling water also prevents infection. Treatment with 5% NaCl for 3 h, 1% HCl for 8 days, 2% acetic acid for 9 days, 3% acetic acid for 6 days and soya sauce for 30 min kills the metacercariae in aquatic plants.

There is an increase in prevalence of foodborne trematodiases, as climate and temperature change can greatly influence various stages in the life cycle and transmission of *F. buski*. Embryonation of eggs, larval development in snail and emergence of cercaria are all dependent on temperature of freshwater and other abiotic factors. Additionally, local climate change affecting the epidemiological factors will increase the transmission window, thereby increasing the prevalence of fasciolopsiasis in both pigs and humans.

Case Study

A 15-year-old young girl, living in a village in Bihar, India, was admitted to a hospital with complaints of recurrent upper and middle abdominal pain since last 2 months. The associated symptoms were nausea, vomiting, and chronic diarrhoea with increase in abdomen size. On physical examination, she was found to be anaemic, malnourished with abdominal distension. The patient gave history of playing near ponds and water bodies and eating the aquatic plants. The patient belongs to low socio-economic strata. On investigation, stool sample showed brown, oval, operculated ova, suggestive of trematode eggs. Subsequently, the patient was treated with praziquantel 75 mg/kg orally. Gradually, over a period of 3–4 weeks her symptoms improved and repeat stool examination was negative.

1. What are the other trematode eggs which can be confused with those of *F. buski*?
2. In the above case, how can you arrive at a definitive aetiological diagnosis?
3. What are the various ways by which foodborne trematode infections can be controlled?

Research Questions

1. What is the true prevalence of fasciolopsiasis in the world? How to use the existing diagnostic methods to map the infection in endemic areas?
2. Why is it important to develop point of care test for fasciolopsiasis?
3. Why is it necessary to study the proteomics of the parasite?

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Paragonimiasis

Jagadish Mahanta

Learning Objectives

1. To be aware of paragonimiasis as a condition which can mimic tuberculosis, particularly in endemic areas.
2. To be familiar with the egg morphology so as to be able to identify it in sputum samples of patients with pulmonary symptoms.
3. To have the knowledge to distinguish between various species using molecular methods.

Introduction

Paragonimiasis is caused by genetically and geographically diverse lung flukes, *Paragonimus* spp., with variable biological features and human infectivity. Paragonimiasis has remained as an important parasitic zoonosis, mainly transmitted through food in Southeast Asia and Far East countries. The cost in terms of disability-adjusted life years for paragonimiasis is more than the combined food-borne trematode infections such as opisthorchiasis, fascioliasis, and intestinal diastome infection. The clinical manifestations of paragonimiasis mimic those of pulmonary tuberculosis causing diagnostic dilemma, especially in smear-negative haemoptysis cases. *Paragonimus westermani* is the best known species to cause pulmonary paragonimiasis. In India, paragonimiasis was recognized as a public health problem from 1981 onwards, after large numbers of cases were reported from Manipur, India. Subsequently, paragonimiasis was reported from all the states of Northeastern India.

History

Historically, in the past, human paragonimiasis was known by several names like endemic haemoptysis, oriental lung fluke infection, pulmonary diastomiasis, parasitical haemoptysis, parasitare haemopte, Gregarinosis pulmonum, etc.

Although Naterer (1828) first detected lung flukes, yet *P. westermani* was first authenticated by Kerbert (1878) from lungs of two Bengal tigers that died in Hamburg and Amsterdam Zoological garden. The species was named after the tiger's zookeeper, C. F. Westerman.

Serveyor (1919) was first to report eggs of *Paragonimus* in sputum of a Chinese man in Bombay (Mumbai), India. Vevers (1923) reported *P. westermani* and *Paragonimus kellicotti* in London Zoo. He also reported *Paragonimus compactus* and *P. westermani*

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from India. Cooper (1926) demonstrated *Paragonimus* in Gauhati (Guwahati), Assam, from the lung of civet cat. Gulati (1926) reported *Paragonimus edwarsi* from a palm civet cat in India. Subsequently, in 1934, *P. westermani* was recorded from lungs of domestic dogs in Madras (Chennai) and Coimbatore; cats, tigers (*Panthera tigris)* in Terai area of the Himalayas; and mongoose in Corbett National Park, India; and in beer cat in zoological park in Chandigarh. *Paragonimus mungoi* and *Paragonimus pantheri* were described in animals from Odisha, India in 1976.

First epidemiological study on human paragonimiasis was conducted in Manipur, during 1986–1987 and Indo-Japan joint research (1990) on *Paragonimus*. In the said study, *Potamiscus manipurensis* (Alcock, 1909) and *Maydelliathelphusa lugubris* (Wood-Mason, 1871) from mountain stream were found to harbour metacercariae of *Paragonimus heterotremus*, *P. westermani* like metacercariae establishing endemicity of paragonimiasis in Manipur, India. Subsequently, several crab host species were identified and cases of human paragonimiasis were documented in all the states of Northeastern region of India.

Taxonomy

Taxonomy of *Paragonimus* spp. is based on morphological and molecular characteristics. Morphological keys include size and shape of different life stages, relative size of suckers (oral and ventral), branching patterns of the ovary and testes, and tegumental spines.

A total of 36 valid species of the genus *Paragonimus* has been recognized. *P. pseudoheterotremus* has been recorded as a new species from experimental cat in Thailand. Recently, four new species, *Paragonimus vietnamensis, Paragonimus proliferus, Paragonimus bangkokenis*, and 1 from Costa Rica, *Paragonimus caliensis* have been have been identified in animals.

The genus *Paragonimus* belongs to family: Paragonomidae; order: Plagiorchiida; class: Rabditophora; and the phylum: Platyhelminthes.

Genomics and Proteomics

The genome of *P. westermani* is about 1.1 Gb long. Approximately, 922.8 Mb genome assembled, which constitutes about 84% of the size. About 45% of the genome has DNA repeats in long interspersed element and long terminal repeat subtypes. About 12,852 protein coding genes were predicted. This shows high levels of conservation among related trematode species. A comparative genomic study of four species of *Paragonimus* (*Paragonimus miyazakii*, *P. westermani*, *P. kellicotti*, and *P. heterotremus)* collected from different countries has demonstrated that the genome size varies between 697 and 923 Mb, containing 12,072–12,853 genes. These draft genomes were estimated to be between 71.6% and 90.1% complete. Nearly 256 lung fluke–specific and conserved orthologous groups with consistent transcriptional adult-stage *Paragonimus* expression profiles have been identified.

Analyses of the 2-DE protein profiles of the excretory–secretory products (ESP) of adult *P. westermani* revealed approximately 147 protein spots, 15 of which were identified as cysteine proteases (CP), with molecular weights between 27 and 35 kDa. Additional three CPs (designated as PwCP3, 8 and 11) were newly recognized by TOF/TOF Mass spectrometry (MS), and majority of these proteases react with sera from patients. In another study, ESP of adult *P. westermani* using 2-DE coupled to MS identified 25 different proteins, some of them are cysteine proteases.

P. heterotremus causing human infection in Northeastern region of India showed genetic homology with isolates from China and Southeast Asian countries. The transcriptome of adult *P. kellicotti* was sequenced, which showed 78,674 unique transcripts derived from 54,622 genetic loci and 77,123 unique protein translations were predicted. A total of 2555 predicted proteins (1863 genetic loci) were verified by mass spectrometric analysis of total worm homogenate, including 63 proteins lacking homology to previously characterized sequences.

The Parasite Morphology

Adult

Adult worms are ovoid, and vary from 7.5 to 12 mm in length and 4–6 mm in breadth with two suckers (ventral and oral). Body of the worm is covered with tegumental spine. They are hermaphrodite with lobed ovaries and branched testes (Figs. 1 and 2).

Eggs

Eggs are golden brown and thick-shelled. Un-embryonated egg measures 80–120 μm in length and 45–70 μm in breadth. Generally, eggs of *P. westermani* and *P. ringeri* are larger than that of *P. compactus*. Egg shell in *P. kellicotti* and *P. ringeri* is markedly thickened at the opposite pole to the operculum, whereas the shell of *P. compactus* is uniformly thickened. A small knob occurs in the opercular shoulder of *P. westermani* egg (Fig. 3).

Metacercaria and Cercaria

Metacercariae are 280–450 μm in diameter with two suckers and spined teguments. They are infective stage of the parasite (Fig. 4). Cercariae have small and short tail with large ventral sucker and tegumental spines.

Cultivation of Parasites

No stage of the parasite can be cultivated in vitro.

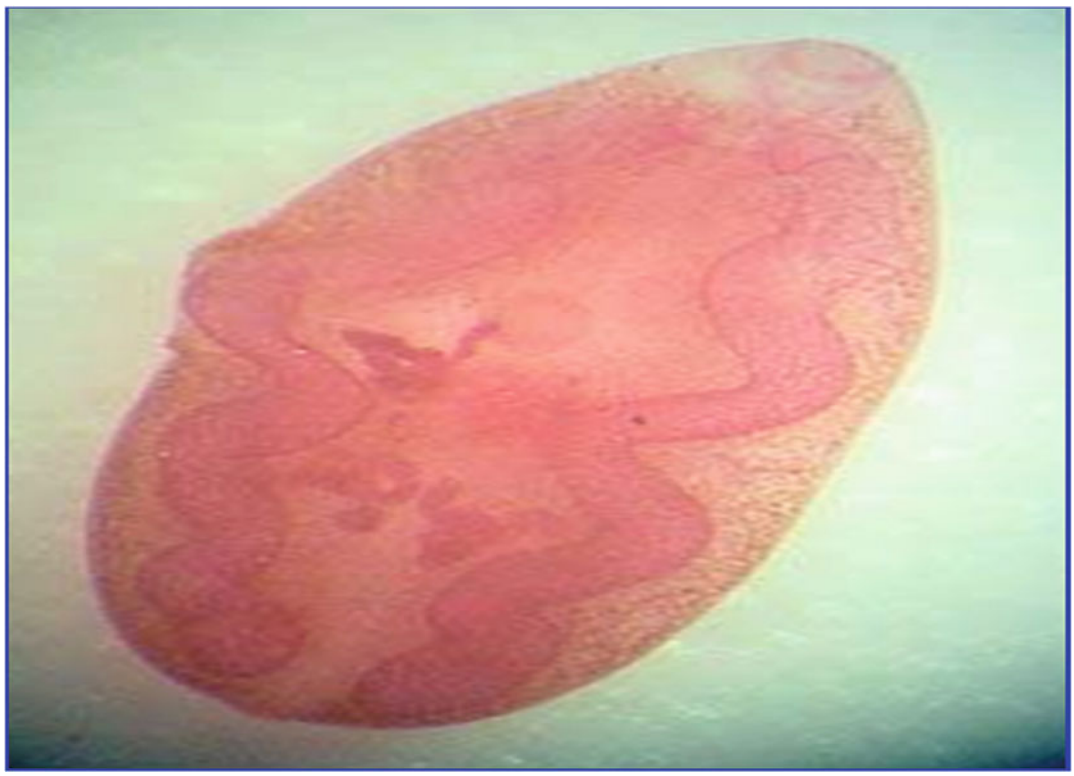

Fig. 1 Adult worm of *Paragonimus* spp. (Acetocarmine mount) (Author's collection)

Fig. 2 Reproductive organs of *Paragonimus* spp. (Acetocarmine mount) (Author's collection)

Fig. 3 Egg of *Paragonimus* spp. in sputum (Author's collection)

Fig. 4 Metacercaria of *Paragonimus* spp. in crab (Author's collection)

Laboratory Animals

Puppies and kittens support development of all *Paragonimus* species. Outbred Wister rat was developed as model to grow Indian strain of *P. heterotremus* and *P. westermani* at Regional Medical Research Centre (ICMR), Dibrugarh, India. Investigators reported that metacercariae took nearly 27–36 days to develop into adults. The worm reaches pleural cavity in 27–81 days. In next 5–7 days, pulmonary cysts appear and the worms start laying eggs. In about 45 days, some parasites also encapsulate in the liver. Although 42.4% of the adults remain in pulmonary cavity, 17% remain free in parenchyma.

Life Cycle of *Paragonimus* Species

Hosts

Paragonimus completes its life cycle in two intermediate hosts and one definitive host (Fig. 5).

Definitive Hosts

Carnivorous or omnivorous mammals such as humans, tigers, lions, cats, dogs, wolves, foxes, leopards, mongooses, raccoons, minks support the growth and sexual maturity as definitive host.

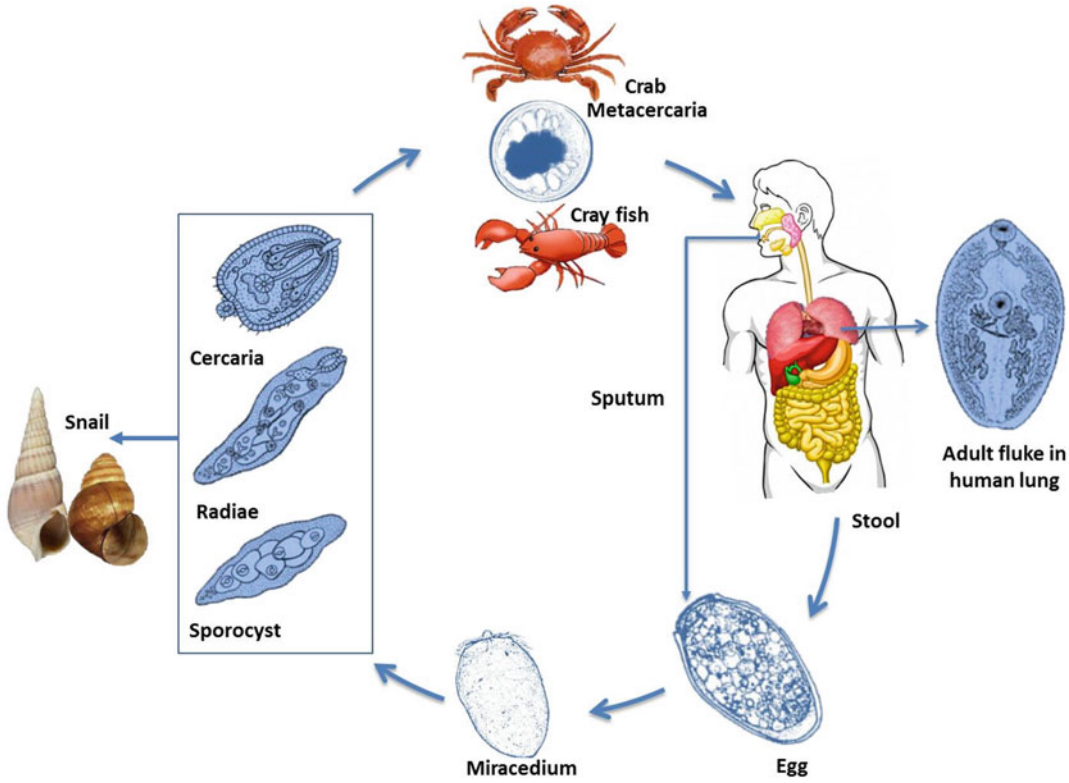

Fig. 5 Life cycle of *Paragonimus* Spp.

First Intermediate Hosts

Freshwater molluscan; *Semisulcospira (Semisulcospira libertina, Semisulcospira cancellata, Semisulcospira amurensis, Semisulcospira pregrinomum*, and *Semisulcospira toucheana), Oncomelania (Oncomelania hupensis nosophora), Melanoides tuberculata, Pomatiopsis lapidaria*, *Aroapyrgus colombiensis* etc. *Brotia*, *Tarebia*, *Assiminea* spp., *Tricula gregoriana, Hemisinus maculatus* and *Thiara* support the growth of miracidium as first intermediate host.

Second Intermediate Hosts

Crabs belonging to *Maydelliathelphusa lugubris, Pseudotelphusa chilensis, Potamiscus manipurensis* and *Potamiscus smithianus, Potamon johorense, Potamon flexum, Geothelphusa dehaani, Parathelphusa incerta, Sundathelphusa philippina, Esanthelphusa dugasti*, *Siamthelphusa paviei, Trichodactylus faxoni, Moreirocarcinus emarginatus, Hypolobocera chilensis* and *Hypolobocera aequatorialis* or crayfish of *Cambaroides dauricus, Oronectes* and *Cambarus* species support the growth of cercariae as second intermediate host.

Transmission of Infection

Metacercariae, the infective stages of the parasite, infect carnivorous mammals, including humans (Table 1). These hosts acquire infection by consumption of raw or undercooked crabs or crayfish with the metacercariae. After entry of the infective metacercaria, excystation occurs in the duodenum and the adolescaria penetrates through the wall of the intestine, traverses the abdominal cavity in 3–6 h and migrates upwards through

Table 1 Distribution of some *Paragonimus* spp. important to humans

Species	Distribution	Intermediate host		Paratenic host	Definitive host
		First	Second		
Paragonimus westermani	India, Sri Lanka, Nepal, Pakistan, China, Taiwan, Korea, Vietnam, Japan, Siberia, Papua New Guinea, USA	**Snail***:* *Semisulcospira libertina, Semisulcospira cancellata, Semisulcospira amurensis, Semisulcospira pregrinomum, Semisulcospira toucheana, Oncomelaniah upensis nosophora, Melanoides tuberculata, Brotia*, *Tarebia*, *Thiara*	**Crab**: *Maydelliathelphusa lugubris, Potamiscus manipurensis, Potamiscus smithianus, Potamon johorense, Potamon flexum, Geothelphusa dehaani, Parathelphusa incerta, Sundathelphusa philippina, Esanthelphusa dugasti*, *Siamthelphusa paviei, Eriocheir* **Crayfish:** (*Cambaroides dauricus, Orconectes* and *Cambarus*) **Shrimps:** (*Acrohrachium, Caridina*)	*Sus scrofa leucomystax* (Japanese wild boar)	Humans, cats, dogs, pigs, mongooses, wild boars, civet cats, Asian tigers
Paragonimus skrjabini	China, Thailand, Japan, Vietnam, India	*Tricula* and *Akiyoshia*	*Potamiscus manipurensis*, *Sinopotamon* sp.	*Sus scrofa leucomystax* (Japanese wild boar), *Rana boulengeri* (frog), rat	Humans, dogs and *cat* (*Paguma larvata*)
Paragonimus miyazaki	Japan	*Bithynella nipponica*	*Geothelphusa dehaani*	*Sus scrofa leucomystax* (Japanese wild boar)	Humans, cats including dogs, weasels, sables, pigs, raccoon dogs and badgers
Paragonimus philippinensis	Philippines, India	*Antemelania asperata* and *Antemelania dactylus*	*Sundathelphusa philippina*	Not recorded	Humans, cats, dogs, field rats
Paragonimus heterotremus	Southeast Asia, China, Thailand, Laos, India	*Tricula gregoriana*	*Maydelliathelphusa lugubris, Potamiscus tannanti*, *Potamiscus manipurensis, Ranguna smithiana, Parathelphus dugasti, Sinopotamon*	Rat, *Sus scrofa leucomystax* (Japanese wild boar)	Humans, rats

(continued)

Table 1 (continued)

Species	Distribution	Intermediate host		Paratenic host	Definitive host
		First	Second		
Paragonimus kelicotti	North America, Canada	*Pomatiopsis lapidaria*, *Oncomelania hupensis nosophora*	Freshwater crayfish: the *Oronectes* and *Cambarus* spp.	Not recorded	Humans, cats, dogs, sheep, skunks, red foxes, coyotes, raccoons, bobcats and mink
Paragonimus africanus	Cameroon, Guinea, Nigeria, Ivory Coast	*Melania* spp., *Homorus striatella* (*Subulinidae*), *Achatinid* spp.	*Sudanonautes africanus, Sudanonautes peli, Sudanonautes africanus, Sudanonautes aubryia*	Not recorded	Humans, dogs, mongooses (*Atilax paludinosus* and *Crossarchus obscurus*), civets (*Viverra civetta*), Mandrill (*Mandrillus leucophaeus*), monkeys, mouse, rats and shrews
Paragonimus uetrobilateralis	Cameroon, Nigeria, Liberia, Guinea, Ivory Coast, Gabon	*Melania* spp., *Homo rus striatella* (*Subulinidae*), *Achatinid* spp.	*Sudanonautes africanus, Sudanonautes aubryi, Liberonautes latidactylus, Liberonautes latidactylus nanoïdes*	Not recorded	Humans, dogs, white-tailed mongooses (*Ichneumia albicauda*)
Paragonimus maxicanus	Mexico, Peru, Ecuador, Costa Rica, Panama, Guatemala	*Aroapyrgus colombiensis*	*Trichodactylus faxoni, Zilchiopsis ecuadoriensis Moreirocarcinus emarginatus, Hypolobocera chilensis* and *Hypolobocera aequatorialis, Hypolobocera guayaquilensis*	Not recorded	Humans, cats, dogs, rats, *Didelphis marsupialis, Felis pardalis*, *Nasua nasua* and *Tayassu pecari*

diaphragm and pleura into the thoracic cavity and the lungs. For cerebral paragonimiasis, adolescaria ascends through peri-vascular tissues around the carotid artery or jugular vein and enters via the foramen in the skull base.

In the lungs it encysts, and attains sexual maturity to lay eggs in about 65–90 days. It is reported that *Paragonimus* worms can lay 9530–18,380 eggs per day per worm in experimental dogs during days 64–151 post-infection. The parasites may survive from 1 to 20 years in mammals without specific therapy. Eggs are discharged either in sputum or faeces to reach water.

Miracidium from eggs is the infective stage for snail. Eggs hatch in about 2 weeks to ciliated miracidia in water, which crawl to reach suitable snail host. Not much work has been done in India on first intermediate host. In the snail, the miracidium develops into a mother sporocyst to produce asexually second generations of rediae and to cercariae in 9–13 weeks.

Cercariae enter the crab or crayfish either by ingestion of snail along with cercariae, or directly by penetrating the soft inter articular space or other soft parts. Cercariae develop into metacercaria and remain encysted in the liver, gills, intestine, skeletal muscles or in the heart.

Infection is transmitted to carnivorous or omnivorous mammals, including humans after consumption of infected crab or cray fishes. Humans can also be infected through contaminated hand or utensils or by application of crab paste in raw ulcers. Sometimes, juvenile parasites remain encysted in muscle of wild boars, bears, rats, mice frogs (China), and they can act as paratenic host for *Paragonimus* transmission. They are often called third intermediate host. Consumption of undercooked meat of such animal results in infection.

Pathogenesis and Pathology

Once infected by ingestion, the juvenile worms migrate through intestinal wall to settle in lungs to form granulomatous or cystic lesions in humans. Some *Paragonimus* species show tropism and locate themselves in different sites of the body to manifest as pulmonary, thoracic, abdominal, cerebral, spinal and cutaneous paragonimiasis (*trematode larva migrans*).

In humans, cyst formation along with tissue necrosis typically occurs in lungs during chronic stage of the infection in pulmonary paragonimiasis. Granuloma formation with leukocyte infiltration is characteristic, which leads to cyst formation. The cysts are superficial and 1–3 cm in diameter, contain viscous fluid, with RBC, eosinophils, necrotic tissue, Charcot-Leyden crystals, often adult worms and ova. This give rise to slate blue coloration of the cyst and black or rusty sputum during cough. The cysts have an outer fibrous wall with macrophages containing hemosiderin and congested blood vessels and inflammatory cells in the inner layer. Small foci of haemorrhage with leukocyte infiltration are present in walls of small intestine marking the areas of larval migration. Small granuloma, abscess around egg may be seen in abdomen and brain. Inflammation may cause intra-abdominal adhesion of organs with peritoneal wall or diaphragm. Muscles of thorax, abdomen, thigh, heart and pericardial cavity, brain, spinal cord, orbital tissue, skin and subcutaneous tissues, mediastinal tissues, breast, bone marrow, peritoneal cavity, intra-abdominal organs like liver, spleen, omentum, adrenal gland, appendix, ovary, uterus, scrotum, inguinal regions and urinary tract have also been reported to be the other ectopic foci of paragonimiasis.

Immunology

Adaptation and long-term survival of *Paragonimus* in humans with active immune responses are fascinating. The parasite releases a few enzymes (27- and 28-kDa) that cleave heavy and light chains of human immunoglobulin. Further, IgG-induced eosinophil degranulation and production/release of superoxide to defend against the pathogen are reduced. Thus, it creates a zone of immune privilege around it. Further, in response to excretory/secretory products (ESP) of the worm, eosinophils become apoptotic which reduces local inflammatory response. Cysteine proteases secreted by PwNEM attenuate both activation and degranulation of eosinophils. Five species of the cysteine proteases of *P. westermani* have been identified and purified. They are 28- and 27-kDa enzymes produced by metacercarial larvae, 15- and 53-kDa enzymes by the juveniles and adults and 17-kDa cysteine proteases by the adults. Of the five cysteine proteases, two cysteine proteases from the metacercarial larvae reveal higher levels of proteolytic activity in cleaving IgG than the others. The inhibitory effect of the ESP on IgG-induced superoxide production is dose dependent.

Infection in Humans

Incubation period of paragonimiasis, on average, varies between 2 and 16 weeks. However, shorter incubation period was reported among 52% cases in an outbreak of *P. westermani* in Harbin, China.

Four clinical forms of paragonimiasis caused by *P. westermani* and other *Paragonimus* spp. are recognized in humans.

Pleuro-pulmonary paragonimiasis presents with chronic cough, recurrent haemoptysis, with signs and symptoms of fever, anaemia, weakness, and weight loss, mild pleural effusion, pneumonitis, bronchiectasis or bronchopneumonia. In cases reported from Manipur (India), 20% showed pleural effusion, and most (60%) cases presented complaints of pain or tightness in the chest with difficulty in breathing. Sputum was black or rusty brown in colour. Recurrent pleural effusions, empyema, constrictive pleuritis and recurrent pneumothorax are the noted complications.

Abdominal paragonimiasis presents with abdominal pain, nausea, vomiting, diarrhoea, dysentery, hepatomegaly, peritonitis and pancreatitis (some cases) etc.

Cerebral paragonimiasis presents with fever, headache, nausea, vomiting, seizures (Jacksonian epilepsy in young), blurred vision, motor weakness and often coma. The condition may lead to eosinophilic meningoencephalitis, hydrocephalus, increased intracranial pressure and blindness. The first case of cerebral paragonimiasis in India was documented from Nagaland in a child. The case was originally thought to be tuberculoma.

Cutaneous paragonimiasis, frequently caused by *Paragonimus skrjabini* infection, shows cutaneous nodules in about 30–60% of infected persons. The nodules are painless, firm and 2–5 cm in diameter and often show migratory character. Studies in Manipur, India, recorded 16% subcutaneous nodules in children.

Though ectopic paragonimiasis may involve any part of the body, yet involvement of heart and pericardium is a serious problem and may even result in death.

Infection in Animals

Disease spectrum caused by *Paragonimus* spp. in wild animals is largely unknown. Post-mortem examination of tiger infected with *P. westermani* reveals numerous cysts, emphysematous lungs with areas of collapse, severe congestion with pneumonia, bronchitis and bronchiectasis. Captive or domestic animals show chronic, intermittent cough and gradually become weak and lethargic; infections in these animals pass unnoticed.

Domestic dogs show mild coughing with serous nasal discharge. Multi-loculated cysts in dogs and interstitial nodules in cat have been recorded on radiological examination. Experimental dogs initially showed pleural effusion and subpleural ground-glass opacities or linear opacities on the 10th day, followed by subpleural and peribronchial nodules, hydropneumothorax, cavity lesion on the 13th day and mediastinal lymphadenopathy on the 60th day. Subpleural ground-glass opacities and nodules with or without cavity persist till 180 days.

Epidemiology and Public Health

Ten *Paragonimus* species are known to cause infection in mammals including humans. Although, *P. westermani* is the most common species causing human infection all over the world, *P. skrjabini*, and *P. hueitungensis* (China), *P. miyazaki* (Japan), *P. philippinensis* (India and Philippines), *P. heterotremus* (South China, Thailand, Laos and India), *P. kelicotti* (North America), *P. africanus* (Nigeria), *P. uterobilateralis* (Africa), and *P. maxicanus* (South and Central America) are other species reported from various parts of the world (Table 1).

Approximately, 293 million people worldwide are at risk of paragonimiasis and more than 23 million people are infected in 48 countries (Fig. 6). Studies carried out in a hyperendemic zone of Arunachal Pradesh, India, recorded *P. heterotremus* infection. While studies on first intermediate host was non-conclusive, *Maydelliathelphusa lugubris* were incriminated as the second intermediate host in the area. A cross-sectional study in Arunachal Pradesh and Assam, among persons with cough >1 week, revealed sero-positivity for paragonimiasis in Arunachal Pradesh as 7.6% and in Assam 1.2%

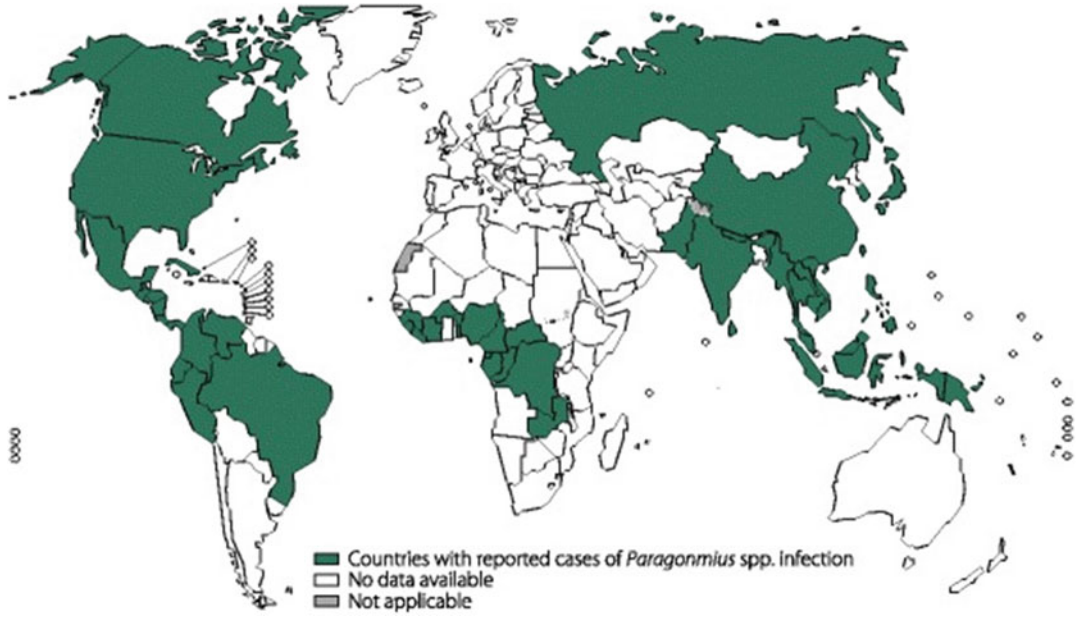

Fig. 6 Global distribution of *Paragonimus* spp. (Source: Lu, XT, Gu, QY, Limpanont, Y. et al. Snail-borne parasitic diseases: an update on global epidemiological distribution, transmission interruption and control methods. *Infect Dis Poverty* **7,** 28 (2018). https://doi.org/10.1186/s40249-018-0414-7)

with a history of eating undercooked crabs and even children consuming raw crabs. However, there was no history of consumption of crayfish.

Diagnosis

Laboratory findings such as leukocytosis, eosinophilia, high erythrocyte sedimentation rate, presence of Charcot-Leyden crystals in sputum, exudative type of pleural effusion or cerebrospinal fluid analysis are frequently helpful to suggest the diagnosis of paragonimiasis.

Microscopy

Morning sputum samples are the recommended specimen of choice for examination by direct wet microscopy for detection of *P. westermani* eggs. However, low egg positivity in sputum (28–39%) and irregular shedding of eggs in sputum often make parasitological diagnosis difficult. Formol-ether concentration of sputum or bronchoscopic washing helps in improving diagnosis by microscopy. Sensitivity of single sputum examination is low (30–40%) but multiple sputum sample examination increases (54–89%).

P. westermani eggs are also demonstrated in stool by direct microscopy, the sensitivity of which in children varied between 25.6% and 60%. About 10% centifuged deposit of pleural fluid shows *Paragonimus* ova. Excision biopsy of the lesion also helps in diagnosis as well as treatment. Adult or immature *Paragonimus* worm may be found in a subcutaneous nodule or cyst.

Immunodiagnosis

Intradermal (ID) test using acid soluble of crude extract of adult worm was earlier used extensively for diagnosis of paragonimiasis, for its rapidity, simplicity and cost-effectiveness in the past in Japan, China and India. After intradermal injection of antigen in the forearm, formation of wheel within 15 min indicates a positive test. However, past and the present infection cannot be differentiated with this test because the test remains positive for 10–20 years even after cure of the disease. Negative skin test in the absence of wheel rules out paragonimiasis.

Complement fixation test was used earlier to confirm *P. westermani* infections. The test quickly reverts back to normal as the flukes die. Counter-immunoelectrophoresis (CIEP), a sensitive and specific test, is used for speciation of *Paragonimus* species by specific precipitation bands. Excretory/Secretory (E/S) antigen was found to be better than adult somatic antigen in the test. Indirect haemagglutination test, latex fixation, bentonite flocculation, indirect immunofluorescence tests are the other tests that have been evaluated in various laboratories with variable results.

Enzyme-linked immune sorbent assay (ELISA) using adult fluke and metacercarial stage-specific antigens has shown a sensitivity of 82–93% and specificity of 98–100%. Use of 27 kDa protein of excretory/secretary product of *P. westermani* and 31.5 kDa of E/S protein of *P. heterotremus* showed high specificity. IgG-based ELISA using E/S antigen for diagnosis of paragonimiasis, developed at the Regional Medical Research Centre, ICMR (RMRCNE), Dibrugarh, India, was reported to be highly sensitive and specific. ELISA, used to detect IgG4 antibodies, was evaluated by using a synthetic peptide, based on the antigenic region of *P. westermani* in the diagnosis of human paragonimiasis. Sensitivity and specificity were reported to be very high (100% and 96.2%). Positive and negative predictive values were also high (100% and 88.9%). But, cross-reactivity was frequent with sera tested from the cases of fascioliasis (75%) and hookworm infections (50%).

An enzyme-linked immuno-electrotransfer blot was developed to differentiate between *P. heterotremus* and *P. westermani* infections. Dot-immunogold filtration assay (DIGFA) for diagnosis of *P. westermani* has been reported to be simple, rapid as well as sensitive (99%) and specific (92%). In this test, ES antigen of *P. skrjabini* was coated on nitrocellulose membrane as the capture line. Recombinant *Staphylococcus aureus* protein A was used to prepare the control line. This immunochromatographic test showed a sensitivity and specificity of 94.4% and 94.1%, respectively.

Molecular Diagnosis

Species-specific PCR assays have been constructed for the specific detection and identification of *Paragonimus* species. The test targets conserved regions within the *Paragonimus* genome. DNA pyrosequencing for *P. bangkokensis*, *P. harinasutai*, *P. heterotremus*, *P. macrorchis*, *P. siamensis*, *P. westermani* has also been attempted. Protein microarray, evaluated recently, in *P. westermani* diagnosis, has shown sensitivity of 86–92% and specificity of 97–100%.

Loop-mediated isothermal amplification (LAMP) *P. Westermani* has been evaluated to detect *P. Westermani in* freshwater crabs, crayfish, and infected human sputum and pleural fluid. The test is sensitive and specific, and also gives result within 45 minutes. Multiplex protein microarray assay was also reported to be 97–100% specific and 85.7–92.1% sensitive. DNA pyrosequencing for species-level identification of *Paragonimus* infections in endemic areas of Thailand successfully discriminated amongst six *Paragonimus* species.

Imaging Techniques

About 10–20% chest X-rays of patients remain normal. A study in South Korea, observed classic 'ring sign' subpleural or subfissural nodule and areas of necrosis, focal pleural thickening in adjacent areas were helpful in diagnosis of pulmonary paragonimiasis. Consolidation, lobar infiltrates, coin lesions, calcified nodules, hilar lymphadenopathy, pleural thickening, pleural effusions and pneumothoraces are common findings in symptomatic Asian or American paragonimiasis.

Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) of brain in patients with paragonimiasis brain show multiple ring-shaped shadows like 'grape cluster' or 'soap bubble appearance'. Iso-dense lesions often show similarity with tuberculoma in one cerebral hemisphere. Presence of a track between a pulmonary nodule and the pleura by chest CT may help in distinguishing paragonimiasis from those of

chronic eosinophilic pneumonia, tuberculosis, fungal infection or malignancy. Table 2 summarizes the diagnostic approaches for paragonimiasis.

Treatment

Praziquantel is the treatment of choice for paragonimiasis and is given at a dose of 75 mg/kg/day for 2–3 days, with a very few side effects.

Table 2 Laboratory diagnosis of paragonimiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Sputum, broncho-alveolar lavage, stool, body fluid, formol-ether concentration, excision biopsy of subcutaneous nodule or cystic lesion	Eggs, adult or immature worm	**Gold standard test** *Limitation*: Low sensitivity, irregular shedding of eggs in sputum or stool
Immunodiagnostics	Intradermal test using soluble extract of adult worm	Hypersensitivity	Simple, easy to perform, cheap and rapid. Negative result excludes infection; positivity suggests past or present infection *Limitation*: Test remains positive for 10–20 years even after cure
	Complement fixation test	Ag-Ab complex	Test quickly reverts after death of the worm
	Counter-immunoelectrophoresis	Precipitation of IgG Ab against E/S protein	Sensitive and specific, can be used for speciation
	ELISA using E/s protein, adult and metacercarial antigens (27 kDa E/S of *Paragonimus westermani* and 31.5 kDa of *Paragonimus heterotremus*)	IgG Ab. IgG4	Sensitivity of 82–93% and specificity of 98–100% **Limitation:** Cross-react with serum from fascioliasis and hookworm infections cases
	Immuno-electrotransfer blot and dot-immunogold filtration assay (DIGFA)	To differentiate between *Paragonimus heterotremus* and *Paragonimus westermani*	Simple, rapid, 99% sensitive and 92% specific
Molecular assays	RFLP, PCR, qPCR	ITS-1, ITS-2 and conserved regions within the genome	High sensitivity and specificity. *Limitations*: Require skilled personnel
	LAMP	ITS-1, ITS-2 and conserved regions within the genome	Can be used for determining infection status of freshwater crabs and crayfish and human sputum and pleural fluid
	Protein microarray and multiplex protein microarray assay	Proteins of *Paragonimus westermani*	Sensitivity of 86–92 and specificity of 97–100 *Limitations*: Require sophisticated equipment and skilled personnel
	DNA pyrosequencing	Genomic DNA	Species-level identification
Imaging technique	X-ray chest, CT scan, MRI	Ring sign, subpleural or subfissural nodule in chest X-ray. 'Grape cluster' or 'soap bubble appearance' in brain imaging	Help in distinguishing paragonimiasis from mimickers, including pulmonary TB, chronic eosinophilic pneumonia, tuberculosis, fungal infection or malignancy

However, about 2% cases need 5 day of therapy for complete cure. Bithionol (adult: 2.0–2.5 g, child 1.5 g/day) on alternate days for 15 doses has also been used with success. Oral triclabendazole at 10 mg/kg twice a day for 1–2 days has been used successfully to treat human paragonimiasis in South America. Oral triclabendazole has also been tried in praziquantel or bithionol failure cases with success. Initial 2 days of praziquantel treatment followed by triclabendazole has shown 100% cure rate.

Prevention and Control

Prevention and control of zoonotic paragonimiasis is often difficult due to the widespread distribution of competent intermediate hosts, large freshwater ecosystems and the variety of domestic and wild animal reservoir hosts consuming infected crustaceans.

Control of human paragonimiasis is best accomplished by public health education and by proper food hygiene. Frequent hand washing during preparation of crustaceans for the food, cleaning of crustaceans, avoiding contamination of utensils and serving platters with metacercariae are important components of prevention. The average survival of *Paragonimus* metacercariae in biogas plant, tap water, dechlorinated water and well water has been recorded as 13, 48.2, 52.14 and 56.21 days, respectively. Metacercariae of *P. westermani* in dead crabs survive for a week in winter, and the cysts in water remain viable for 2–3 weeks. *P. kellicotti* lived for 5 days in the viscera of dead crayfish, at 12–21 °C. Boiling or cooking crustaceans at 55 °C for 10 min is adequate to kill all the metacercariae.

Treatment and community education method have shown to bring a sharp decline in prevalence of pulmonary paragonimiasis within 6 years (2005–2011) in hyper endemic areas of Arunachal Pradesh, India. World health organization suggested preventive chemotherapy among others to combat paragonimiasis and approximately 75% population at risk should be covered by 2020. However, usefulness of preventive chemotherapy remains to be evaluated.

Case Studies

In 2001, people and healthcare professionals of Changlang district of Arunachal Pradesh, India, expressed concern about rising numbers of acid-fast bacilli (AFB) negative tuberculosis not responding to anti-tubercular treatment. They requested us to investigate the problem.

We investigated the cases from broad category of haemoptysis. Clinical history and examination of the patients revealed history of chronic cough, occasional fever and haemoptysis. Acid-fast bacilli in sputum were negative in most of the cases. Patients had adequate treatment as per category III tuberculosis of Government of India (Revised National Tuberculosis Control Programme). We started exploring other aetiology of haemoptysis and observed that no survey on paragonimiasis (one cause of haemoptysis) was done in Arunachal Pradesh. We interviewed the local health practitioners and found very low index of suspicion regarding paragonimiasis and did not consider it as a cause for haemoptysis.

We started epidemiological study with clinical profile of untreated cases. Arunachal Pradesh shares international boundary with Bhutan, China and Myanmar. Changlang district is mostly hilly with an area of 4662 km^2 at 200 to 4500 m height above sea level. The district is thinly populated (27 persons/km^2) inhabited by tribal population (125,334 persons) (http://changlang.nic.in). Subsistence farming and fishing are the primary occupation in the areas. The district has a diverse wildlife including the tiger (*Panthera tigris*), leopard (*Panthera pardus*), snow leopard (*Panthera uncia*) and the clouded leopard (*Neofelis nebulosa*). In the cross-sectional study, six villages were selected randomly. A total of 675 individuals including 263 (39%) children were studied. History revealed chronic cough (97.2%) followed by haemoptysis (83.3%), chest and abdominal pain (68.1% and 43.5%).

Sputum and blood were collected for detection of AFB using Ziehl-Neelsen stain and wet microscopic examination after decontamination. Blood samples were processed for detection of

Paragonimus antibody against excretory secretory protein antigen by ELISA (developed in house).

Light microscopic examination of sputum revealed presence of characteristic operculated golden brown eggs of *Paragonimus*. *The* eggs were later on identified as *P. heterotremus*. Children (<15 years) showed 20.9% egg positivity in sputum and 51.7% antibody positivity in blood, while 4.1% of adults (>15 years) showed egg in sputum and 18.7% of them antibody in blood. All samples were negative for AFB on smear microscopy. Children and adults showed 51.7% and 15.3% antibody positivity, respectively. This study may be the tip of the iceberg as many of the interior areas and other districts of Arunachal Pradesh are yet to be explored. All people eat crabs, often cooked inadequately. About 40% of freshwater crab (*M. lugubris)* collected during the study were found to harbour metacercariae of *Paragonimus*. Patients attending Miao Hospital for tuberculosis (clinical) revealed 17.3% prevalence of paragonimiasis and 12.8% prevalence of tuberculosis (sputum positive for AFB).

1. Which parasites can cause pulmonary infections, sometimes resembling tuberculosis?
2. How can you determine the species of *Paragonimus* spp.?
3. What are the hurdles in controlling paragonimiasis?

Research Questions

1. Operational study to address awareness, prevention of paragonimiasis addressing, food habit and food processing, food handling to relate with transmission of food-borne parasitic zoonosis like paragonimiasis among general population and health professionals.
2. Systemic study to assess health system preparedness for tackling (etiological diagnosis and appropriate treatment) the cases of chronic cough and endemic haemoptysis in the peripheral healthcare setup.
3. We do not have a good (cheap, quick, easy to handle) point-of-care test system at RNTCP for differential diagnosis of chronic cough and haemoptysis cases. Research addressing development of such test kit is a priority.

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Echinostomiasis

Rajendran Prabha

Learning Objectives

1. To make the reader aware of the helminth parasites that can cause dysentery-like illness with anaemia in long-standing cases.
2. To have a knowledge of the diversity of species causing echinostomiasis with their host specificities.

Introduction

Echinostomiasis is an intestinal food-borne infection in birds and mammals, including humans caused by Echinostomatidae or echinostomes. The infection is frequently seen among populations living near freshwater bodies and in people who have the practice of eating raw or undercooked aquatic bivalve molluscs or fish and snails dipped in a salt and vinegar mixture. *Echinostoma* spp. adult worms are distinguished by the presence or absence of the single or double crown of large circumoral spines on a disc surrounding the oral suckers.

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History

In 1907, Garrison discovered the eggs of *Echinostoma* spp. in five prisoners in Manila. Adult worms were recovered from one patient after treatment and were identified as *Echinostoma ilocanum*. Thereafter, several cases have been reported from many other countries.

Taxonomy

The name *Echinostoma* (*Echino*: spiny; *stoma*: mouth) is derived from *echino* meaning spiny and *stoma* meaning mouth due to the presence of characteristic spines around the oral suckers in the adult worm.

The genus *Echinostoma* belongs to the class: Trematoda; order: Echinostomida and family: Echinostomatidae; in the phylum: Platyhelminthes of the kingdom: Animalia. The genus *Echinostoma* has more than 56 species that cause infection in birds and mammals including humans. *Echinostoma revolutum* is the type species.

Human infections are caused by *Echinostoma trivolvis, Echinostoma hortense*, *Echinostoma echinatum, Echinostoma ilocanum, Echinostoma cinetorchis,* (= *lindoense*]) and *Echinostoma fujianensis.* Sporadic infections are also caused by members of other echinostomids

S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
https://doi.org/10.1007/978-981-16-7204-0_23

including *Echinoparyphium, Acanthoparyphium, Artyfechinostomum, Episthmium*, *Himasthla, Hypoderaeum* and *Isthmiophora*.

Genomics and Proteomics

The mitochondrial genome sequences of several *Echinostoma* species have been sequenced. For *E. revolutum*, the entire mitochondrial genome sequence was 15,714 bp in length, including 12 protein-coding genes, 22 transfer RNA genes, 2 ribosomal RNA genes and 1 non-coding region (NCR). It has a 61.73% A + T base content.

A total of 39 parasite proteins have been accurately identified in the excretory/secretory proteome of *Echinostoma caproni* adults. The metabolic enzymes, and particularly glycolytic enzymes, constitute the largest protein family in the excretory/secretory proteome of the adult worms. Moreover, representative proteins involved in parasite structure, response against stress, chaperones, calcium binding and signal transduction have also been identified.

The Parasite Morphology

Echinostoma spp. are distinguished by the presence or absence of the single or double crown of large circumoral spines on a disc surrounding the oral suckers. Identification of *Echinostoma* spp. is based on the position and number of suckers, sucker ratio, the form and arrangement of the reproductive organs, the shape of the gut, excretory vesicle and egg size.

Adult Worm

The length and width of adult *Echinostoma* spp. vary among different species. They usually measure 5–10 mm in length and 1–2 mm in width.

Adult *Echinostoma* is large, flattened, often broad and sometimes narrow. The adult has well-developed anterior oral sucker and a ventral sucker that are adjacent to each other (Fig. 1). The oral sucker is surrounded by head collar with spines. The number, size and arrangement of collar spines vary for each *Echinostoma* species (Fig. 2). Echinostomes possess one or two circumoral collar of spines which vary among different species from 27 to 51. *E. trivolvis* in North America has four 37 collar-spined species.

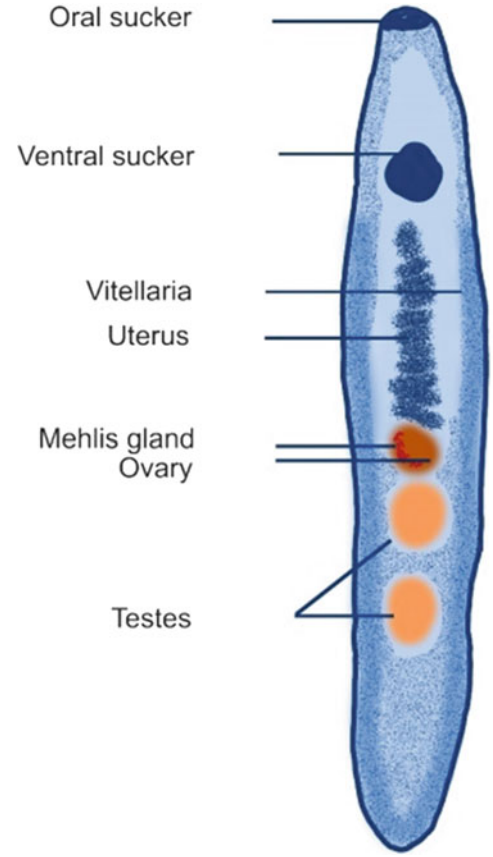

Fig. 1 Schematic diagram of adult *Echinostoma revolutum*

Oral sucker lies in the centre of the circumoral disc and the ventral sucker is present at the anterior portion of the body. The forebody is short. Tegument is armed with small spines; intestinal ceca is present with or without dendritic lateral branches. Two testes are present posterior to the ovary and are deeply lobed, the uterus is intercaecal and pre-ovarian. The vitellarium is follicular, and is present in two lateral fields,

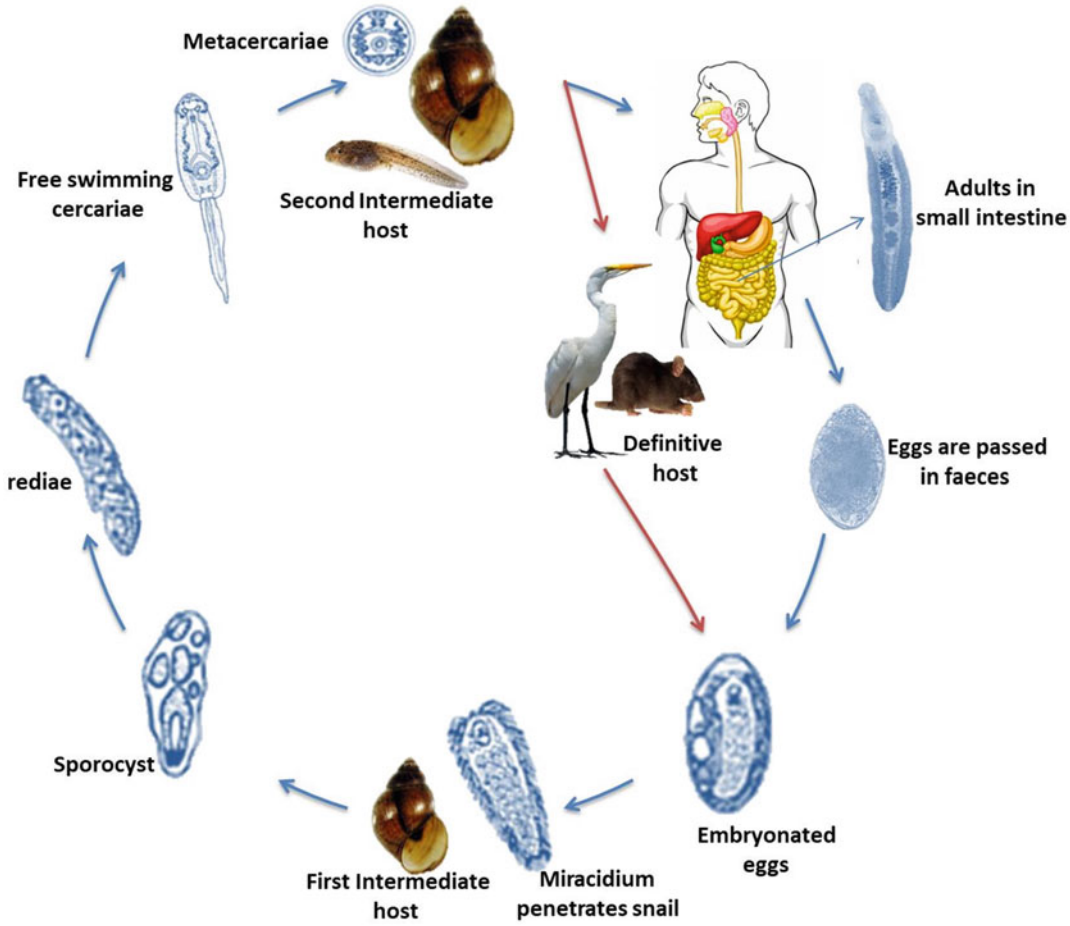

Fig. 2 Life cycle of *Echinostoma* spp.

usually in the hind body but may extend into the forebody. Alimentary canal consists of pharynx, oesophagus and an excretory pore. Intestinal coeca is present. *Echinostoma* spp. have both male and female reproductive organs (hermaphrodite) and are capable of self-fertilization.

Echinostoma malayanum adult has 43–45 collar spines, rounded posterior end, two large testes with 6–9 lobes arranged in tandem.

Eggs

Echinostoma spp. eggs are straw-coloured, thin-walled, operculated, large and ovoid. They measure 83–116 μm × 58–69 μm in size. They are non-embryonated when shed in the faeces of definitive hosts.

Metacercariae

In the second intermediate hosts, the cercaria encysts to become metacercaria, which is the infective stage of the parasite.

Cultivation of Parasites

Locke's solution, medium 199 and RPMI 1640 have been employed as media for growth and development of parasite. The highest encystment and normal metacercariae development have been demonstrated in the RPMI 1640 medium. The addition of fetal bovine serum to RPMI 1640 increases the level of encystment and normal metacercariae development. The 0.5× media induced higher encystment and normal

metacercaria formation than the 1× media. Encystment was highest in the mixture of 0.5× RPMI 1640 and 10% fetal bovine serum.

Laboratory Animals

In the laboratory, life cycle has been studied using Lymnaea and Radix snails as the first intermediate hosts, tadpoles as the second hosts and rats as the definitive hosts.

In experimental infection of mice with adult *E. caproni*, mesenteric lymph node, during the first 3 weeks of infection, produced IFN-γ and to a lesser extent IL-4. IL-5 levels were elevated throughout the period studied. The humoral response was consistent with a Th1 cytokine pattern showing elevated antigen-specific IgG2a antibodies.

Life Cycle of *Echinostoma* spp.

Hosts

Echinostoma is a digenean trematode parasite that invades the intestines and bile duct of the definitive hosts.

Definitive Hosts

Aquatic birds, carnivores, rodents and humans are the definitive hosts. Echinostomes dwell in the intestine and bile ducts of hosts like birds, mammals and humans.

Intermediate Hosts

Snail species such as *Lymnaea* spp., bivalve molluscs, planorbids, lymnaeids and bulinids. Fish and tadpoles may also act as intermediate hosts.

Infective Stage

Metacercaria is the stage infective for definitive hosts.

Transmission of Infection

Humans acquire infection by ingestion of metacercariae which are found encysted in freshwater snails, tadpoles or fish (Fig. 2). Humans and animals get infected by eating secondary hosts which are infected with *Echinostoma* metacercariae. The metacercariae excyst in the jejunum or ileum of the infected host and is influenced by intestinal pH, temperature or bile salt concentration. After excystation, the young flukes using the spiny tegument, large suckers and collar spines attach to the wall of the lower small intestine. Spines along with the large oral and ventral suckers cause damage to the intestinal mucosa leading to the inflammation and ulceration. Adult worms begin to lay unembryonated eggs that are excreted in the faeces.

Echinostoma eggs in coming in contact with water in ponds, streams and lakes, become embryonated, and develop in 2–3 weeks at 22 °C to miracidia, which are free-swimming larva of *Echinostoma*. The miracidium is active free-swimming larva of *Echinostoma* that penetrates and infects snails of *Lymnaea* spp., the first intermediate host of the parasite. In these snail hosts, the miracidium undergoes asexual reproduction for several weeks, undergoes development into sporocyst, rediae and cercariae. Miracidia enters the snail and develops to mother sporocysts in the heart of the snail. The germinal cells of the sporocyst develop into mother rediae. Mother rediae undergo asexual reproduction and produce daughter rediae, which in the digestive gland of the snail develop further into cercaria. The cercariae emerge and infect the snails, frogs, tadpoles and fish, the second intermediate hosts, where they encyst to become metacercariae, the infective stage of the parasite. Snails of the genera *Pila* and *Corbicula* are important as they are eaten raw. A freshwater fish is also a suitable host.

Pathogenesis and Pathology

Echinostoma spp. are not highly pathogenic. Pathogenic lesions of greater severity occur with high parasite load. The worms invade the gut wall

by their oral suckers and cause damage to the intestinal mucosa, resulting in inflammation and ulceration. Heavy *Echinostoma* spp. infections cause ulceration of the bowel, diarrhoea and abdominal pain. General intoxication occurs due to absorption of metabolites of the worms.

Decrease in villus/crypt ratio, villous atrophy, crypt hyperplasia and inflammation of the stroma are the typical pathology of the intestinal tract in infected animals. *E. hortense* infection in rats is characterized by villus atrophy and crypt hyperplasia. Severe enteritis is observed in fowls infected by *E. revolutum* and *Himasthla conoideum*.

Immunology

IgA, IgG, IgM antibodies are detected in sera of mice and golden hamsters infected with *E. caproni* and *E. trivolis*. Antibodies were detected within a few weeks of infection in mice, whereas in hamsters it took several weeks to produce a comparable response. Hence, it is rather difficult to predict the kinetics of humoral response in an echinostome infection in humans.

Infection in Humans

Eechinostomiasis in humans has a prolonged latent phase, a short acute phase and is usually asymptomatic.

The spectrum of clinical manifestations in symptomatic cases correlate with the worm load; the greater the worm burden, more serious is the disease. A mild infection is usually asymptomatic. In light to moderate infections, anaemia, headache, dizziness, passage of loose stools, tiredness and weight loss are present. Heavy infection is characterized by severe abdominal pain associated with ulcerative lesions in the duodenum, leading to haemorrhagic enteritis and general intoxication.

Infection in Animals

Light infections do not cause significant pathology or symptoms in animals, but heavy infections are associated with serious pathology, disease and death. Haemorrhagic diarrhoea, progressive emaciation, weakness in flight and death have been demonstrated in fowls. The flukes feed on the tissues with their oral suckers by inserting their anterior ends deep between the villi. This results in desquamation of the epithelium and the villi, and marked cellular reaction with oedema and thickening of the mucosa. *E. hortense* infection in rats is associated with histopathological changes in the intestines showing villous atrophy, crypt hyperplasia and stromal inflammation.

Epidemiology and Public Health

Human echinostomiasis is seen most frequently in countries where the habit of eating raw or undercooked freshwater snails, clams, fish or amphibians is seen. Population, pollution, lack of sanitation and poverty are contributory factors for echinostomiasis. In the Far East, echinostomiasis is seen. The human infection in Taiwan is estimated as 2.8–6.5%. In northern Thailand, *H. conoideum* is a trematode parasite of fowls causing infection among people having the habit of eating raw snails (Table 1).

Echinostoma spp. infections in wildlife and domestic animals are prevalent worldwide. They are most common in South Korea, Thailand, Philippines, Japan, Singapore, Indonesia, India, Romania, Hungary, Italy and in a few European countries. *E. hortense* and *E. revolutum* sensu lato are most common species that cause infections in mammalian (rat, dog) and avian hosts, respectively. *E. malayanum* infection is found in the Philippines, India, Malaysia, Indonesia (Sumatra), Thailand, and Singapore infecting dogs, mongooses, pigs, cats and rats. *E. ilocanum* is distributed in the Philippines, Indonesia (Java and Sulawesi), parts of southern China, India, and Thailand infecting dogs, murid rodents and cats. *E. hortense* is found in Japan and in the Republic of Korea. *E. trivolvis* is distributed in North America, which infects 26 fowl species and 13 mammalian species. *E. echinatum* causes infection among anseriform fowls in Indonesia, Brazil, India, Malaysia and the Philippines. *E. revolutum* is found in the Far East and Europe, infecting geese and ducks.

Table 1 Human Echinostomiasis and their geographical distributions

Echinostoma species	Transmission to humans	Prevalence
Echinostoma revolutum	Snails, frogs	Southeast Asia, Australia, Egypt, Russia
Echinostoma ilocanum	Snails	East and Southeast Asia including India
Echinostoma hortensae	Freshwater fish	China, Japan
Echinostoma echinatum	Mussels	Europe, South America, Japan, Southeast Asia

Diagnosis

Microscopy

Demonstration of *Echinostoma* spp. eggs and adult by stool microscopy confirms diagnosis of echinostomiasis. Kato-Katz procedure is frequently used to demonstrate unembryonated eggs that are operculate, ellipsoidal and yellow to yellow-brown in colour (Fig. 3). *Echinostoma* eggs appear to be morphologically similar to the eggs of *Fasciola*, *Fasciolopsis* and *Gastrodiscoides*. Hence definitive diagnosis of genus- and species-level identification of *Echinostoma* spp. is based on the detection and identification of *Echinostoma* adults excreted in the faeces (Table 2).

Examination of echinostome metacercariae in the second intermediate host, mainly in fish, is a frequently used method to detect infection in fish for the purpose of control. These include: (1) muscle compression method, in which sample from different parts of the fish (e.g. muscle, gill, head, intestine, fin, scale) or any other host is compressed between two glass slides and examined under the stereomicroscope for the presence of echinostome metacercariae and (2) pepsin-HCl artificial digestion method. In this method, small pieces of flesh are minced and mixed in a beaker with artificial gastric juice and incubated at 37 °C for 2 h with stirring in between. This is followed by filtration (1 × 1 mm of mesh) to remove larger particles, and addition of 0.85% saline and then allowed to stand for a while. The supernatant is then discarded and the sediment kept. This procedure is repeated till the supernatant becomes clear. Finally, the sediment is collected in a Petri dish containing physiological saline and examined under the stereomicroscope for the presence of metacercariae.

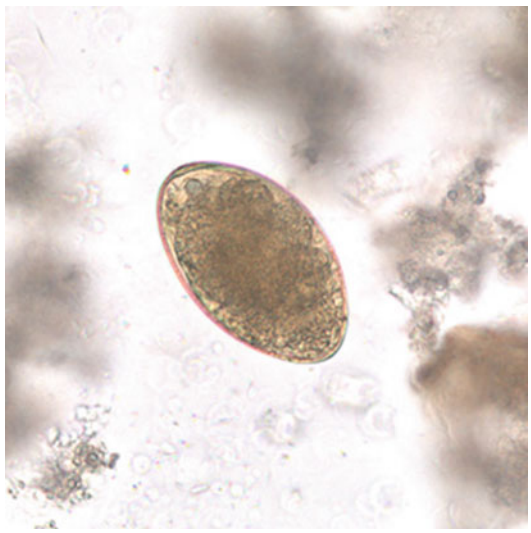

Fig. 3 Echinostomid egg in an unstained wet mount of stool. Image taken at 400× magnification (Courtesy: DPDx, CDC)

Serodiagnosis

IgM, IgG ELISA is an antibody-based method frequently used for serodiagnosis of echinostomiasis in humans.

Molecular Diagnosis

DNA sequencing, over the last few years, was used to differentiate 37 collar-spined species of Echinostomes. Currently, molecular techniques have been increasingly used for species identification, phylogenetic studies and systematic studies of *Echinostoma* spp. However, PCR for detecting DNA of echinostomes in human faecal sample is yet to be developed.

Other Methods

Counting chromosomes and comparing iso-enzyme patterns were used earlier for detection and identification of Echinostomes.

Table 2 Laboratory diagnosis of human echinostomiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Kato-Katz technique Biopsy	Stool or duodenal contents Larval sections in tissues/fluids	Gold standard test Drawback: Invasive and insensitive
Immunodiagnostics	Antigen detection (Sandwich ELISA)		Confirms active infection
	Antibody (ELISA)	Ig M; Ig G antibodies	Good sensitivity and specificity
Molecular assays	PCR, DNA sequencing	The internal transcribed spacers (ITS-1 or ITS-2) of nuclear ribosomal DNA (rDNA), cox1, Rn1 and nad2 genes	High sensitivity and specificity *Limitations:* Require skilled personnel

Treatment

Praziquantel given in a single oral regimen of 10–20 mg/kg is the drug of choice. Mebendazole, albendazole, niclosamide, tetrachloroethylene, etc. are the other drugs that have been evaluated for treatment of infection in humans and animals. Tetrachloroethylene given in a dose is 0.1 ml/kg body weight (maximum adult dose is 5 ml) is effective. Nausea, abdominal pain, headaches or dizziness are the noted side effects.

Tetrachloroethylene and carbon tetrachloride have been used successfully to treat intestinal trematodes of birds and mammals. Nevertheless, relatively less toxic and more effective compounds such as niclosamide, oxyclozanide, rafoxanide or praziquantel have been recommended for treatment of infection in animals.

Prevention and Control

Avoiding consumption of raw or undercooked meat of molluscs, fish, crustaceans and amphibians is essential to prevent human infection. Treatment of infected people and livestock, appropriate changes in diet and food preparation and implementation of community-based education campaigns are useful control measures. Examination of echinostome metacercariae in the second intermediate host, mainly in fish, is a good alternative for identification of infected fish and effective control.

Case Study

A 40-year-old fisherman presented with complaints of fever and loose stools associated with blood of 5 days duration. It was associated with abdominal cramps and nausea. On clinical examination, patient was febrile without any evidence of dehydration. Abdominal system examination revealed diffuse tenderness and no organomegaly. With clinical diagnosis of infective diarrhoea, stool sample was collected and sent to microbiology laboratory for microscopy and culture. Peripheral blood examination showed eosinophilia. On stool culture, faecal coliforms were grown. On stool wet mount, eggs of *Echinostoma* species were seen. The patient was then treated with praziquantel and improved symptomatically.

1. What are the food items that can transmit this infection?
2. What precautions one should take to prevent echinostomiasis?
3. What are the other parasites which can infect humans in a similar manner?

Research Questions

1. What antigens can be used to develop effective serological test for echinostomiasis?
2. What tests can be used in field conditions?
3. How to examine the fish and snail population in a region is necessary to find out the infection risk from *Echinostoma* in that area?

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Dicrocoeliasias

V. Samuel Raj, Ramendra Pati Pandey, Rahul Kunwar Singh, and Tribhuvan Mohan Mohaptara

Learning Objectives

1. To gain knowledge of a rare parasite that can cause hepatic manifestations.
2. To distinguish between true and spurious infections by eliciting proper history and careful examination of eggs.

Introduction

Dicrocoeliasis is a disease of ruminants (e.g. cattle, goats, sheep, deer) including wild ruminants (camelids in South America and yaks and buffalos in India) caused by *Dicrocoelium* Spp. The animals suffering from dicrocoeliasis reveal iron deficiency, leanness and in severe cases, cirrhosis, scarring of the liver surface, and occlusion of nerve channels. *Dicrocoelium* spp. infection in humans usually is not fatal, unless the infection of the liver is very severe.

History

Rudolphi in 1819 discovered the parasite *Dicrocoelium dendriticum*. The full life cycle was described by Krull and Mapes during 1951–1953. The discovery that snail is the first intermediate host was followed by the finding that the slime of the snail can potentially transmit the parasite. The ant, *Formica fusca* was later found to be the intermediate host infecting sheep.

Taxonomy

The genus *Dicrocoelium* belongs to the family: Dicrocoeliidae; order: Plagiorchiida in the phylum: Platyhelminthes. *D. dendriticum* and *Dicrocoelium hospes* are two important species that cause infections in humans and animals.

Genomics and Proteomics

D. dendriticum genome is 548 Mb in size and GC content is 47%. The mitochondrial genome is of 14,884 bp; sequencing of partial 18S rDNA, ITS-1 and ITS-2 has been primarily done for species differentiation. Proteomic analysis of

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major tegument and excretory-secretory products identified a total of 29 proteins in the excretion-secretion products and 43 proteins in the teguments, many of which were antigenic. These proteins are involved in various activities of the parasite like metabolism, detoxification, and transport or as structural molecules. A 25–27 kDa polypeptide has been identified as immunodominant protein, of potential use in the diagnosis of dicrocoeliasis and for inducing protective immunity against *Dicrocoelium* infection.

The Parasite Morphology

Adult Worm

D. dendricitum has a fixed, lancet-shaped, tapered and flattened transparent body (Fig. 1). The body measures 6–10 mm, and 1.5–2.5 mm in width and morphologically very similar to that of *Clonorchis sinensis.* The body characteristically tapers at both the anterior and posterior ends. They have two prominent suckers, the oral sucker and the ventral sucker, both on the anterior surface. *D. dendriticum* is distinguished by the presence of lobed testes in the anterior surface of the body. The uterus lies posteriorly, and the vitelline glands are found in the midsection of the worm and take part in egg production.

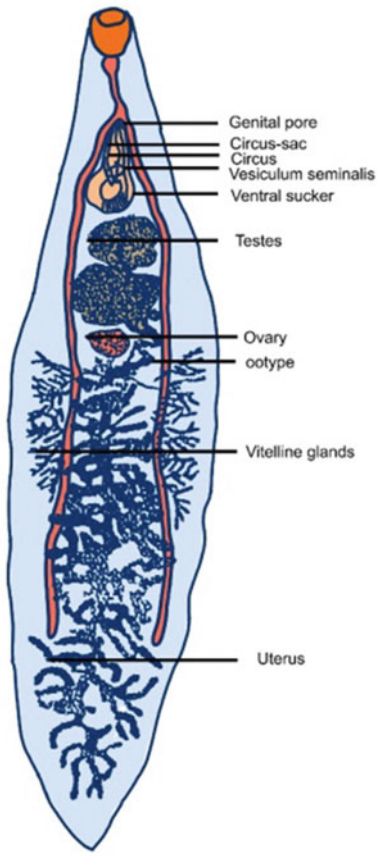

Fig. 1 Schematic diagram of adult *Dicrocoelium dendriticum*

Eggs

The eggs have a conspicuous operculum measuring 36–45 μm in length and 20–30 μm in width. They are thick-shelled, brownish in colour and are embryonated when laid.

Cultivation of Parasites

The adults can be maintained in RPMI 1640 medium, pH 7.4 at a temperature of 37 °C. The adults remain viable in this medium for prolonged periods and continuously shed eggs up to 4 days.

Laboratory Animals

Golden hamster is used as an experimental animal model in which the parasites produce an active infection showing histopathological changes in the liver. Laboratory weaned sheep is also used as experimental model. In experimental infections in hamsters, parasite-initiated changes showed channel multiplication and broadening, invasion by lymphocytes, macrophages and eosinophils resulting in liver necrosis.

Life Cycle of *Dicrocoelium dendriticum*

Hosts

Definitive Host

Ruminants like cattle, goat, sheep and other animals like pigs or cervids. Humans are the accidental hosts.

Intermediate Hosts

First Intermediate Host – Land snails.

Second Intermediate Host – Ants (*Formica fusca*).

Infective Stage

Metacercaria present in the infected ants is the infective stage of the parasite.

Transmission of Infection

The infection begins when a snail, the first intermediate host, ingests the embryonated eggs of *D. dendriticum* (Fig. 2). The egg hatches to the miracidium in the snail's intestine. The miracidium passes through the intestinal wall and reaches the digestive gland, in which it is transformed into mother sporocyst, subsequently daughter sporocysts and finally the cercaria with tail. The cercariae gather on the body surface and the mantle cavity of snails and are surrounded by a mucus or slime coat. The slime balls that may contain up to 500 cercariae are shed by the snails and are ingested by the ant.

Ants acquire the infection by ingesting the slime balls of the snails. Inside the ant the cercariae mature into metacercariae. The metacercaria encyst in haemocoel, and more than 100 metacercaria may be found in a single ant. Definitive hosts acquire the infection by ingestion of ants in the pasture. On ingestion, the metacercariae excyst in the duodenum. Bile appears to act as an attractant for the metacercaria, the larval form travels to the bile duct and eventually to the liver where it matures into the adult worm. In 6–7 weeks, the fluke attains maturity and starts producing eggs after another 1 month.

Pathogenesis and Pathology

In animals, the larval form of *Dicrocoelium* rarely causes any damage in the intestines. The pathological changes in the liver depend on worm load and extent of injury caused by these worms. In the infected host, the biliary pathology includes inflammation of bile duct, hepatic fibrosis and degeneration of hepatocytes. The pathological changes in ruminants in dicrocoeliasis may sometimes be overshadowed by concurrent liver infections.

Dicrocoelium infections in humans are confined to the distal parts of the bile ducts that result in minor symptoms.

Immunology

The role of host immunity is unclear in *D. dendriticum* infection, in part due to the high interspecific variation and also to the nature of parasite-induced effects. The humoral immune reactions have been observed in sheep infected with a large number of *D. dendriticum* parasites.

Infection in Humans

Most *D. dendriticum* infections caused by a few numbers of flukes are asymptomatic and not associated with any overt clinical symptoms. In symptomatic cases, the clinical symptoms are mostly similar to that of fascioliasis. The clinical manifestations in severe infections include eosinophilia, cholecystitis, liver abscesses, diarrhoea and generalized gastrointestinal/abdominal distress. Consuming raw or undercooked liver of animals suffering from dicrocoeliasis may lead to a spurious infection in humans, in which the eggs of the parasite may be found in the stool of the infected hosts.

Infection in Animals

Most *D. dendriticum* infections in animals, particularly in bovines, are asymptomatic. Few infected animals show iron deficiency, weight loss, and liver cirrhosis. Sheep infected with *D. dendriticum* are often co-infected with other parasites (e.g. gastrointestinal and bronchopulmonary nematodes) making it quite difficult to identify the specific outcomes of each

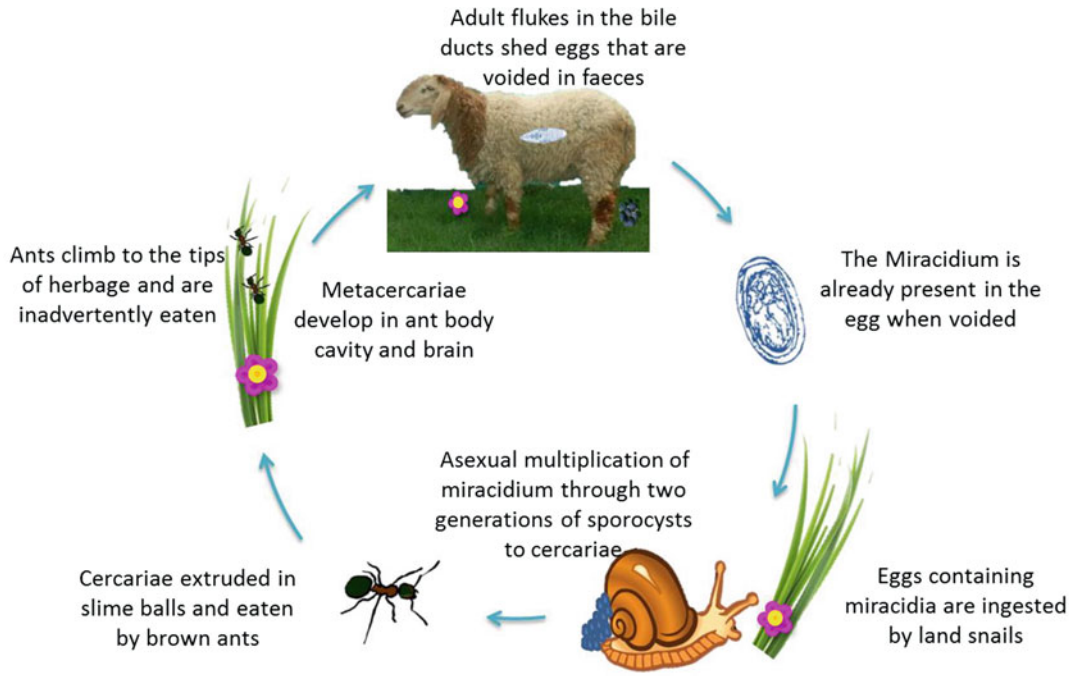

Fig. 2 Life cycle of *Dicrocoelium dendriticum*

individual parasitosis. Liver abscesses, granulomas and fibrosis, as well as bile duct proliferation have also been described in the New World camelids. Occasionally, *Dicrocoelium* spp. can also infect rabbits, pigs, dogs and horses.

Epidemiology and Public Health

Dicrocoeliasis is endemic or apparently endemic in 30 countries across the world. *D. dendriticum* is distributed in the countries of Asia, Europe, Australia, Africa, North America, and South America. The majority of the reports of dicrocoeliasis have originated from North Africa and Middle East (Table 1). *D. hospes* cases have been reported in Africa. The parasite is commonly found in regions with dry, chalky and alkaline soils that favour the sustenance and life of intermediate hosts. Forests inhabited by snail population or dry pastures with limited biodiversity with increased colony of the ants increase the prevalence of dicrocoeliasis.

Diagnosis

Diagnosis of dicrocoeliasis in humans is primarily based on detection of eggs in the stool by microscopy. Serological tests are used, primarily for diagnosis of infection in animals (Table 2).

Microscopy

Diagnosis of dicrocoeliasis depends on finding the eggs of *D. dendriticum* eggs in the faeces of humans or animals. The eggs are oval, asymmetrical and operculated about 35–50 μm size with a brownish thick shell (Fig. 3). The embryonated eggs are surrounded by a uniform dark brown shell and contain a ciliated embryo inside. Demonstration of embryonated eggs or transit eggs in the stool by microscopy helps in the diagnosis of true infection and spurious infection, respectively.

Serodiagnosis

ELISA, Western blot and other tests using the excretory-secretory protein antigens have been evaluated for diagnosis of dicrocoeliasis in

Table 1 Epidemiology of dicrocoeliasis

Species	Definitive host	First intermediate host	Second intermediate host	Geographical distribution
Dicrocoelium dendriticum	Domesticated and wildlife ruminants, pigs	Land snails: *Gastropoda*, *Cionella lubrica*	Ants: *Formica fusca, Lasius* spp.	Russia, Europe, Asia, North Africa
Dicrocoelium hospes	Cattle	Snails: *Limicolaria* spp. or *Achatina* spp.	Ants: *Dorylus* spp. or *Cematogaster* spp.	Africa

animals. The specificity and sensitivity of ELISA have been shown to be 95% and 94% and are found more useful compared to eggs count of faeces by microscopy in diagnosis of dicrocoeliasis. The serum in infected animals becomes positive for antibodies almost 1 month before the appearance of eggs in the stool. Serological tests for diagnosis of dicrocoeliasis in humans are yet to be evaluated.

Molecular Diagnosis

PCR using a fragment, approximately 963 bp, of the 28S rDNA marker has been evaluated in a study for specific identification of *D. dendriticum* adult worm.

Treatment

The treatment of human dicrocoeliasis is yet to be standardized because of rarity of the cases. Oral praziquantel at 25 mg/kg, three times on a single day has been found to be effective. Treatment with triclabendazole has also shown to be equally useful. Treatment by albendazole at 15–20 mg/kg in a single dose or two doses of 7.5 mg/kg on successive days, or netobimin at 20 mg/kg, is effective for both cattle and sheep.

Prevention and Control

Control of the snail population is most important in prevention of dicrocoeliasis in animals. The land snails need humidity for development and survival, hence good drainage system to reduce the humidity and maintenance of dry grazing areas can decrease the snail population. Chemical control of snails with copper sulphate or sodium pentachlorophenate has been tried to control the snail population in isolated water bodies such as small ponds, or water holes where the animals regularly assemble for drinking. However, this measure is impractical in large pastures or grazing areas. Cleaned pastures can become re-infested very fast and there is associated problem of ecological hazards. Similarly, control of ant population with insecticides is not advisable. In 2007, the World Health Organization (WHO) has included *D. dendriticum* in the list of pathogens, in the Foodborne Disease Burden Epidemiology Reference Group.

Case study

A 52-year-old man presented with chronic diarrhoea for 3 months and weight loss. He had five to

Table 2 Laboratory diagnosis of dicrocoeliasis

Diagnostic approach	Methods	Targets	Remarks
Microscopy	Stool microscopy	Eggs	Embryonated eggs signify true infection. Should be distinguished from spurious infection
Immunodiagnostics	ELISA	Antibody against excretory-secretory proteins	Available for animals. High sensitivity and specificity
Molecular methods	PCR	28S rDNA	Identification of adult worm

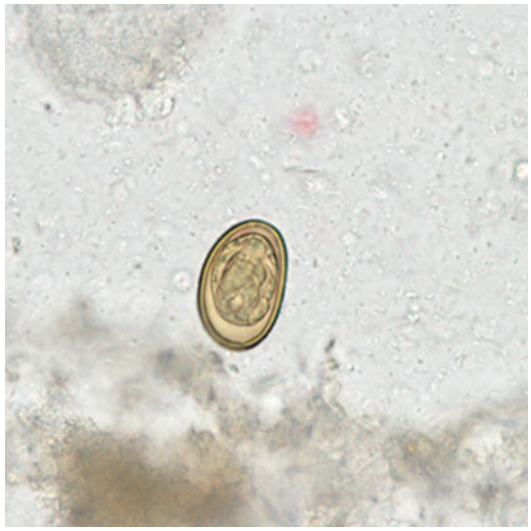

Fig. 3 Egg of *Dicrocoelium* in unstained wet mount of stool (Courtesy DPDx, CDC)

ten loose, non-bloody bowel movement per day, accompanied by lower abdominal and perianal pain. He admitted eating beef liver several times. The stool examination for ova and parasites revealed *D. dendriticum* eggs. He was asked to stop eating liver items of any animal. Four weeks later, it was reviewed and he felt much better and gained weight. This illustrates a case of pseudo-infection where beef liver containing the adult worm with eggs were entering the body and not the infective metacercarial form present in ants. The eggs are released in the intestine and are passively shed with stool giving the appearance of infection.

1. What are the various methods for diagnosis of dicrocoeliasis?
2. What behaviour of the ants helps in transmitting the parasite to the grazing animals?
3. What are the various parasitic diseases for which snails act as intermediate hosts?

Research Questions

1. What is the optimum treatment regimen for dicrocoeliasis?
2. What is the best method for controlling snail population to prevent the spread of dicrocoelia infection?
3. Is it possible to utilize the animal serodiagnostic kit for human infections by *Dicrocoelium* spp.?

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Opisthorchiasis

S. Pramodhini and Tapashi Ghosh

Learning Objectives

1. To understand the importance of fish as a source of infection of various trematode and other parasitic infections.
2. To list the areas of endemicity for this parasite.
3. To suggest about the preventive measures to be adopted for fish consumption.

Introduction

Opisthorchiasis is an infection caused by *Opisthorchis viverrini*, the Southeast Asian liver fluke or *Opisthorchis felineus* also known as cat liver fluke. The infection is acquired by ingestion of infected raw or undercooked fish, crabs, or crayfish harbouring the metacercariae which is the infective stage of the fluke. Most cases of opisthorchiasis in humans are asymptomatic or are subclinical. The liver, gallbladder, and bile duct are the most common sites infected by liver flukes in humans. Cholangitis, cholecystitis, and cholangiocarcinoma (CCA) are some of the rare manifestations of this infection. Direct demonstration of *Opisthorchis* spp. eggs or the adult worm in stool confirms the diagnosis of opisthorchiasis. Praziquantel is the drug of choice for treatment of opisthorchiasis.

History

In 1884, Sebastiano Rivolta, an Italian scientist, described the liver fluke in the liver of a cat and a dog and named it as *O. felineus*. K.N. Vinogradov at the Tomsk University first demonstrated the fluke in the human liver in the year 1891. He also suggested the mollusc *Bithynia leachii* as the first intermediate host of the fluke, which was subsequently proven experimentally by Vogel in Germany. Many human cases of opisthorchiasis were reported during 1892–1929 from Tomsk, Biysk, Novosibirsk, Tyumen Oblast, and various other places. An autopsy of a Russian soldier from Siberia, who died during World War II, demonstrated nearly 42,000 liver flukes in his liver and pancreas. Brown, in 1893, first suggested fish as the source of liver fluke infection, which was experimentally proved by Askanazy in 1904. Both Plotnikov and Zerchaninov demonstrated and identified the larvae of liver fluke in the muscle of fish in 1932. In

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1973, autopsy done on the liver of a cat demonstrated eight cysts containing liver flukes in the erstwhile USSR.

Taxonomy

The genus *Opisthorchis* belongs to family: Opisthorchiidae; order: Plagiorchiida; class: Rhabditophora and phylum: Platyhelminthes. The genus *Opisthorchis* includes *Opisthorchis chabaudi, O. felineus, Opisthorchis gomtii, Opisthorchis parasiluri*, and *Opisthorchis viverrini* that cause infections in a variety of mammals including humans.

Genomics and Proteomics

The nuclear genome of *O. felineus* liver fluke has 684 million base pairs and 30.3% of the genome is representative of repeating elements, mainly retro-transposons. The genome of *O. felineus* resembles those of *O. viverrini* and *Clonorchis sinensis* in the family Opisthorchiidae. O. *felineus* genome contains 11,455 annotated protein-coding genes and 55 genes encoding microRNAs. The total number of *O. felineus* genes approximates the number of genes present in *Schistosoma mansoni* and *Fasciola hepatica,* but nearly one-third less than that of *O. viverrini* and *C. sinensis.* Four genes (GRN-1–GRN-4) that encode single-domain granulins and one multi-domain progranulin (PGRN) gene have been demonstrated in *O. felineus*, as well as in *O. viverrini* and *C. sinensis*.

Highest expression for genes encoding proteases, myoglobin, egg shell protein, glutathione S-transferase, and also proteins modulating antigen processing by the host immune cells have been demonstrated in adult worms of the fluke. Opisthorchiid metacercariae show high level of expression of 'housekeeping genes' encoding for proteins like ribosomal proteins, ubiquitin, and heat shock. Excretory secretory products (ESPs) of *O. felineus* express various protective proteins against reactive oxygen species, associated with proteolytic enzymes, carbohydrate metabolism enzymes, and protective proteins against the host immune system.

The Parasite Morphology

Adult Worm

Adult *Opisthorchis* spp. infecting humans measure 7–12 mm in length, and are slightly smaller than the adult worms isolated from the feline hosts. They have two testes lying one behind the other in the posterior end of the body. The ovary is situated anterior to the testes with a uterus coiled between the ovary and ventral sucker. In definitive hosts, adult worms are found in the bile ducts (Figs. 1 and 2).

Metacerceria

The opisthorchiid metacercariae are elliptical in shape and measure around 0.19–0.25 × 0.15–0.22 mm in size (Fig. 3). The oral and ventral suckers present in the metacercariae are equal in size. Part of its

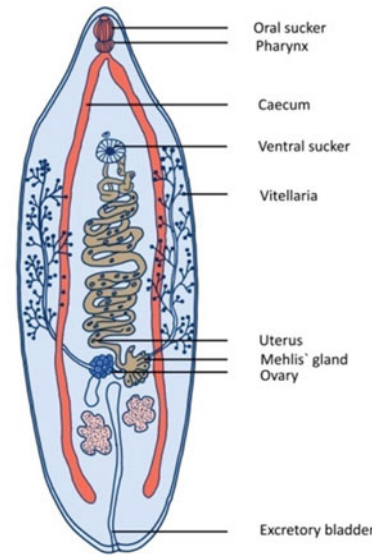

Fig. 1 Schematic diagram of adult worm of *Opisthorchis felineus*

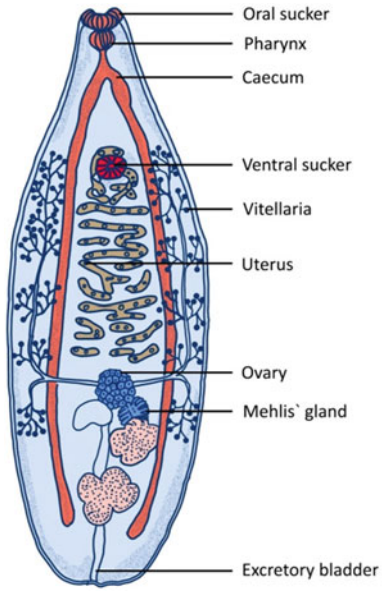

Fig. 2 Schematic diagram of adult worm of *Opisthorchis viverrini*

posterior body is largely occupied by a circular excretory bladder which is filled with black excretory granules. Though morphologically similar, the size of cyst and thickness of cyst wall are used as reference points for differentiating metacercariae of *Opisthorchis* species.

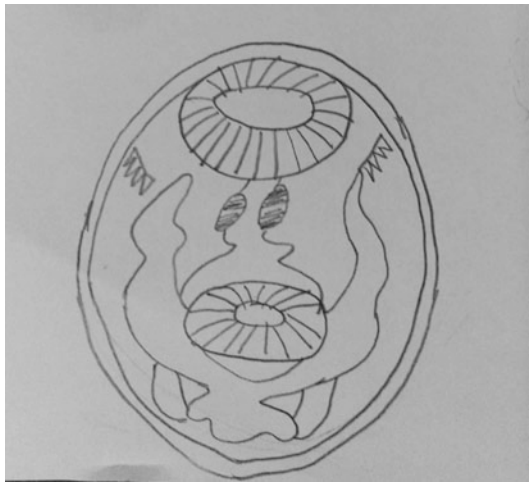

Fig. 3 Opisthorchiid metacercariae

Egg

Eggs of *Opisthorchis* spp. are operculated and measure 19–30 μm in length and 10–20 μm in width. They have prominent opercular 'shoulders' and abopercular knob. The eggs excreted in the faeces are embryonated (Fig. 4).

Cultivation of Parasites

The adult worms of *O. viverrini* remain active metabolically for 7–10 days in Earle's basal medium. The medium supplemented with 5% normal human bile or 1% normal human or hamster sera has been used to maintain the adult worm metabolically active for more extended period of time.

Laboratory Animals

Syrian hamsters and gerbils were used to study the pathogenesis of *O. viverrini* infection. Gerbils were found more suitable to study susceptibility to infection and study the pathogenesis of cholangiocarcinoma of opisthorchiasis.

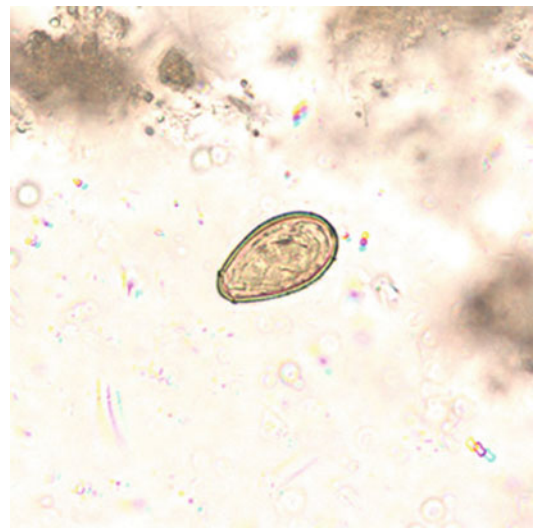

Fig. 4 Egg of *Opisthorchis viverrini* in an unstained wet mount of concentrated stool. Image taken at 400× magnification (CDC)

Life Cycle of *Opisthorchis* spp.

Hosts

Definitive Hosts

Cats, dogs, and many fish-eating mammals as well as humans are the definitive hosts.

Intermediate Hosts

The parasite completes its life cycle in two intermediate hosts.

First intermediate host: Freshwater snail of the genus *Bithynia.*

Second intermediate hosts: Freshwater fish (Cyprinidae fish).

Transmission of Infection

The humans and other definitive hosts acquire the infection by ingestion of raw or undercooked freshwater fish containing infectious metacercariae (encysted stage) (Fig. 5). Further excystation of metacercariae occurs in the duodenum, which then ascends to the biliary ducts through the ampulla of Vater. In the host biliary and pancreatic ducts, it develops into adult worm by attaching to the mucosa. They start laying eggs after 3–4 weeks of infection, and are excreted in their faeces.

Embryonated eggs of the parasites are passed through the faeces of an infected mammal and enter the water habitat of the first intermediate host, a freshwater snail of the genus *Bithynia*. Inside their snail host, the eggs hatch in the alimentary canal of the snail into miracidia. The miracidia penetrate the intestine and develop into sporocysts. The sporocysts develop into rediae and mature into 4–50 pleurolophocercus cercariae in the digestive gland (hepatopancreas) of the snail. An infected snail may release 500–5000 cercariae in the water every day.

The cercariae leave the snail and in the water can survive up to 24 h at temperatures 12–27 °C. They then enter a freshwater fish of *Cyprinoid* family, the second intermediate host. The cercariae, after contact with the fish, penetrate under the scales, lose their tails and encyst, mainly in the muscles, subcutaneous tissues, and to a lesser degree in the fins and gills. The cercaria transforms into metacercariae in about 5–6 weeks' time and is infective for the definitive host.

Pathogenesis and Pathology

Opisthorchis spp. cause the disease primarily by causing trauma as well as by producing toxins.

Opisthorchis infection is associated with cholangitis, cholecystitis, periductal fibrosis, and cholangio-carcinoma (CCA). As there is no tissue migration phase and these flukes reside in the intrahepatic bile ducts, their metabolic (excretory and secretory) products stimulate and produce chronic inflammation of biliary epithelium, resulting in oxidative and nitrative DNA damage of the biliary epithelium. Proinflammatory cytokine/chemokines, especially IL-6 and IL-8 activate biliary TLR4 produce inflammatory changes of the infected bile ducts. Elevation of IL-6 is associated with advanced periductal fibrosis in infected hosts. IL-6 induces further anti-apoptosis, cell transformation, and eventually malignancy. These stimulated cells hyperproliferate, thereby inflamed epithelial cells become neoplastic and may ultimately end up with opisthorchiasis-associated cholangiocarcinoma.

Hyperplasia of the epithelial cells lining the bile ducts is the main pathological lesion in opisthorchiasis. Biliary tract obstruction, biliary fluid retention, severe hyperplasia of the biliary system are the other pathological changes in severe infections. Glandular proliferation, either papillomatous or adenomatous, cholangitis, eosinophilic periductal infiltration, necrosis, and atrophy of hepatic cells are also characteristic. The bile ducts initially get dilated, may become saccular or cystic, eventually ending in large cysts, during the course of infection. The liver profile, in contrast, appears normal.

Pathogenesis of CCA in opisthorchiasis is multifactorial including the mechanical damage, parasite secretions, and immune-mediated pathology. Epithelial desquamation occurs due to mechanical irritation caused by the adult worm and its metabolic products. Other predisposing lesions, such as periductal fibrosis, epithelial

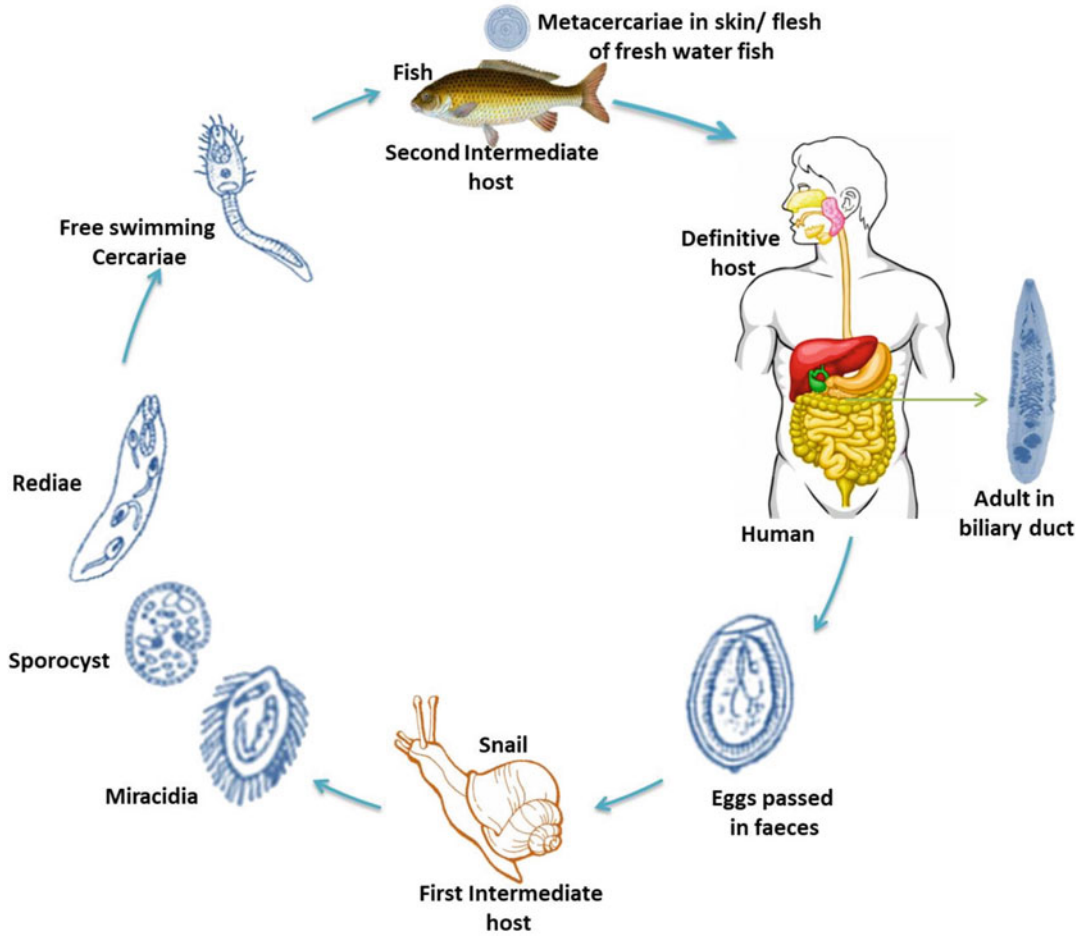

Fig. 5 Life cycle of *Opisthorchis* spp.

hyperplasia, goblet cell metaplasia, and adenomatous hyperplasia, increase the susceptibility of DNA to carcinogens, thereby increasing the possibility of cell transformation, leading to CCA.

Immunology

Evidence of persistence of liver fluke infection for decades in the infected host suggests that neither mucosal nor tissue immune responses protect or cause death of the parasite. Specific immune mechanisms that promote persistence of parasite in the infected host have been suggested. Immune response of the host against the parasite and parasite products contributes to the pathogenesis of the biliary epithelium fibrosis and even carcinogenic changes. Serum IgG levels were found to be increased in patient infested with opisthorchiasis and were found to be associated with gall bladder abnormalities.

Infection in Humans

The majority cases of human opisthorchiasis are asymptomatic. Acute opisthorchiasis commonly presents as acute abdominal pain in the right upper quadrant, associated with signs and symptoms of cholestasis. In addition, *O. felineus* infections, as documented in Italy, may also

present as febrile eosinophilic syndrome with cholestasis instead of hepatitis-like syndrome.

Abdominal pain, anorexia, weight loss, malnutrition, and tender hepatomegaly are present in chronic and heavy infections. Recurrent bacterial cholangitis, cholecystitis, liver abscess, and pancreatitis are also present in some cases. Rarely, progression to cholangitis, cholecystitis, and cholangiocarcinoma may appear as sequelae of infection.

Infection in Animals

Opisthorchis spp. infections in dogs and cats are mostly asymptomatic. Apparently, clinical manifestations in symptomatic animals are very mild showing periductal inflammation, biliary hyperplasia, and periductal fibrosis. So far no reports of *O. viverrini*-associated cholangiocarcinoma have been documented in animal hosts.

Epidemiology and Public Health

As per an estimate by WHO, 56 million people are infected by the food-borne trematode infections and nearly 750 million people at the risk of infection. Among these, 80 million are infected by *Opisthorchis* spp., including 67 million infected with *O. viverrini* in the countries of Southeast Asia. Nearly 13 million people are infected with *O. felineus* in Kazakhstan, Ukraine, and Russia including Siberia (Fig. 6).

Opisthorchiasis in humans is more prevalent in areas where there is large consumption of raw Cyprinidae fish (*Carassius*, *Channa*, *Cyclocheilicthys*, *Hampala*, *Esomus, Osteochilus, Puntioplites*, and *Puntius*) as part of the diet. Human infection by *O. viverrini* is a major public health problem in Mekong countries of Thailand, Laos, Cambodia, Vietnam, and Myanmar with more than 10 million people being infected through consumption of fish containing the infective metacercariae. Infection is highly endemic in lower Mekong River basin, and in some areas of northeast Thailand prevalence rate reaches 60%. In Thailand, a major source of infection is consumption of raw or inadequately cooked, frozen, salted, or smoked fish. *O. felineus* infection was the major liver fluke infection documented in Eastern Europe. Sporadic cases have been reported from Malaysia, Singapore, and the Philippines. In Italy, *O. felineus* human outbreaks occurred due to the consumption of marinated tench fillets. *O. viverrini* infection in humans rises with an increase in age. Children under the age of 5 years are unlikely to be affected. Males are infected more than the females.

Fish-eating mammals, especially cats and dogs, are the potential reservoir hosts of *O. viverrini*. These animals acquire infection by consuming fish, either raw or undercooked, harbouring the metacercariae which are the infective stage of the parasite. The infected animals maintain the infection in the community by contaminating the environment with the parasite egg shed in their faeces. Table 1 lists the important hosts and geographical distribution of *Opisthorchis* spp.

Diagnosis

Diagnosis of *Opisthorchis* infection is based on the clinical diagnosis supported by various diagnostic techniques (Table 2).

Microscopy

Direct demonstration of *Opisthorchis* spp. eggs or the adult worm in stool by microscopy confirms the diagnosis of opisthorchiasis infection and is the gold standard. A single stool sample is inadequate to demonstrate the presence of egg or the parasite, hence collection and examination of multiple stool specimens are needed to increase the sensitivity of stool microscopy. Formalin–ethyl acetate sedimentation concentration technique (FECT) is recommended for concentration of *Opisthorchis* eggs in stool specimen. Although the eggs of *Opisthorchis* resemble those of *Clonorchis*, it can be differentiated by some specific microscopic features.

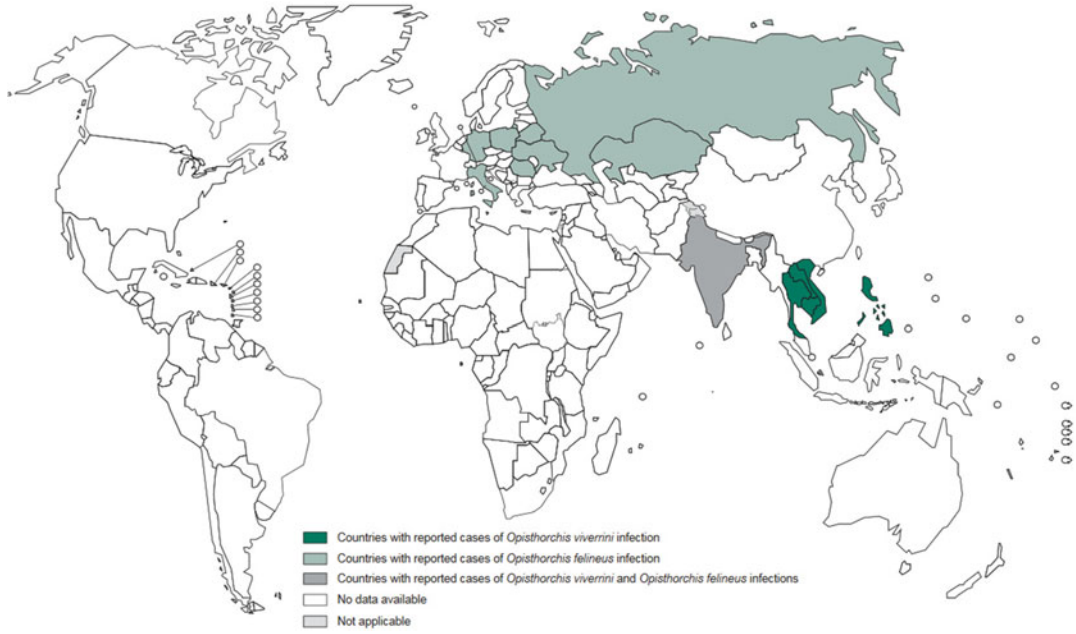

Fig. 6 Distribution of opisthorchiasis (Source-WHO)

Kato-Katz thick smear examination is a simple procedure frequently used to estimate intensity of the infection by counting the eggs present in the faeces and quantifying the same in stool.

Serodiagnosis

Serodiagnosis of opisthorchiasis is based on demonstration of *Opisthorchis*-specific antibodies in serum or antigen in serum or stool.

ELISA has been used to detect specific *O. viverrini* antibodies in serum or even in urine by using either crude somatic extract, tegument extract, and excretory secretory product (soluble metabolic products) antigens from adult worms, metacercariae, and egg and with variable sensitivity and specificity. Inability to differentiate between the recent and past infections and also failure to quantify the intensity of infection are the major limitations of antibody-based serological tests.

A monoclonal antibody-based enzyme-linked immunosorbent assay (MAb-ELISA) has been evaluated to detect *O. viverrini* metabolic antigens in faeces using a single clone of a specific monoclonal antibody (MAb). It is sensitive enough to detect the antigen excreted even by a single mature fluke present in the intestine. Detection of serum antigen by MAb-ELISA indicates

Table 1 Distribution of *Opisthorchis* spp. of human importance

Species	Distribution	Definitive host	First intermediate host	Second intermediate host
Opisthorchis viverrini	Southeast Asian countries like Thailand, Laos, Cambodia, Vietnam, and Myanmar	Fish-eating mammals, including dogs, cats, rats, and pigs	Freshwater snail (*Bithynia siamensis)*	Freshwater fish (cyprinoid fish)
Opisthorchis felineus	Kazakhstan, Ukraine, and Russia including Siberia	Fish-eating mammals such as dogs, foxes, cats, rats, pigs, rabbits, seals, lions, wolverines, martens, polecats, and humans	Freshwater snail of the genus *Bithynia*	Freshwater fish (cyprinoid fish)

Table 2 Diagnostic methods in human opisthorchiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Stool microscopy	Direct demonstration of ova and parasite	Multiple stool specimens are needed to increase the sensitivity
Immunodiagnostics	Antigen detection (monoclonal antibody-based enzyme-linked immunosorbent assay) (MAb-ELISA)	Metabolic antigens of adult worms	Sensitive enough to detect the antigen excreted even by a single mature fluke in the intestine Mab-ELISA also does not cross-react with other flukes and detects current infections
	Antibody (ELISA)	IgG, IgE antibodies	Variable sensitivity and specificity *Limitation*: Inability to differentiate between the recent and past infections and also failure to quantify intensity of infection
	Detection of biomarkers	Bile acids, glycine-conjugated bile acids, products of oxidative DNA damage like 8-nitroguanine and 8-oxodG level	Recommended to access the risk of developing cholangiocarcinoma and to monitor the progress of disease
Molecular assays	PCR	ITS1, ITS2, and cox1	High sensitivity and specificity, used for detecting light infections and for therapeutic monitoring of the disease. *Limitation:* Require skilled personnel

recent infections. The test is specific and does not cross-react with other flukes.

Molecular Diagnosis

PCR for the detection of *O. viverrini* eggs in the stool shows high sensitivity (100%) and specificity and can even detect a single egg in artificially inoculated faeces. The test is highly specific and no cross-reaction is observed with heterophyid flukes. PCR-RFLP analysis of the ITS2 region has been utilized to speciate *Opisthorchis* eggs. Sequencing of cytochrome c oxidase subunit I and NADH dehydrogenase subunit 1 is also used to detect *O. viverini*. Therefore, PCR is useful for detecting light *O. viverrini* infections and for therapeutic monitoring of the disease.

Other Tests

Biomarkers are being evaluated to access the risk of developing cholangiocarcinoma and to monitor the progress of opisthorciasis. Biomarkers, such as primary and glycine-conjugated bile acids, products of oxidative DNA damage which includes 8-nitroguanine and 8-oxodG level are shown to rise significantly in leukocytes and in the urine of patients with cholangiocarcinoma and also correlate well with progression of disease.

Imaging methods such as ultrasonography, CT, MRI, cholangiography, or endoscopic retrograde cholangiopancreatography (ERCP) are used to demonstrate any biliary tract abnormalities, if any, in chronic opisthorchiasis.

Treatment

Praziquantel is the drug of choice for treatment of *O. viverrini* infection. It is effective in a dosage of 25 mg/kg three times daily for 2–3 consecutive days or in a single dose of 40 mg/kg body weight. Albendazole given in a dosage of 10 mg/kg/day for 7 days is also effective for treatment of opisthorchiasis. The drug given along with food in fatty meal increases the bioavailability of the drug.

Prevention and Control

Health education, adopting safe food practices, improved sanitation, and veterinary public health measures are essential to effectively reduce transmission rates in the community. Diagnosis of opisthorchiasis at the district level, followed by chemoprophylaxis with the administration of praziquantel in a single dosage of 40 mg/kg is the method recommended by WHO for effective control of opisthorchiasis in the community.

Freezing the fish at −10 °C for a minimum period of 5 days or by salting in a 10% saline solution that kills the metacercariae is effective in preventing transmission of infection. Avoidance of eating raw or undercooked freshwater fish or salted, smoked, or pickled fish is another preventive measure followed in opisthorchiasis.

Case Study

A 42-year-old female presented with complaints of fever, nausea, vomiting, icterus for 10 days period. On examination, palpable enlarged liver, elevated liver enzymes, and eosinophilia were the positive findings. The patient had consumed smoked fish 10 days before in her relative's house. A diagnosis of opisthorchiasis was made based on laboratory investigations and patient recovered following appropriate treatment.

1. What were the relevant laboratory investigations carried out to confirm the diagnosis of opisthorchiasis?
2. Mention the differential diagnosis of this condition and how would you confirm the diagnosis?
3. What is the treatment protocol of this clinical condition?
4. Would you like to advice any screening protocol of other family members, if so why?
5. What advice should be given to the patient to prevent similar infection in future?

Research Questions

1. How to overcome the problem of structural similarities and presence of cross-reacting antigens between liver and intestinal flukes in the development of sensitive and specific immunodiagnostic tests?
2. What is the role of protective immunity after primary infections when opisthorchiasis reinfection has been reported even after effective treatment?
3. What strategies can be adopted to change or modify human behaviour so as to achieve complete control of *Opisthorchis* infection since consumption of raw food is still considered as one of the traditional- and culture-specific values in few areas?

Further Readings

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Clonorchiasis

Rajendran Prabha

Learning Objectives

1. To have an understanding of the risk of development and aetiopathogenesis of malignancy in chronic clonorchiasis.
2. To emphasize the importance of early diagnosis using immunological or molecular methods in endemic areas to prevent future malignant transformations.
3. To know about the various control measures that should be undertaken to minimize the risk of infection.

Introduction

Clonorchiasis is a common zoonotic and foodborne neglected parasitic disease caused by the liver fluke *Clonorchis sinensis.* It is a fish-borne trematode infection, which is a serious public health problem in the endemic countries. Due to the highly practiced cultural habit of eating raw fish containing the infective larvae and the prevailing social-ecological systems, this infection is common in the countries of East Asia. It is estimated that over 15 million people are infected worldwide, and over 200 million people are in danger of this infection.

Clonorchiasis in humans is frequently associated with liver and biliary disorders, including cholangiocarcinoma (CCA), fibrosis and other hepato-biliary conditions. The International Agency for Research on Cancer (IARC) has classified the trematode as group 2A biocarcinogen. The infection rate is more among males, people who eat raw or undercooked fish, elderly people (40–60 years), businessmen, fishermen, farmers and food handlers.

History

The parasite was first recovered from a 20-year-old Chinese carpenter and identified by James McConnell, a professor of pathology at Medical College Hospital, Calcutta (India), in the year 1874. On autopsy, the patient had swollen liver blocked by 'small, dark, vermicular-looking bodies' and distended bile ducts. In 1877, the first case in Japan was identified by Kenso Ishisaka, and it was named as Oriental liver fluke. Fish as the first host was discovered by Kobayashi in 1911, and snail as the second host was discovered by Masatomo Muto in 1918.

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S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
https://doi.org/10.1007/978-981-16-7204-0_26

Taxonomy

Genus *Clonorchis* belongs to the phylum Platyhelminthes; class Rhabditophora; order Plagiorchiida; and family Opisthorchiidae. *C. sinensis* is the important species that causes infection in humans and animals.

Genomics and Proteomics

C. sinensis has 28 pairs of chromosomes ($2n = 56$). The chromosome pairs are 8 large groups and 20 small groups. Structural variations are seen amongst isolates in various geographical distributions. The whole genome size is 580 Mb, and the GC content was 43.85%. Out of 16,000 predicted genes, 13,634 genes have been identified. There are 50,769 protein domains which are involved in various biological processes. The proteomic analysis of excretory-secretory products of adult worms has shown a total of 110 proteins, including glycolytic enzymes (such as fructose-1,6-bisphosphatase andenolase) and detoxification enzymes such as glutamate dehydrogenase, dihydrolipoamide dehydrogenase and cathepsin B endopeptidase.

The Parasite Morphology

Adult Worm

C. sinensis adult is a narrow fluke, 10–25 mm in length, with a leaf-shaped body which is flattened dorsal-ventrally (Fig. 1) having a life span of about 20 years. It has no cardiovascular system and no body cavity. The common genital pore is anterior to the acetabulum. The fluke is tapered at the anterior end and rounded at the posterior end. A thick and elastic cuticle lacks spines or scales and may either be of translucent grey or yellow colour. It narrows at the anterior end into oral sucker. Caeca, a two-tube structure of digestive and excretory tracts, runs along the length of the body. The intestine is bifurcated and ends blindly. The posterior end is broad and blunt.

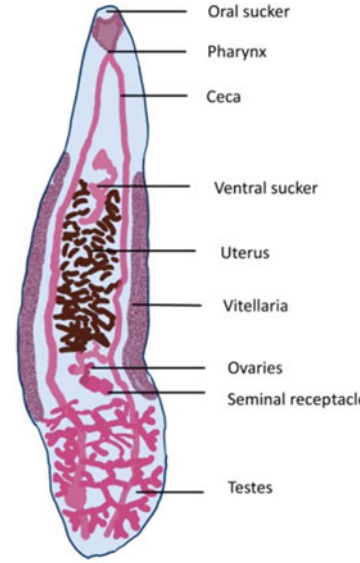

Fig. 1 Adult worm of *Clonerchis sinensis*

Ventral sucker which is poorly developed lies behind the oral sucker, at the linear distance of about one-fourth from the anterior end. A standard genital pore opens just ahead of it. As a hermaphrodite, it has both male and female reproductive organs. An ovary is present at the centre and two branched testes are present at the posterior end. The uterus and seminal ducts meet and open at the genital pore.

Metacercaria

The metacercaria is an oval structure with body folds, the later helps the larva for active movement. It contains oral and ventral suckers that are present with the caeca and excretory bladder. Organelles of the systems are underdeveloped.

Eggs

The egg measures 26–30 μm in length, oval in shape and is surrounded by thick brown shell. The egg has an operculum covered with a rim at the anterior end. The curved spine is present on the posterior end. Miracidium is present inside a

fertilized egg. The egg does not float in saturated solution of common salt.

Cultivation of Parasites

Tissue culture medium containing Locke's solution or RPMI 1640 with 0.005% bovine bile supplementation has been used for maintenance of the adult *Clonorchis* worms for up to 3 months or more.

Laboratory Animals

Rats, mice, hamsters, rabbits and guinea pigs have been used for experimental studies.

Life Cycle of *Clonorchis sinensis*

Hosts

Definitive Hosts

Carnivorous mammals such as dogs and cats and other fish-eating animals act as reservoirs. Humans are the accidental definitive hosts. The adult worm resides in the biliary tract and the liver.

Intermediate Hosts

First intermediate hosts – Freshwater snails (*Parafossarulus striatulus*, *Parafossarulus manchouricus*, *Bithynia fuchsianus* and *Alocinma longicornis*).

Second intermediate host: Fish belonging to members of the genus *Cyprinidae.*

Infective Stage

Metacercaria.

Transmission of Infection

Infection in the definitive hosts is acquired by ingestion of raw or undercooked fish containing the metacercariae. The life cycle occurs in three host systems: a snail is usually the primary host, fish is generally the second host and animals and humans the definitive hosts (Fig. 2).

Freshwater snail of the species *P. manchouricus* is the most important first intermediate host, and is common in East Asia, although other freshwater snail species can also act as intermediate hosts The eggs containing a well-developed miracidium, which is an oval-shaped ciliated larval stage, is ingested by a suitable snail. Subsequently, hatching of the miracidium occurs within the alimentary tract of the snail, facilitated by the digestive enzymes. It develops to a sporocyst in about 4 h time. The sporocyst remains attached to the intestinal wall and in the next 17–20 days metamorphoses into a hollow saclike structure, the *redia*. The rediae are subsequently released after rupture of the sporocyst to develop into the next-stage larvae called cercaria. Each redia produces 5–50 cercariae. The mature cercariae has two eyespots, penetration glands and a stylet at its anterior end, and a cuticle with small spines. The cercariae are shed from the snails, swim freely in water and come to lie upside down in water and slowly sink to the bottom.

The cercaria when it comes in contact with a fish attaches itself with its suckers, loses its tail, and penetrates the skin and comes to lie as a cyst in muscle or under the scales. Fish of *Cyprenidae* group like carp and also crayfish are infected. The cercaria transforms into metacercaria, which is the infective form of the parasite. Hundreds of metacercaria may be found in a single fish.

Humans or other animals are infected on consumption of the raw fish infected with the metacercaria. *C. sinensis* metacercaria excysts within the alimentary canal and enters the common bile duct through the ampulla of Vater and migrates to smaller intrahepatic ducts or occasionally the gall bladder and pancreatic ducts, where it matures into adults in about 1 month. In the duodenum, the young fluke excysts and migrates to the liver through the bile duct. It matures into an adult worm, and being hermaphrodite starts producing the eggs. Each adult yields a daily production of 4000 eggs for a minimum of 6 months, and the eggs are excreted out in the faeces of the infected host.

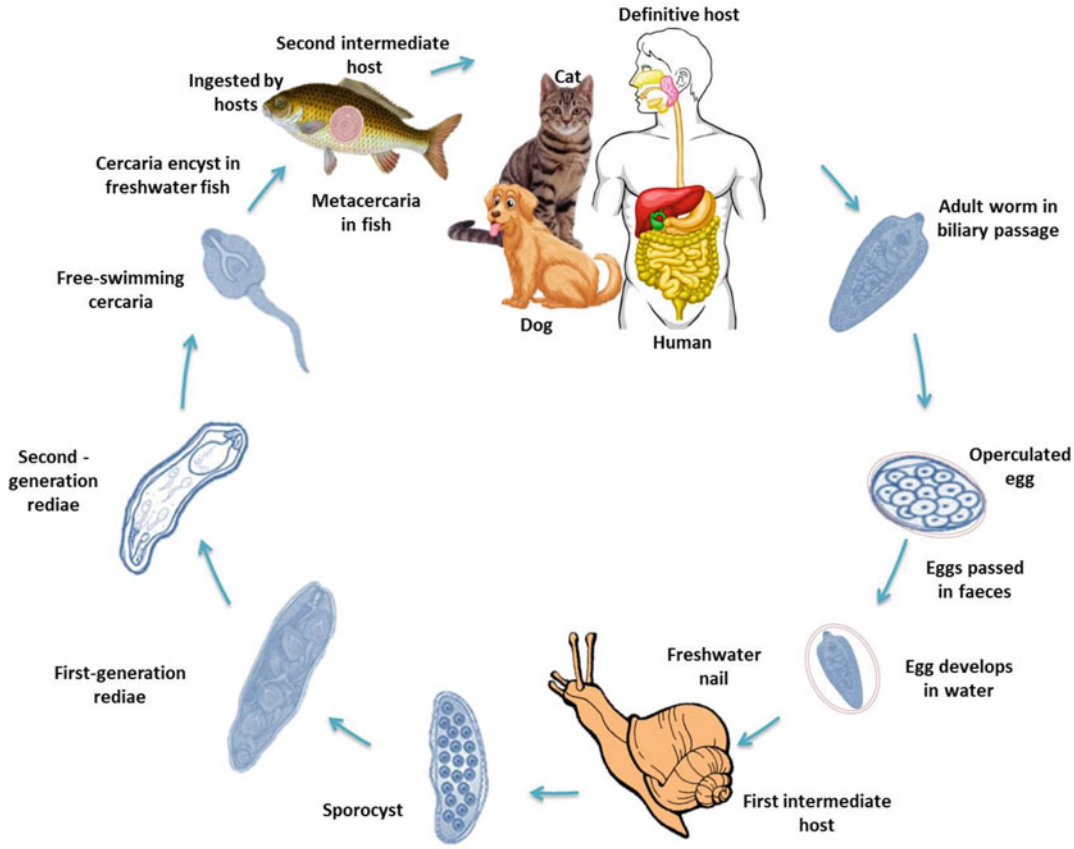

Fig. 2 Life cycle of *Clonorchis sinensis*

Pathogenesis and Pathology

The adult worm of *C. sinensis* resides in the bile ducts of liver and attaches itself to the duct epithelium. It consumes blood and tissue fluids and also feeds on the cells resulting in an inflammatory reaction. Biliary epithelium undergoes hyperplasia and then metaplasia resulting in cholangitis and hepatic enlargement. Metaplasia of mucin-producing cells in the mucosa is a constant feature, and proliferation of these cells results in development of small glandular elements with attendant excessive mucus content of bile. The surrounding stroma becomes fibrotic. Thus, fibroplasia of the bile duct followed by that of liver parenchyma is the hallmark of *Clonorchis* infection. The bile ducts become dilated, thick-walled, and may contain large number of worms. Eggs deposited in the hepatic parenchyma get surrounded by a fibrous and granulomatous reaction. Eggs and worms that migrate out of the biliary system into gall bladder will become nuclei for gallstones. The pancreatic duct may also be invaded and become dilated, thickened and lined by metaplastic epithelium. Secondary bacterial infection of bile duct is commonly seen and is of enteric origin, *Escherichia coli* being the most common organism.

On a molecular level, excretory secretory products (ESPs) from *C. sinensis* play a major role in the disease progression and host-parasite interactions. Components of ESPs activate human hepatic stellate cells and other liver cells for the assembly of collagen resulting in fibrosis. There is activation of TGF-β/Smad signalling pathway which results in the synthesis of collagen type I, fibroplasias and degeneration of hepatocytes. In the genesis of cholangiocarcinoma, biliary stasis

or chronic infection of biliary tract is considered a risk factor. The development of cholangiocarcinoma in experimental hamster model has shown that there is upregulation of cancer-promoting *PSMD10* and *CDK4* genes while the tumour suppressor genes, namely, *p53* and RB protein in addition to *BAX* and *caspase 9* were downregulated. ESPs may also suppress the apoptosis of malignant/abnormal cells and induce tumours.

Immunology

C. sinensis infection, similar to other helminthic infections, induces a marked Th2 response. Experimental studies with mice have revealed elevated levels of Th2 cytokines such as IL-4, IL-5 and IL-13, IL-10 and TGF-β in response to ESP components or crude worm antigens. In infected humans as well as mice, IL33 levels are abnormally high, and this cytokine acts as an important cause of proliferation and fibrosis of bile duct. Experimental mice also show elevated Treg/Th17 ratio. TGF-β, IL-13 and IL-10 are well-known cytokines that can activate Th2 response.

TGF-β, IL-13 and IL-10 cytokines activate stellate cells to supply collagen types I and III. These cytokines help the worm to survive in bile ducts by evading the immune reaction and causing chronic inflammation and resulting in fibrosis. Innate immune response in the form of upregulated TLR2 and TLR4 activities may act as defence mechanism against *C. sinensis*, as demonstrated in experimental mouse model. High TLR4 expression also induces the production of pro-inflammatory cytokines, which may contribute to pathogenicity of the trematode.

Infection in Humans

The clinical symptoms depend upon the fluke burden, frequency of infection and host immunity. At the early stage of infection, the patient may not show any symptoms.

Mild infection (with fewer than 100 flukes): Patients with mild infection present at times with epigastric discomfort, anorexia, dyspepsia, etc.

Moderate infection (generally fewer than 1000 flukes): Patients present with fever and chills, discomfort, fatigue, anorexia, diarrhoea, weight loss and abdominal distension.

Severe infection (up to 20,000 flukes): Patients may develop hepatitis, liver abscess, pancreatitis and pyogenic cholangitis. Hepatic cirrhosis, with oedema, ascites and gastrointestinal bleeding may be the other manifestations. Cholangitis and cholecystitis with bile stasis, obstruction, periductal fibrosis and hyperplasia are also seen.

Chronic infection is associated with cirrhosis of liver, malignant hypertension and ascites. Recurrent pyogenic cholangitis acts as a nidus for stone formation, predisposing to stasis and secondary bacterial infection with resultant damage of the bile ducts with stricture formation. The lining epithelium of bile duct is altered biochemically, and may pass through the stages of mucosal adenomatous hyperplasia and dysplasia finally resulting in carcinomatous transformation leading to cholangiocarcinoma.

Infections in Animals

Clonarchiasis in animals may present with manifestations of cholangitis, cholecystitis, bile stasis, obstruction, bacterial infections, inflammation and periductal fibrosis.

Epidemiology and Public Health

Clonorchiasis is an important food-borne zoonotic disease and is prevalent in low- or middle-income Asian countries. The largest population of infected people is present in China. It is also seen in South Korea, Vietnam and far-eastern Russia (Table 1 and Fig. 3). The risk of disease transmission in other countries is increased by travellers from endemic areas.

Thirty-five million people are estimated to be infected with *C. sinensis* worldwide. It is estimated that 12.49 million individuals are

Table 1 Epidemiology of clonorchiasis

Species	Definitive host	First intermediate host	Second intermediate host	Geographic distribution
Clonorchis sinensis	Humans, carnivores (cats, dogs)	Snails*: Parafossarulus manchouricus, Parafossarulus sinensis, Melanoides tuberculata, Bithynia fuchsianus, Bithynia misella, Alocinma longicornis*	Freshwater fish: *Pseudorasbora parva, Ctenopharyngodon idellus, Carassius auratus, Cyprinus carpio, Hypophthalmichthys nobilis* and *Saurogobio dabryi*	Asia: China, South Korea, Vietnam, and far eastern Russia

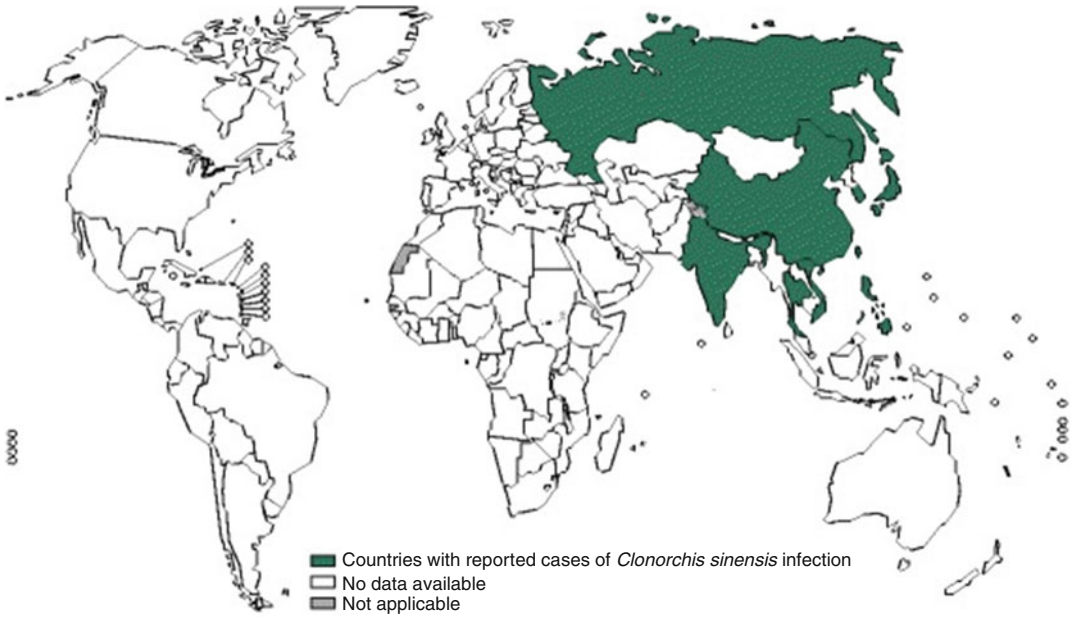

Fig. 3 Global distribution of *Clonorchis sinensis* (Source: Lu XT, Gu QY, Limpanont Y, et al. Snail-borne parasitic diseases: an update on global epidemiological distribution, transmission interruption and control methods. *Infect Dis Poverty* **7**, 28 (2018). https://doi.org/10.1186/s40249-018-0414-7)

infected with *C. sinensis* in mainland China alone with the highest (16.4%) prevalence in Guangdong province according to a survey conducted in 2001–2004. In South Korea, an estimated 1.4 million people, and about 3000 in the Amur River basin in Eastern Russia have been found to be suffering from clonorchiasis. Clonorchiasis has also been reported from North America particularly among the immigrants from endemic areas. It is an uncommon infection in India due to the practice of thorough cooking of fish, although few cases have been reported. The estimated global burden of clonorchiasis is 275,370 disability-adjusted life years (DALYs) and around 5591 people die from the infection annually.

Clonorchiasis is higher in older age groups and in males compared to younger age groups and females. This may be linked to the dietary habits of eating raw or undercooked fish along with alcoholic drinks and the practice of frequently eating outside.

Diagnosis

Laboratory diagnosis of clonorchiasis relies heavily on detection of eggs in the stool specimen

Table 2 Laboratory diagnosis of clonorchiasis

Diagnostic approach	Methods	Target	Remarks
Microscopy	Stool examination	Eggs	Gold standard
	Histopathology	Biliary, hepatic tissues	Can diagnose early cholangiocarcinoma
Immunodiagnostics	IgY (egg yolk immunoglobulin)-based immunomagnetic bead enzyme-linked immunosorbent assay system (IgY-IMB-ELISA)	Circulating antigen detection	Optical density of ELISA correlates with egg counts
	Immunoassays	Antibody to crude extract or excretory-secretory products	Cross-reaction with other trematodes
Molecular diagnosis	PCR, PCR-RFLP and FRET-PCR, real-time PCR	Internal transcribed spacers (ITS-1 or ITS-2) of nuclear ribosomal DNA (rDNA), cox1, Rn1 and nad2 genes	Sensitive but not widely used

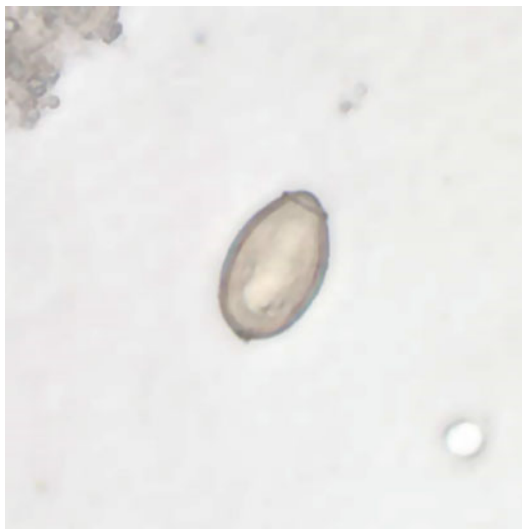

Fig. 4 *Clonorchis sinensis* egg; image taken at 400× magnification (Courtesy: DPDx, CDC)

by microscopy. The egg count in stool is carried out to assess the severity of infection. Antigen detection or molecular diagnosis (Table 2) is helpful but may not be available in the endemic areas of the world.

Microscopy

Microscopic examination of stool or duodenal contents using the Kato Katz method to detect and count *C. sinensis* eggs (Fig. 4) is the common method for diagnosis and remains the gold standard. The cellophane thick-smear method is used for mass screening of the disease.

Histopathology

Histopathological examination of biopsy specimen shows proliferation of the ductal epithelium with metaplastic cells (adenomatous hyperplasia) and periductal fibrosis in patients in endemic areas which is suggestive of clonorchiasis. Adult flukes may be observed in surgical specimens or during percutaneous transhepatic cholangiography.

Serodiagnosis

Crude extracts of *C. sinensis* have been found to be sensitive for the demonstration of serum antibodies in the serodiagnosis of clonorchiasis, but cross-reaction with other trematode infections is a noted problem. A number of excretory-secretory proteins (ESPs), including 21.1-kDa tegumental protein and cathepsin L proteinase, have been evaluated as antigens in the antibody-based immunoassays for serodiagnosis of clonorchiasis.

An IgY-IMB-ELISA has been found to be a sensitive and specific assay for the detection of circulating antigen in human clonorchiasis, and a significant correlation has been found between ELISA optical density and egg counts.

Molecular Diagnosis

Various molecular assays such as real-time PCR, multiplex PCR, PCR-RFLP and FRET-PCR have been used for targeting the internal transcribed spacers (ITS-1 or ITS-2) of (rDNA), cox1, Rn1

and nad2 genes in the diagnosis of clonorchiasis. These tests are highly specific. Multiplex PCR is a more sensitive method for differentiating C. *sinensis* from that of *O. viverrini*. Nevertheless, many of these tests are technically demanding as well as difficult to optimize the time, temperature and the reagents needed to amplify multiple genes or genetic loci. Many of these tests even are not available in resource-challenged regions.

Treatment

Praziquantel is the drug of choice for the chemotherapy of clonorchiasis. Dosage is 25 mg/kg of body weight, thrice daily for 2 days and has a cure rate of 93.9–100%. The drug is often used as single dose regimen in the area endemic for the disease. The drug has mild adverse effects, such as dizziness, headache and abdominal discomfort. Tribendimidine is another new drug that has been evaluated and found to be effective in clonorchiasis. Artemether, artesunate and mebendazole are the other drugs that have also been evaluated for treating *C. sinensis* infection in animal models. Biliary obstruction in clonorchiasis may require surgery.

Prevention and Control

Preventive methods are based on the strategies to decrease transmission of infections that include health education on safe food practices, environmental and individual sanitation, treatment of manure with ammonium sulphate to kill eggs of parasite, eradication of snails and public health measures such as government-driven mass chemotherapy. Non-polluted fish culture with the utilization of fish vaccines is another approach to reduce transmission of the infection.

Case Study

A 35-year-old businessman with frequent international travel presented with complaints of right upper abdominal pain, fever of 10 days duration and yellowish discoloration of eyes of 2 days duration. He was vaccinated with hepatitis B vaccine, and there was no co-morbidity. He was an occasional alcoholic. On clinical examination, patient was febrile, with pulse rate of 110/min and BP of 124/80. He had jaundice and tender hepatomegaly of 4 cm enlargement. Other systemic examination was normal. With the clinical diagnosis of acute hepatitis, the patient was subjected for laboratory investigations. The faecal sample was collected and subjected to direct microscopy and culture. Spontaneous sedimentation and floatation techniques were also performed. On culture, faecal coliform was grown. On stool microscopy, egg of *C. sinensis* was seen. A detailed history revealed that he has taken pickled fish a few times while travelling to China and Korea. The patient was started on praziquantel and improved clinically.

1. What factors are responsible for malignancy in case of chronic *C. sinensis* infection?
2. Why the highest prevalence of clonorchiasis is seen in the fifth decade of life?
3. What are the preventive methods that should be advised to the population at risk?

Research Questions

1. How to utilize genomic and proteomic studies for better understanding of pathogenesis and parasite biology of *C. sinensis*?
2. Why is it necessary to develop cheap, point-of-care tests for effective diagnosis of clonorchiasis in endemic regions?
3. Is there any drug resistance for praziquantel and why an alternate drug should be developed and properly evaluated?

Further Readings

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Amphistomiasis

Nonika Rajkumari

Learning Objectives

1. To make the reader aware about an unusual group of parasites that can cause severe gastrointestinal manifestations and even death.
2. To know the names of the various parasite species which can cause amphistomiasis and its diagnosis.

Introduction

Trematodes form an important group of helminthic parasites causing serious infections in humans and in the livestock animals of economic importance. Amphistomes, which are digenetic trematodes, cause amphistomiasis in a variety of hosts including humans and in both domesticated and wild animals. In livestock industry, huge economic loss occurs due to poor production of milk, meat, wool and other animal products because of animals suffering from amphistomiasis. *Gastrodiscoides hominis* and *Watsonius watsoni* are two important human pathogens that cause infection in humans.

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History

Amphistomiasis in domesticated animals is a well-known entity for a long time in India and in some parts of the country; the condition has been known as 'Pitto' and 'Gillar'. Lewis and McConnell first described an amphistome parasite in 1876 from a patient in Assam, India. Later, this parasite was identified as *G. hominis* by Buckley in 1939 who elucidated the life cycle of the parasite and also mapped the disease prevalence in Assam.

Taxonomy

The genus *Gastrodiscoides* belongs to family Paramphistomatoidea/Gastrodiscoides; superfamily Paramphistomatoidea; class Trematoda and phylum Platyhelminthes. *Gastrodiscoides hominis* (Leiper 1913) and *Watsonius watsoni* (Conyngham, 1904) (Stiles and Goldberger (1910)) are two important species that cause infection in humans. Amphistomiasis in farm and wild mammals is caused by species of *Paramphistomum*, *Calicophoron*, *Cotylophoron*, *Pseudophisthodiscus*, etc. Among 40 species of amphistomes, four common species causing infections in domestic ruminants include *Gastrothylax crumenifer, Fischoederius cobboldi, Fischoederius elongatus* and *Paramphistomum*.

S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
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Genomics and Proteomics

The genomics of *Gastrodiscoides* species of human importance is yet to be known. Proteomic studies of *Gastrodiscoides* species are largely unreported.

The Parasite Morphology

Adult Worm

Amphistomes are endoparasites. The adults in the infected hosts parasitize the intestine, liver and bile duct. Living adults are light reddish, brownish or whitish in colour and are elongated, conical or flat in shape (Fig. 1). The body has thick or thin cuticle, which may be smooth or papillated. Some are provided with ventral pouches. The muscles are sub-cuticular, and the highly muscular organs include oral sucker, oesophagus, oesophageal bulb, acetabulum and genital atrium.

The body consists of an oral sucker at the anterior end and a large acetabulum at the posterior end. These two suckers may be located terminal or subterminal. These two suckers act as adhesive organelles of attachment. The mouth opens terminally or sub-terminally, followed by an oesophagus. The oesophagus may be short and thin or thick with oesophageal bulb at the posterior end in certain species. The oesophagus may be curved and bifurcates at the posterior end into two intestinal caeca. The caeca may be straight, wavy or form loops and end blindly.

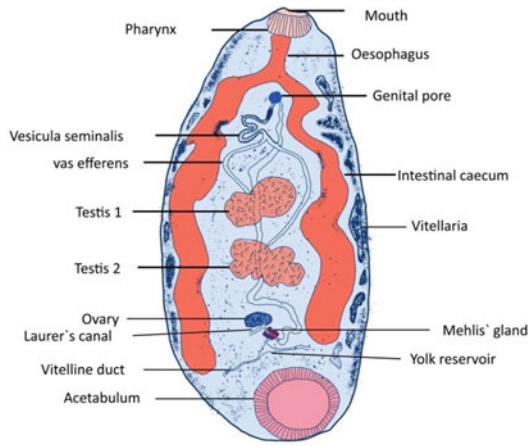

Fig. 1 Schmatic diagram of adult Amphistome

Like most trematodes, amphistomes are hermaphrodites and have a common genital pore. Two, and sometimes one testis comprise the male reproductive organ. The vas efferens originating from the testis joins to form the vas deferens and opens at the common genital pore. The vas deferens has a swollen vesicular seminalis and pars prostatica and a cirrus which is a muscular organ. The ovary is situated either at the anterior or posterior end of the testes or in between the two testes. The oviduct originating from the ovary joins the ducts of the vitelline glands. A small thin Laurer's canal can be found opening a little near this junction. The receptaculum seminis can be seen near the joining of the Laurer's canal and oviduct. The reproductive ducts of the testis and ovary open into the common genital atrium which opens outside as genital pore situated at the ventral aspect of the parasite. A set of longitudinal lymph vessels are present in these parasites which take part in distribution of food materials and excretory substances. The lymphatic vessels may occur in one to three pairs.

Eggs

Eggs measure about 140 by 65 μm in size and are rhomboidal in shape. They are transparent, and green in colour. Each egg contains an unembryonated ovum (Fig. 2).

Metacercaria

Metacercariae are the infective stage of the parasite to humans and other mammals. They are oval or round in shape, surrounded by a single-layered cyst. They contain oral and ventral suckers that are poorly developed with small pharynx and intestinal caeca. Testes are two in number, small anterior testis is placed laterally or sub-medially and large posterior testis is present transversely.

Fig. 2 Egg of *Gastrodiscoides hominis*

Ovary is situated sub-median, lateral or diagonal to the anterior testis.

Cultivation of Parasites

Amphistomes have not been cultivated in any cell-free culture media so far.

Laboratory Animals

Studies or data pertaining to animal models for demonstration of immunopathogenesis of amphistomes are yet to be available.

Life Cycle of Amphistomes

Hosts

Definitive Host

Ruminants, pigs and monkeys. Humans are the accidental hosts.

Intermediate Hosts

Snails belonging to genus *Bulinus, Planorbis, Physa Stagnicola* and *Pseudosuccinea* act as an intermediate host in particular to seven species of significance including *Bio pfeifferi, Bulinus forskalii, Bulinus globosus, Bulinus nasutus, Bulinus tropicus, Ceratophallus natalensis* and *Galba truncatula*. Fish may or may not act as second intermediate hosts.

Transmission of Infection

Humans acquire infection by the ingestion of vegetables or raw fish contaminated with the viable infective metacercaria (Fig. 3). Inside their duodenum and jejunum, the metacercaria excyst to release the immature flukes, which migrate and subsequently penetrate the intestinal wall by destroying the mucosa. Five to nine months after infection, the immature flukes develop to sexually mature adults. The gravid mature adult worm migrates to the caecum and ascending colon where it attaches and starts laying eggs, which are then excreted in the faeces. The faeces contaminate the water bodies such as river, lake richly inhabited by freshwater snail species.

The miracidia hatch out from the eggs and swim actively in the water until they are ingested by the snail intermediate hosts of the species *Bio. pfeifferi, Bul. forskalii, Bul. globosus, Bul. nasutus, Bul. tropicus, Cer. natalensis* and *Galba truncatula*. Inside the snail, the miracidium metamorphoses into the sporocyst stage followed by redia. The redia then undergoes further metamorphosis to enter the cercarial stage in a period of about 3 weeks. Mature cercariae contain two eyespots and a long slender tail. It gets attached to aquatic plants where they get encysted into metacercariae. At some instances, the cercaria released from the snail may penetrate a second intermediate host, such as fish or water vegetation and encyst as metacercaria. Humans or other hosts ingest the metacercaria with fish or contaminated vegetables and the cycle is repeated.

Pathogenesis and Pathology

Generally, the adult flukes are harmless; they do not cause any pathological changes in the caecum or colon of the infected while the severe pathology occurs due to heavy infections by migrating

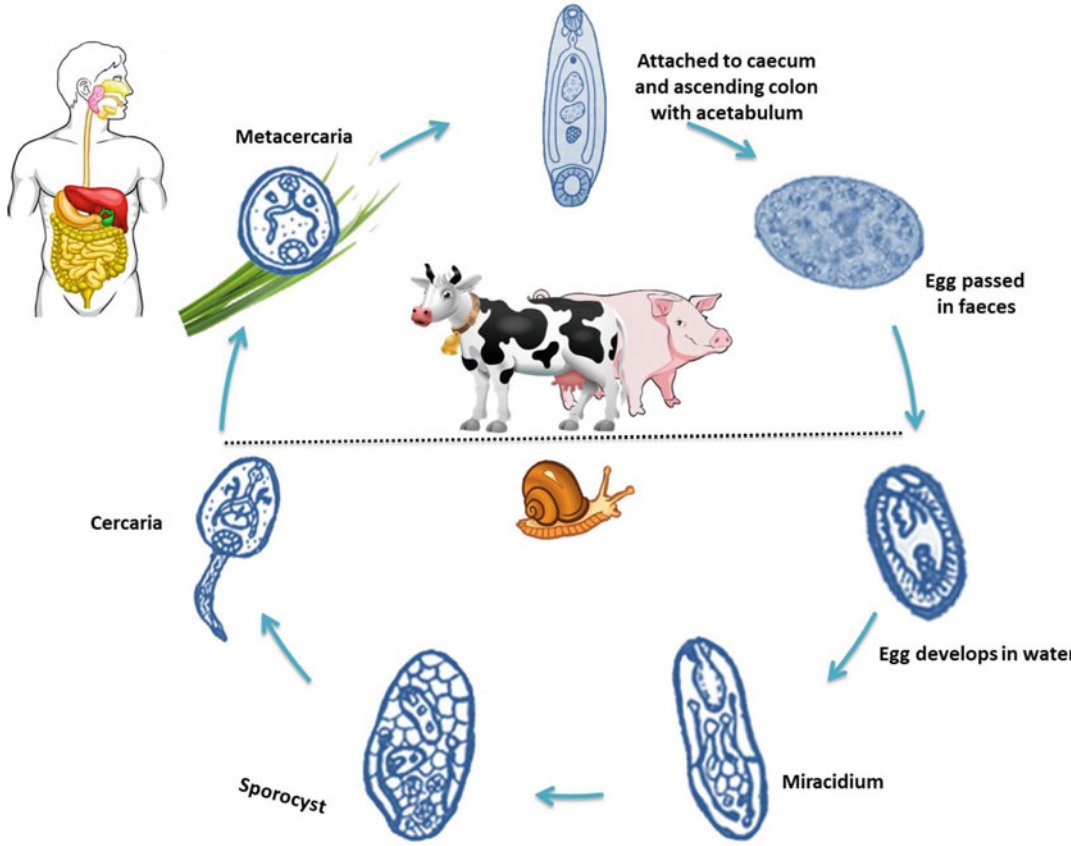

Fig. 3 Life cycle of an amphistome

immature flukes. The worm attaches itself to the small intestine causing inflammation and haemorrhage at the site. The lamina propria is infiltrated with macrophages and lymphocytes.

Adult flukes, residing in the bile duct of ruminants, cause mild infection whereas immature flukes cause severe infections resulting in active sloughing and necrosis of the intestinal wall. The necrosis is manifested as bloody dysentery with signs and symptoms of severe enteritis. Anorexia and lethargy along with signs of dehydration due to copious diarrhoea and weight loss are features of amphistomiasis in animals. Sheep develop profuse diarrhoea 2–4 weeks after exposure. In chronic infection, there is weight loss, emaciation, anaemia, along with thickening of the duodenum.

Immunology

During the course of development, amphistomes in the infected host undergo antigenic polymorphism that brings about significant alterations in immune response. Furthermore, antigens released during developmental stages initiate varying immune responses in the infected host.

Infection in Humans

Clinical manifestations in ruminants and humans depend on the infecting dose, species pathogenicity of the metacercariae in the small intestine. Minimal pathogenicity is noticed in light infections, although inflammation of mucosa occurs at the site of attachment or by the large

posterior sucker of the fluke. Heavy infestations result in intestinal oedema and diarrhoea.

The clinical manifestations of infections caused by the amphistomes in humans range from severe diarrhoea to inflammation of the mucosa of duodenum, ileum, caecum and colon. The infection may even prove fatal in certain cases. In Assam, India, there have been reports of death among children suffering from gastrodiscoidosis caused by *G. hominis.*

Infection in Animals

Paramphistomum cervi is the most common rumen flukes that causes infection in ruminants. The infection is asymptomatic in most cases. The presence of adult worms in their natural hosts such as pigs and monkeys sometimes presents as mild diarrhoea. The immature flukes usually cause severe disease in the infected animals. In ruminants, disease manifestations include general weakness, polydipsia, oedema of submandibular region, anaemia, hypoproteinemia, foul-smelling diarrhoea, reduction in feed conversion, weight and milk production leading to mortality in young animals. Terminally sick animals are immobile, and completely emaciated till their death. Liver cirrhosis and nodular hepatitis in buffaloes may precipitate massive haemorrhages. Untreated, it is often fatal within 2–3 weeks and high mortality up to 80% has been observed in livestock farms among cattle and sheep.

Epidemiology and Public Health

Amphistomiasis in humans is a neglected tropical disease prevalent globally, but the highest prevalence of the disease has been recorded in the countries of Africa, Asia, Australia, Eastern Europe and Russia (Table 1 and Fig. 4). The disease also has been documented from India, Bangladesh, Myanmar, China, Kazakhstan, Philippines, Thailand and Vietnam. In India, human gastrodiscoidiasis is endemic in Assam, and is attributed to inadequate sanitation and use of night soils as manure. Figure 4 shows the global distribution of various amphistomes.

Diagnosis

A list of important diagnostic methods is summarized in Table 2.

Microscopy

Diagnosis of infections is based on microscopy of stool for the detection of eggs and adult worms. The amphistome eggs are identified by their characteristic rhomboid shape and distinct green colour. Adult worms are identified by their distinctive appearance.

Serodiagnosis

Serodiagnosis is important for early diagnosis and treatment of amphistomiasis to prevent irreparable damage to the small intestine. ELISA, western blot and other serological tests employing either adult worm somatic antigen or excretory/secretory antigen are used to demonstrate circulating antibodies in the sera of infected hosts. An indirect ELISA has also been evaluated to demonstrate coproantigens in faecal samples for the diagnosis of intestinal amphistomiasis. Detection of coproantigen in stool has been proved to be highly specific and immensely useful for the diagnosis of immature amphistomiasis, in which the amphistome eggs cannot be demonstrated in stool by microscopy.

Molecular Diagnosis

PCR-based techniques using rDNA ITS2 sequences have been identified as a reliable molecular marker in identification of the causative species of both adult amphistome and cercariae and to study their phylogenetic relationships.

Treatment

There is no standard treatment for amphistomiasis, but the home remedy of soap enema has been very effective in expelling the

Table 1 Hosts and geographic distribution of amphistomes

SN	Amphistomes	Hosts	Geographic distribution
1	*Fischoederius elongatus*	Molluscan intermediate host like snails (larval forms) Rumen hosts like cattle, sheep, etc. (adult parasite)	India, Sri Lanka, China, Russia, Indonesia, Laos, Cambodia, Japan, UK
2	*Gastrodiscoides hominis*	Molluscan intermediate host like snails (larval forms) Definitive host is usually pig (adult parasite) Man—accidental host Reservoir—pigs and monkeys	Pakistan, Thailand, Myanmar, Cambodia, Malaysia, Laos, Philippines, Vietnam, USA, Kazakhstan, Nigeria, Zambia
3	*Watsonius watsoni*	Molluscan intermediate host like snails (larval forms) Definitive host is usually monkeys (adult parasite) Man—accidental host	Laos, Senegal, Nigeria, Zambia, China

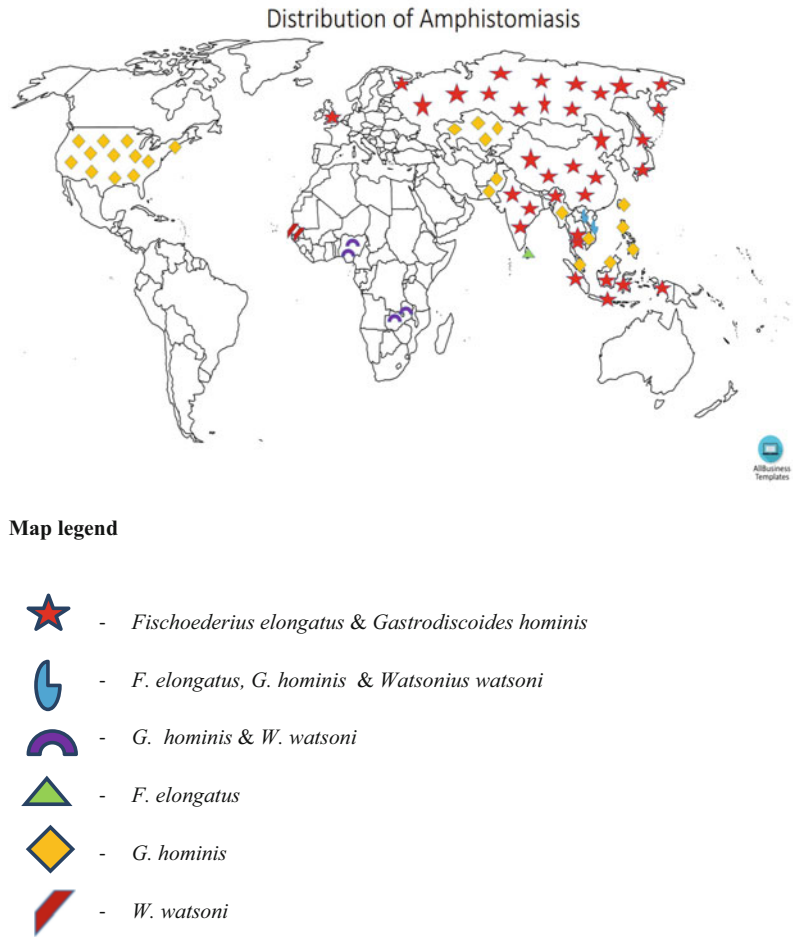

Fig. 4 Geographical distribution of amphistomes

Table 2 Laboratory diagnosis of amphistomiasis

Diagnostic approach	Method	Targets	Remarks
Microscopy/ morphological analysis	Stool examination	Immature flukes and eggs (rarely)	• These can be seen in severe cases • Differentiating it from other types of intestinal flukes like *Fasciola hepatica*, schistosomes etc. is very important
Post-mortem analysis/histological analysis	Biopsy or tissue sections	Adults or eggs	Species identification
Serodiagnosis	Indirect ELISA	Coproantigens in faecal samples	Helpful in diagnosis of immature amphistomiasis where eggs cannot be demonstrated in stool examination
Molecular analysis	Tissue of adult of *Fischoederius elongatus*	ITS-2 and COI	Tried in research settings
Egg hatching	Tap water	Eggs	Helpful for species identification

parasite from the intestine due to the flushing action. Certain drugs are shown to be effective, which include resorantel, oxyclozanide, clorsulon, ivermectin, niclosamide bithional and levamisole. Drugs effective against immature flukes and recommended for massive drenching include oxyclozanide in humans and niclosamide in sheep.

Prevention and Control

Prevention and control of amphistomiasis depends on reduction of mollusc hosts such as nails by using molluscicides like copper sulphate to eradicate the entire population. This also includes discouraging the use of night soil as manure in organic farming, avoidance of eating fish or vegetables raw, and thorough washing of raw vegetables.

Case Study

A farmer from rural Assam, India, comes to the OPD with history of pain in abdomen, uneasiness and passage of some blood tinged in his stool off and on. He gives a history of keeping livestock like cattle and sheep besides cultivating crops. Stool examination results were negative for any ova or cysts and bacterial cultures were negative. Colonoscopy picture reveals black tadpole-like worm attached to the intestinal walls. On further enquiry, he gave history that some of his cattle were suffering from loose motion, bloating, restlessness and anorexia too.

1. What is the most probable diagnosis?
2. Which parasitic infections should be ruled out first?
3. What are the various species which can cause zoonotic infections in human?
4. Give two preventive measures for this parasitic infection.

Research Questions

1. Are there more species of amphistomes which can infect humans?
2. Though they are parasites of animals, can humans be directly infected?
3. Have the species of amphistomes got outbreak potential in humans?
4. Why it is important to study parasite proteomics and its modulation?

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Heterophyiasis

Jagadish Mahanta

Learning Objectives

1. To understand the importance of ectopic egg deposition in various organs with development of severe and life-threatening illness.
2. To identify the population at risk and fish preparation and eating practice in the community.

Introduction

Heterophyes are one of the smallest intestinal flukes to infect humans. Most of the people infected with *Heterophyes* live in Africa, and Asia including India. Food-associated parasitic infections are increasing across the globe due to some traditional food habits and changing preferences to exotic food. Further, close association with domestic animals, encroachment into wildlife habitat, experimental cooking methods have also increased vulnerability to zoonotic food-borne diseases such as heterophyiasis.

History

Theodor Maximilian Bilharz first described *Heterophyes* fluke in an Egyptian mummy, and Von Siebold in 1852 named it as *Distoma heterophyes*. Cobbold in 1866 proposed the genus *Heterophyes* with *Heterophyes aegyptica* as the type species, but subsequently, the species was synonymized with *Heterophyes heterophyes*. Ohdner named the family as Heterophyidae in 1914, including few morphologically similar genera and reorganized the family into five sub-families. Rao and Ayyar reported heterophyes from dogs in Madras (Chennai), India in 1931. They observed that the parasite was morphologically similar to *Heterophyes persicus* (Braun 1901), a synonym of *Heterophyes heterophyes.* Subsequently, Bhaleraoin 1934 and Sen in 1965 also demonstrated *Heterophyes* from dogs in Madras (Chennai) and Bombay (Mumbai), respectively.

Taxonomy

This parasite belongs to phylum Platyheminthes; class Trematoda; subclass Digenea; order Opisthorchiida; family Heterophyidae and genus *Heterophyes.* Six valid species of genus *Heterophyes* have been described, namely, *Heterophyes heterophyes* (von Siebold, 1852), *Heterophyes nocens* (Onji and Nishio, 1916), *Heterophyes dispar* (Looss, 1902),

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Heterophyes aequalis (Looss, 1902), *Heterophyes indica* (Rao and Ayyar, 1931) and *Heterophyes pleomorphis* (Bwangamoi and Ojok, 1977). Out of these, only four (*H. heterophyes, H. nocens, H. dispar* and *H. aequalis*) are known to cause infection in humans. *H. heterophyes* is the type species of the genus and pathogenic to both humans and animals.

Genomics and Proteomics

Reports on the studies of genomics in *Heterophyes* are rare. The mitochondrial cytochrome *c* oxidase 1 (CO1) and nuclear ribosomal gene (28S rRNA) of *H. nocens* have been studied earlier, and more recently, the sequences of internal transcribed spacer 2 (ITS2) and 28S rRNA have been analysed for *H. heterophyes*, *H. nocens* and *H. dispar.*

The Parasite Morphology

Adult Worm

The adult *Heterophyes* are minute, ovoid to elliptical, elongated or pyriform in shape (Fig. 1). They measure about 1.4 × 0.5 mm in size. The body surface is covered with minute spines and 50–80 scale-like spikes. The adult possesses an oral sucker and a ventral sucker. Pharynx is well developed and connected to the intestinal caeca.

The oral sucker is small and covered with spikes, but ventral sucker is large. They possess a large sub-median prominent genital sucker, armed with 22–85 chitinous rodlets. The number of chitinous rodlets varies (*H. heterophyes,* 70–85, *H. nocens,* 50–62, *H. dispar,* 22–35) with the species and it acts as a diagnostic marker for morphological differentiation of species. Genital organs and vitelline glands are situated in the posterior part of the body. Testes are placed side by side and the vas deferens expands to form a seminal vesicle and then narrows to form ejaculatory duct. One ovary of the fluke is located just above the testes. The long tube-like uterus joins with the ejaculatory duct to form the genital duct. This gives rise to genital sinus and genital pore. The uterine loops in gravid worms are placed in between the long intestinal caeca.

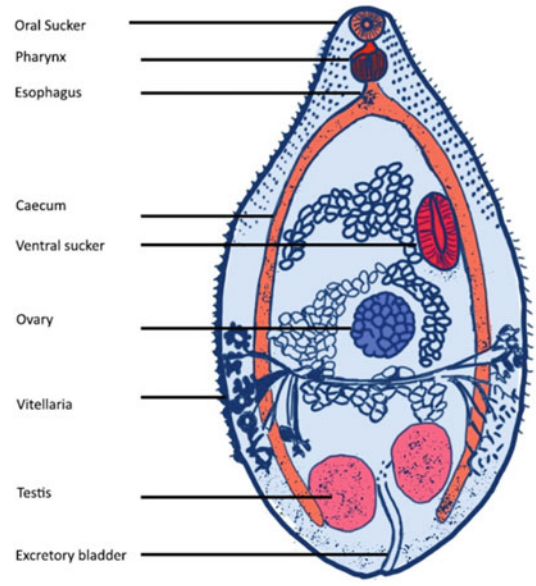

Fig. 1 Schematic diagram of adult *Heterophyes heterophyes*

Metacercaria

Infective stage of the parasite for humans and other definitive hosts is the metacercariae. They are yellowish brown in colour, spherical and measure 0.13–0.20 mm in diameter. They have a bilayered structure and have a ventral and an oral sucker. The genital sucker is placed posterior to the ventral sucker.

Eggs

Mature eggs are operculated, 30 × 16 μm in size, yellowish brown in colour and contain miracidium (Fig. 2). Opercular shoulder is inconspicuous, and there is no abopercular knob.

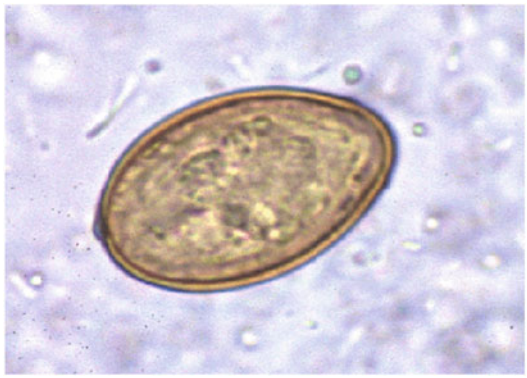

Fig. 2 Egg of *Heterophyes heterophyes* with inconspicuous opercular shoulder. (Courtesy: DpDX, CDC)

Cultivation of Parasites

The parasite has not been cultivated in vitro.

Laboratory Animals

Heterophyes develop well in laboratory animals such as mice, rats, dogs (puppies), cats etc. Foxes, badgers, pigs, macaques, and gulls also support in its growth and development. In experimentally infected mice, the kidneys show mild glomerular congestion with lymphoid aggregation. The brain shows capillary haemorrhage with focal accumulation of endothelial cells and histiocytes.

Involvement of Peyer's patches and mesenteric lymph nodes is frequently seen in experimentally infected dogs and cats with *H. heterophyes*. In rats, eggs and/or immature worms of *H. heterophyes* are found in the intestinal wall, lymph nodes, liver and spleen. In seagulls, frequent invasion of intra-abdominal organs such as liver, pancreas and bile duct by the flukes is documented.

Life Cycle of *Heterophyes heterophyes*

Hosts

Definitive Hosts

Various species of fish-eating mammals (e.g. dogs, cats, wolves, bats, rats, foxes, including humans) and birds (e.g. seagulls and pelicans).

First Intermediate Hosts

Snails (mainly *Pirenella conica, Cerithidea cingulata, Cerithidea fluviatilis*).

Second Intermediate Hosts

Fish particularly mullets, tilapia and gobies (*Mugil cephalus, Mugil capito, Mugil auratus, Mugil saliens, Mugil chelo, Liza menada, Liza haematocheila, Acanthogobius flavimanus, Tilapia nilotica, Tilapia zilli, Aphanius fasciatus, Barbuscanis, Sciaena aquilla, Solea vulgaris,* and *Acanthogobius* spp., *Glossogobius giuris, Tridentiger obscurus, Glossogobius brunnaeus, Therapon oxyrhynchus* and *Scartelaos* sp).

Infective Stage

Eggs containing miracidium are infective for snail; cercariae for fish; and metacercariae present in fish are infective for fish-eating mammals, birds and humans.

Transmission of Infection

Heterophyes infection is acquired by consumption of raw, undercooked or pickled fish. Infection is also acquired by coming in contact with knife or chopping board contaminated with the metacercariae (Fig. 3). On ingestion of the fish, the metacercariae excyst in the small intestine to form juveniles and start living in inter-villous space. They subsequently develop to sexually mature adult worms and remain attached to the intestinal wall. They produce embryonated eggs, which are excreted along with the faeces, which in turn contaminate water bodies including fresh or brackish water.

Infection is transmitted to snail, the first intermediate host after ingestion of eggs containing miracidium. The eggs hatch in the intestine to release miracidium. Miracidium, in turn form sporocysts. Within the sporocyst, radiae develop in two generations to daughter radiae, and transform into cercariae. The cercariae are ophthalmo-pleuro-lophocercous type with a primitive gut and

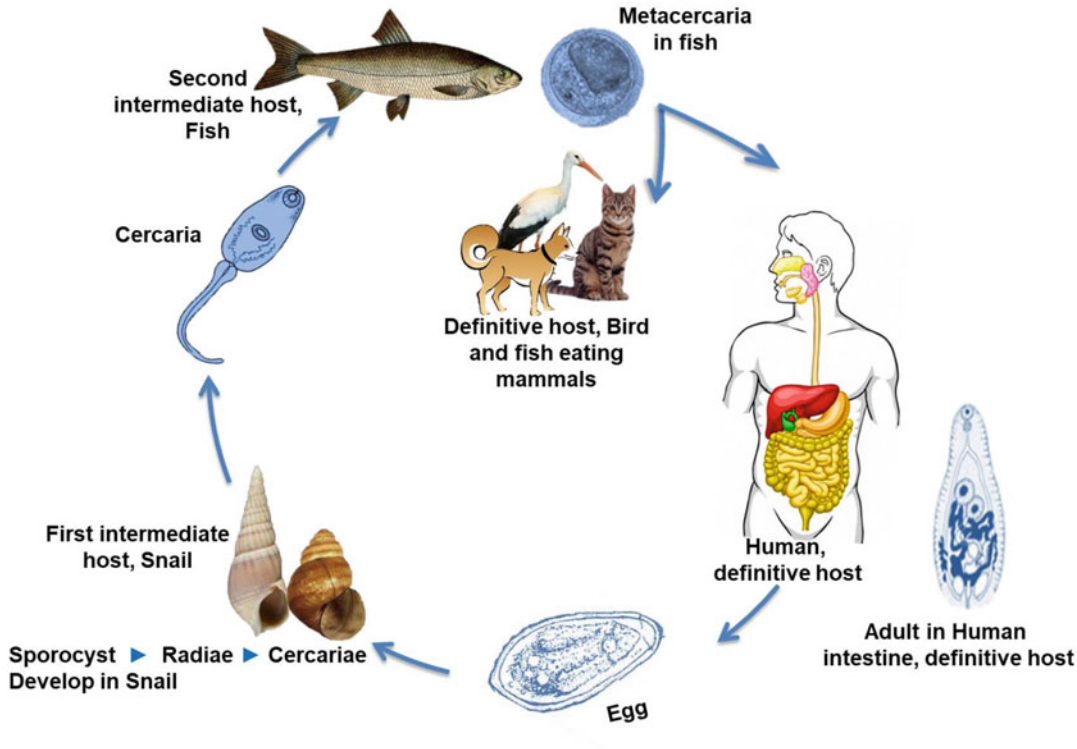

Fig. 3 Life cycle of *Heterophyes heterophyes*

two dark eye spots. They measure about 185 × 90 μm in size and consist of an oral sucker and seven pairs of penetration glands surrounding it. These cercariae, released to the aquatic environment, swim in water and infect the freshwater fish, the second intermediate host of the parasite. They penetrate the skin between the scales of fish and encyst in the tissue and form metacercaria, the infective stage for humans and other hosts.

Pathogenesis and Pathology

Immature developing *Heterophyes* cause intestinal epithelial cell apoptosis in the infected host, during early stages of infection. The villi are swollen, the columnar epithelial cells disintegrate, intestinal crypts enlarge and underlying submucosa becomes oedematous with hyperplastic Peyer's patches at the sites of invasion by the larvae in the intestinal mucosa. Cellular atrophy, fusion and shortening of the intestinal villi are also observed. Immature flukes are sometimes found in the lymphoid follicles and Peyer's patches. Mesenteric lymph glands enlarge and become hyperplastic. At times, mature flukes are seen in the gland. Possibly, the young flukes migrate from the sinuses in Peyer's patches, via the lymphatics. Immune complex deposits are also seen towards later part of infection. Encapsulated *H. heterophyes* eggs in the brain, or eggs of *H. nocens* within an intestinal tumour are rare findings.

Immunology

Levels of serum and intestinal immunoglobulins (IgM, IgG and IgA or IgE) show an inverse relation with the intensity of the infection caused by the developing fluke in the gut in heterophyiasis. Serum IgG, IgM and IgE are elevated in heterophyiasis. Although serum shows an elevated IgE, yet the IgE level in the intestinal secretions remains normal. On the other hand, serum IgA level remains normal in serum but is

always elevated in the intestinal secretions. Similarly, the cell-mediated immune response of the host also shows an inverse relation with the intensity of infection. In experimentally infected puppies with *H. heterophyes,* T-cell count and leucocyte migration are increased. Eosinophil counts of the host increase as a part of immune response against the worm. Spontaneous restoration of damaged intestinal mucosal in heterophyiasis possibly is caused by the strong protective immunity of the host.

Infection in Humans

Incubation period in heterophyiasis varies from 1 to 3 weeks. The infection may remain asymptomatic. In symptomatic infections, abdominal discomfort is the main manifestation along with anorexia and mucous diarrhoea. If untreated, the disease may last for nearly 2–6 months.

Ectopic deposition of eggs resulting in extra-intestinal heterophyiasis has been demonstrated in immunocompromised subjects including HIV-positive patients with involvement of heart valves, myocardium, brain and spinal cord with fatal outcome. Cerebral involvement may result in epilepsy, brain abscess or cystic granuloma.

Infection in Animals

Disease spectrum caused by *Heterophyes* infection in animals is by and large unknown. Histopathology of the intestine of infected cats reveals close association of the parasite with the villi. At the sites of infection, the villi are swollen with destruction of columnar epithelium and swelling of the underlying submucosa and hyperplasia of the Peyer's patches. Immature flukes are found in the lymphoid follicles and Peyer's patches. Mature flukes are also detected within mesenteric lymph nodes.

Epidemiology and Public Health

Heterophyiasis in both humans and animals is widely reported from the countries of Asia, Europe and Africa. The condition has been reported from Egypt, Sudan, Greece, Turkey, Palestine, Italy, Tunisia and India. Although animal infection has been frequently reported in the Middle East, the human infections are infrequent in Saudi Arabia, Iran, Iraq, United Arab Emirates and Kuwait. Sporadic cases of human heterophyiasis have been reported from several countries such as Greece, Turkey, Italy, Spain, Tunisia, Yemen and Sri Lanka. Imported human cases have also been seen in France, Korea and Japan. Heterophyiasis has been reported from the USA after eating 'Sushi' prepared with imported fish. Certain *Heterophyes* species have been found to be more prevalent in specific countries such as *H. heterophyes* from Egypt, Sudan, Palestine, Turkey, India, Middle East, Japan, Korea; *H. nocens* from Korea and Japan; *H. dispar* from Egypt, Middle East and Korea; *H. aequalis* from Egypt, Middle East, and *H. indica* from India (Table 1).

In endemic areas, people who live near brackish water, lake or bank of river usually show higher prevalence and intensity of infection. It may be an occupational hazard among fishermen due to their activities in handling and processing of fish along with cooking and eating behaviours. The practice of eating undercooked fish or salted fish is a risk behaviour. People normally start eating salted fish from third day onwards and the metacercariae of heterophyes survive up to 7 days in salted fish. Therefore, there is a fair chance of transmission of the parasite to humans from salted fish. In endemic areas, open field defecation or defecation from the boats during fishing frequently contaminates water. Fish nurseries are often infected with heterophyid trematodes and spread the infection with the fish seed to different places. Role of nursery fish in spread of heterophyid infection has immense epidemiological importance.

Table 1 Distribution of *Heterophyes* spp. of importance infecting humans

Species	Distribution	1st intermediate host	2nd intermediate host	Definitive host
Heterophyes heterophyes	East Africa, Egypt, Israel, Kuwait, Greece, Turkey, Spain, Sudan, Palestine, India, Middle East, Japan, Korea	**Snail:** *Pirenella conica, Certhideopsilla cingulata*	**Fish:** *Mugil cephalus, Mugil capito, Mugil auratus, Mugil saliens, Mugil chelo, Tilapia nilotica, Tilapia zilli, Liza haematocheila, Acanthogobius flavimanus, Glossogobius giuris, Tridentiger obscurus, Aphanius fasciatus, Barbus canis, Sciaena aquilla, Solea vulgaris*	Humans, dogs, cats, wolves, bats, rats, foxes, badgers, pigs, macaques, seagulls, pelicans
Heterophyes nocens	Korea, Japan, China, Taiwan	**Snail***: Tricula, Akiyoshia, Cerithidea cingulate*, *Cerithidea fluviatilis*	**Fish:** *Mugil cephalus, Liza menada, Tridentigerobscurus, Glossogobius brunnaeus, Therapon oxyrhynchus, Acanthogobius flavimanus, Boleophthalmus pectinirostris, Scartelaos* sp.	Humans, cats, dogs, rats, mice
Heterophyes dispar	Egypt, Middle East, Korea	**Snail:** *Pirenella conica*	**Fish:** *Mugil* spp., *Epinephelus enaeus*, *Tilapia* spp., *Lichia* spp., *Barbus canis*, *Solea vulgaris*, *Sciaena aquilla*	Humans, dogs, cats, wolves, jackals, foxes, kites, rabbits, rats, red foxes
Heterophyes aequalis	Egypt, Middle East	**Snail:** *Pirenella conica*	**Fish:** *Mugil cephalus, Mugil auratus, Mugil capito, Tilapia simonis, Lichia glauca, Lichia amia* and *Barbuscanis*	Humans, cats, dogs, Persian wolves, foxes, red foxes, rats, pigs, rabbits, pelicans, kites, herons

Diagnosis

Microscopy

Microscopy to demonstrate eggs of heterophyes in stool is widely used for diagnosis. However, the eggs show similarity with other members of heterophyid family. It is often difficult to distinguish eggs of heterophyes from those of *C. sinensis* (except the abopercular knob). Specific diagnosis is made by demonstration of adult flukes during gastro-duodenoscopy, surgical procedures in the intestine or after autopsy. Examination of adult flukes in stool, following anti-helminthic treatment and purging is a practical but tedious way in the field or laboratory.

Serodiagnosis

Serodiagnosis has been tried by several workers. Counter-current immunoelectrophoresis (CIEP), intradermal test (ID) and indirect fluorescent immunoassay (IFI), etc. have been evaluated for the diagnosis of *Heterophyes* infection in humans as well as in animals. ID becomes positive early (2 weeks) in comparison to other serological tests and performs well (sensitivity and specificity: 100% and 90%, respectively). Sero-conversion was detected with CIEP and IFI after 3 weeks. Sensitivity of IFI and CIEP was 40% and 20%, respectively; however, specificity for both tests was reported to be 100%. Enzyme-linked immunosorbent assay (ELISA) often shows cross-reactivity with other trematodes.

Molecular Diagnosis

Molecular diagnostic tool for detecting heterophyiasis in animals and humans is yet to gain popularity. PCR for detecting heterophyes in fish has been successfully developed. Sequencing of ITS2 of rDNA can be used to detect and identify species of the *Heterophyes*. Sequencing also helps in tracing the source of infection in imported fish. Multiplex PCR for detecting intestinal parasites including *Heterophyes* has also been evaluated with success (Table 2).

Treatment

Praziquantel is the drug of choice for infection caused by *H. heterophyes*. It disrupts the tegument of *H. heterophyes* in vitro. It is given in a dose of 75 mg/kg orally in three divided doses for 1 day. Cure rate with praziquantel is over 95% for *H. heterophyes*. However, bioavailability of praziquantel is low and erratic because of its poor water solubility. The drug is usually administered with liquids during meal. Studies have shown that niclosamide in a dosage of 14–17 mg/kg bodyweight given twice daily for 3 days cured 74% people infected with *H. heterophyes.*

Prevention and Control

Heterophyiasis and other fish-borne trematode infections have got desep roots in the cultural habit of the population in endemic areas. The practice of eating raw or undercooked fish in these countries and the recent trend of eating exotic dishes containing raw fish in non-endemic areas are difficult to control although health education can produce temporary reduction in cases. Similarly, mass drug treatment has got limited efficacy since, with its discontinuation, cases start rising.

Screening of population at risk with prompt treatment of cases can help in control of heterophyiasis to a certain extent. Control of snail with molluscicides, public health education to create awareness for proper handling and cooking of fish in endemic areas are several suggested public health measures to control heterophyiasis in a community. In fish, the metacercariae survive for about 2.5 years or throughout the lifespan of the fish; therefore, handling during salting, drying or pickling needs utmost care. Smoking the fish at 65 °C kills the metacecariae. Similarly, storing of pickled fish beyond 3–4 days also destroys the *Heterophyes* metacercariae. Water sanitation also reduces fish-borne trematode infections in endemic areas. Prevention of open field defecation to prevent faecal contamination of water sources can interrupt transmission of infection to snail population in water bodies. Biological control of snails in nursery ponds with Indian carp (*Labeo rohita)* was tried with successful reduction of snail populations in field conditions.

Case Study

A 40-year-old fisherman presented with severe abdominal pain, vomiting and diarrhoea. He had vague abdominal discomfort for about 2 months before this acute episode. Routine stool examination for bacteriological examination did not return any bacterial pathogen, but stool microscopy revealed suspected Heterophyid eggs. The patient admitted occasional eating of raw freshwater fish.

1. How can you arrive at the species of this parasite?
2. What advice is to be given to the fishermen for control of the infection in the community?
3. How can you treat this condition?

Research Questions

1. What are the heterophyid parasites that can be acquired through fish?
2. Development of a definitive species-level diagnostic tool.
3. What are the treatment options for heterophyiasis?
4. What is the burden of heterophyiasis in India where fish is extensively consumed?

Table 2 Diagnostic methods for human heterophyiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Microscopic examination Direct visualization in stool after purging, visualization during endoscopy, surgical procedures in the intestine or at autopsy	Eggs and adult in stool and in intestine	**Gold standard test** Drawback: Similarity with eggs of *Clonorchis sinensis* and other members of Heterophyidae is a major drawback for species identification with microscopy. Difficult to differentiate metacercariae in fish
Immunodiagnostics	Intradermal test (ID)	Hypersensitivity	ID shows good sensitivity and specificity and becomes positive early (2 weeks)
	ELISA with (excretory-secretory proteins and somatic protein of adult)	IgGAb against E/S protein and somatic Ag	Sensitivity and specificity good *Limitation*: Cross-react with other trematodes
	Counter-immune-electrophoresis in experimental animal	Precipitating Ab	Sensitivity is 20% but specificity is 100%
	Indirect fluorescent immunoassay in experimental animal	IgGAb	Sensitivity is 40% but specificity is 100%
Molecular assays	Polymerase chain reaction-based RFLP in fish and sequencing of PCR product	ITS 2 of rDNA	Help in identification and speciation of *Heterophyes*. Help in tracing the source of infection in imported fish *Limitations:* Require sophisticated equipment and skilled personnel

5. What is the prevalence of infection in fish nurseries and how to overcome it?
6. How to develop modules on zoonotic diseases for public health intervention which can create awareness in controlling infection by *Heterophyes* spp.?

Further Readings

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Metagonimiasis

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Learning Objectives

1. To know about the parasite as this can cause non-specific abdominal manifestations.
2. To emphasize the importance of molecular methods in the diagnosis.

Introduction

Metagonimiasis is one of the neglected food-borne disease among fish-eating mammals, including humans and birds. Out of seven *Metagonimus* species reported from Asia, *Metagonimus yokogawai*, *Metagonimus takahashii* and *Metagonimus miyatai* are the species that cause infection in humans. Cases of metagonimiasis are mostly reported from South and North Korea, Japan, China, Taiwan, Vietnam, Laos, Thailand, Malaysia, Indonesia, Philippines and India. Traditional fish processing and eating behaviour of humans, preference for exotic food and change in cooking methods expose humans to such food-borne infections including metagonimiasis.

History

Evidence of *Metagonimus* infection in humans existed during the fifteenth century as reported by Pyo Yeon Cho. They demonstrated eggs of *Metagonimus* in the soil along with other parasites, during a construction work near Sejong-ro, Jongro-ku, Seoul, South Korea, where leaders of the Yi dynasty and others lived. However, Fujiro Katsurada, in 1912, first authentically described *Metagonimus* eggs in Taiwan and Japan and proposed a new species, *Heterophyes yokogawai* (synonyms: *Loxotrema ovatum* Kobayashi, 1912 and *Yokogawa yokogawai* Leiper, 1913), currently known as *Metagonimus yokogawai.* Subsequently *M. takahashii* was described by Suzuki in 1930. Miyata (1941) described a fluke in Japan, with widely separated testes, but the taxonomic status as a new species was established only in 1997 as *M. miyatai Saito,* Chai, Kim, Lee & Rim, 1997. Subsequently, *M. minutus* was described from Taiwan by Katsuta (1932), *M. katsuradai* from Japan and Russia by Izumi (1935), *M. ovatus* from Japan by Yokogawa (1913), *M. otsurui* was described by Saito and Shimizu (1968) and *M. hakubaensis* by Shimazu (1999) from Japan. *M. suifunensis* was reported by Shumenko et al. (2017) from Russia.

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Taxonomy

The genus *Metagonimus* belongs to family Heterophyidae; order Opisthorchiida; subclass, Digenea; class, Trematoda; sub-phylum, Neodermata and phylum: Platyhelminthes.

M. yokogawai is the type species. Molecular studies have supported the taxonomic classification. Numerical taxonomy was also applied to study other *Metagonimus* species and proposed *M. miyatai* as a subspecies of *M. takahashii.*

Genomics and Proteomics

The genome of *M. yokogawai* has not been sequenced. Genomic studies using PCR-RFLP of ITS1 and Cytochrome oxidase 1(COX1) have confirmed that *M. yokogawai, M. takahashii* and *M. miyatai* could be genetically separated from each other. Studies of chromosomes and karyotypes as well as sequence of 28S ribosomal DNA (rRNA) and CO1 have also supported the view. Phylogenetic analysis based on 28S rRNA, ITS2 and COX1 sequence placed *M. yokogawai, M. takahashii, M. miyatai, M. hakubaensis,* in one clade and *M. katsuradai, M. otsurui* in another. Sequencing of ITS1–5.8S–ITS2 region and 28S nuclear rRNA of adult has added another new species (*M. suifunensis*) of *Metagonimus*. A detailed study about proteomics of *M. yokogawai* is lacking.

The Parasite Morphology

Adult Worm

Adult *Metagonimus* species vary within 0.8–2.320 × 0.4–0.75 mm in their size; *M. yokogawai* (0.800–1.320 mm), *M. takahashii* (0.863–1.193 mm) and *M. miyatai* (0.998–1.300 mm) and *M. minutus* (0.457 mm). *Metagonimus* differs morphologically from *Heterophyes*, as they have a smaller, sub-median ventral sucker and no genital sucker (Fig. 1). The ventral sucker is relatively larger than the oral sucker. They have paired testes and ovaries. Both the testes of *M. yokogawai* are closely placed in the posterior end of the adult worm. The vitelline follicles are placed between the posterior testis and the ovary. Testes of *M. takahashii* are placed wide apart, and vitellaria is distributed beyond the posterior testis. However, distribution of vitellaria is lacking in *M. miyatai*. The uterine tubule in *M. takahashii* and *M. miyatai* crosses the anterior testis but in *M. yokogawai,* it does not overlap the testis.

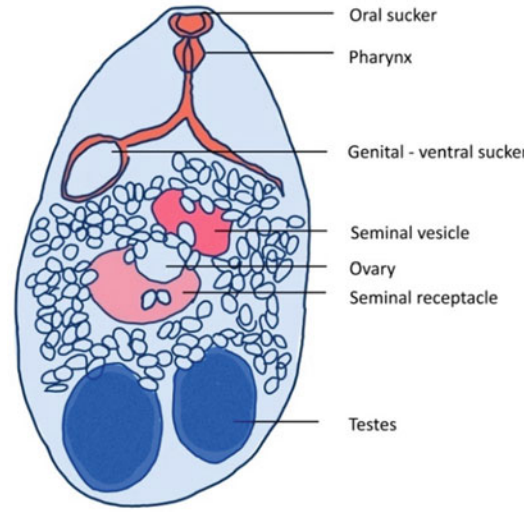

Fig. 1 Schematic diagram of adult *Metagonimus* spp.

Eggs

Metagonimus eggs are yellowish brown, operculated with inconspicuous shoulder (Fig. 2). The eggs of various species vary between 28.4–32.6 and 16.1–18.9 μm in their sizes (*M. yokogawai,* 28.4 ± 1.3 × 16.1 ± 1.4 μm; *M. takahashii,* 32.6 ± 0.8 × 18.9 ± 1.3 μm; *M. miyatai,* 30.8 ± 0.9 × 17.8 ± 0.8 μm; *M. minutus* 23 μm). Surface of the egg is covered with multiple ridges.

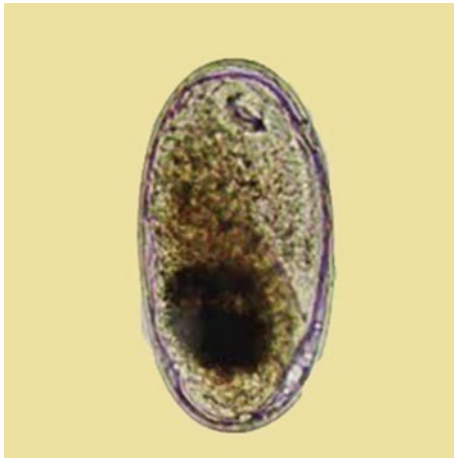

Fig. 2 Morphology of egg of *Metagonimus* spp. (Courtesy: CDC DPDx)

Metacercaria

Metacercariae, the infective stage of the parasite, are round or slightly elliptical and measure 0.14–0.16 mm in diameter. The larva within metacercariae reveals oral and ventral suckers, and excretory granular vesicle.

Cultivation of Parasites

Metagonimus species has not been grown in vitro.

Laboratory Animals

Golden hamster is a good laboratory animal model for *Metagonimus* spp. Mice, rat, gerbil etc., are other animals that have been used for development of *Metagonimus* species. Worm recovery after feeding *M. yokogawai* metacercariae orally in hamsters was reported to be 75.3%, 70.0% in mice, 23.3% in rats and 6.0%in gerbils.

Life Cycle of *Metagonimus* spp.

Hosts

Life cycle of *Metagonimus* is completed in three hosts (Fig. 3).

Definitive Hosts

Humans, dogs, cats, rats, mice and fish-eating birds are the definitive hosts.

First Intermediate Host

Snail (*Semisulcospira libertina*, *Semisulcospira coreana*, *Semisulcospira reiniana*).

Second Intermediate Host

Fish (golden carp (*Carassius auratus*), common carp (*Cyprinus carpio*), *Plecoglossus altivelis, Tribolodon hakonensis*, *Tribolodon taczanowskii, Tribolodon ezoe*, *Lateolabrax japonicus, Zacco temminckii, Protimus steindachneri, Acheilognathus lanceolata* and *Pseudorashora parva.*

Transmission of Infection

Humans, other mammals and birds acquire infection on consumption of raw, pickled or undercooked fish containing metacercariae of the *Metagonimus* spp. Metacercariae present in the fish excyst in the small intestine and develop into an adult in about 7 days. Adults attach to the intestinal lumen in the crypts of Lieberkühn or in inter-villous space to cause disease. *Metagonimus* are hermaphroditic and eggs are self-fertilized. These eggs which are excreted in the faeces contaminate freshwater bodies or brackish water.

Operculated and yellowish brown eggs (28.4–32.6 μm long) passed by definitive host are ingested by snails. The eggs hatch in the snail's intestine and penetrate to develop into sporocysts. The miracidium in the eggs develops into sporocyst and subsequently transformed into multiple rediae, and cercariae. The cercariae are ophthalmo-pleurolophocercous type with prominent eye spot. These cercariae coming out of snail infect the fish by penetrating the skin in between scales of fish (second intermediate host) to form metacercariae. These metacercariae remain encysted under the scale, fins, tail and gills or in tissue cause infection in humans, mammals and birds.

Pathogenesis and Pathology

The juvenile worms reside in the crypts of Lieberkühn of small intestine of the definitive

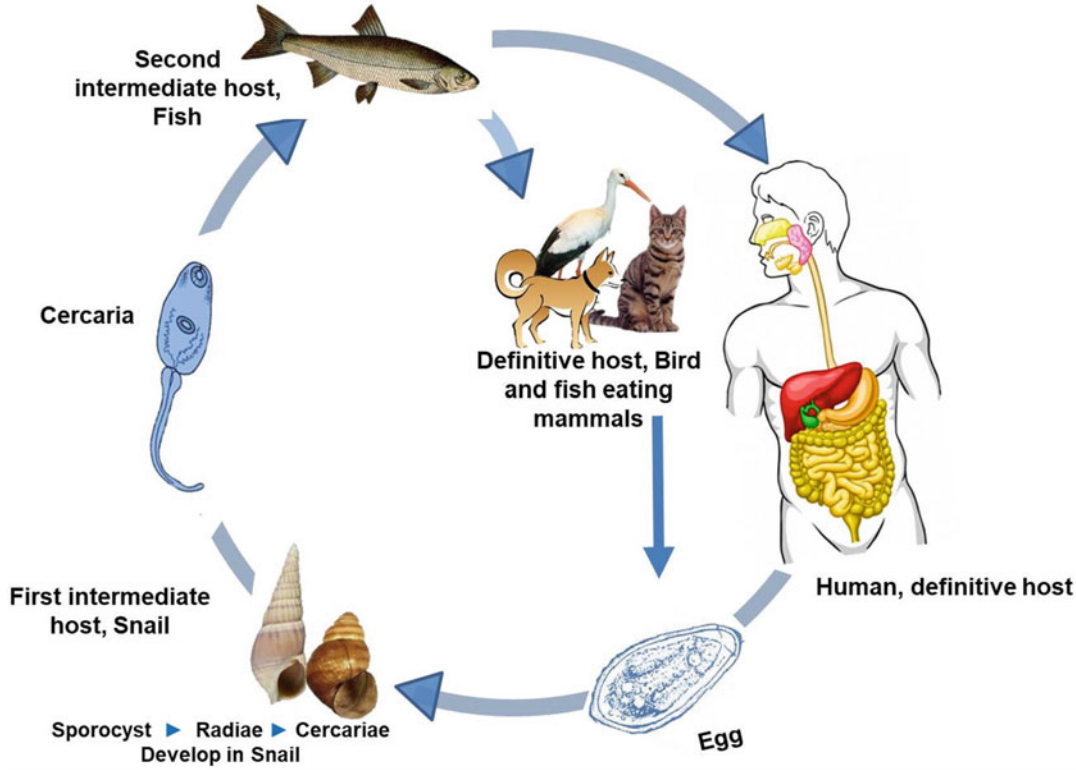

Fig. 3 Life cycle of *Metagonimus* spp.

hosts and in about 5 days develop into adult worms. Chai in his series of experimental observations recorded that the adult worm causes mucosal damage mechanically and excretory-secretory proteins (ESP) of the adult cause toxic reaction with variable degree of inflammations in the tissue. Intestinal villi show atrophy, blunting, fusion, oedema of tips with hyperplasia of crypts. Intestinal mucosa shows congestion, goblet cell hyperplasia, mastocytosis with inflammatory cell infiltrations in the stroma. Mucosal change prevents the absorption of nutrients, hence an increase in permeability of body fluid results in watery diarrhoea. Malabsorption and decreased enzyme activities are seen in acute infection. In animals, the intestinal pathology is normalized by 3–4 weeks after the infection. In immunocompromised host, the adult worm even can invade the deeper tissues.

Immunology

In immunocompetent hosts, deeper invasion of the worm is prevented by host's strong innate immunity and can normalize the pathology in 3–4 weeks' time. The intestinal intraepithelial lymphocytes (CD8 + cytotoxic T-cells), lymphocytes of lamina propria (IgA secreting B-cells), mucosal mast cells, goblet cells and circulating eosinophils are the main components of the host immunity. Experimental infection of animals by *M. yokogawai* has shown different parts of the parasite such as syncytial tegument, tegumental cell cytoplasm, vitelline cells and epithelial lamellae of the caecum to be antigenic. SDS-PAGE analysis has demonstrated 14 immunogenic proteins, of which 66 kDa and 22 kDa proteins were found to be parasite-specific. Another protein (100 kDa) of the tegument of

M. yokogawai is also shown to be of promise in immuno-diagnosis.

Infection in Humans

M. yokogawai infection in humans is a self-limiting disease, nevertheless the symptoms persist for a year or more.

Incubation period is 14 days.

Clinically, *M. yokogawai* infection in humans presents with epigastric pain, fatigue, malaise, watery diarrhoea, nausea, lethargy, anorexia, prostration and abdominal pain of various degrees. Rarely, eggs enter circulation through intestinal lymphatics and reach brain, spinal cord or heart showing egg associated granulomas. Such patients present with seizures, neurologic deficits or cardiac insufficiency. Routine blood examination shows eosinophilia.

In endemic areas, certain infected patients may progress to become a carrier for the disease.

Infection in Animals

Disease spectrum in animals is not well understood. Dogs, experimentally infected with *M. yokogawai,* and mice infected with *M. miyatai* often develop watery diarrhoea with signs and symptoms of malabsorption.

Epidemiology and Public Health

Due to lack of systematic study, the accurate data on global burden or prevalence of metagonimiasis are not available. However, different reports suggest that metagonimiasis is widely prevalent in about 19 countries. High prevalence of metagonimiasis has been recorded in areas of intense poverty in low- and middle-income countries in tropical and subtropical regions, sub-Saharan Africa, Asia, Latin American countries and among immigrants and refugees of North America and Europe (Table 1). Human

Table 1 Distribution of *Metagonimus* spp. of human importance

Species	Distribution	First intermediate host	Second intermediate host	Definitive host
Metagonimus yokogawai	Far East, Siberia, the Balkans, Spain Russia, Korea, Japan, China, Hong Kong, Taiwan, India	**Snail:** *Semisulcospira libertina*, *Koreanomelania* spp. *Scoreana*, *Thiara granifera*	**Fish**: *Plecoglossus altivelis*, *Odontobutis obscurus*, *Salmo perryi*, *Tribolodon hakonensis,* golden carp (*Carassius auratus*), common carp (*Cyprinus carpio),* *Opsariichthys pachycephalus*, *Zacco platypus, Zacco temminckii, Distoechodon turmirostris, Varicorhinus barbatulus, Hemibarbus labeo, Acrossocheilus formosanus, Sinibrama macrops, Lateolabrax japonicus, Protimus steindachneri, Acheilognathus lanceolata*, *Pseudorashora parva*	Humans, dogs, cats, rats, mice, gerbils, golden hamsters, fish-eating birds
Metagonimus takahashii	Japan, Republic of Korea	**Snail:** *Semisulcospira coreana, Koreanomelania nodifila*	**Fish:** *Carrassius carassius, Cyprinus carpio, Tribolodon taczanowskii, Lateolabrax japonicus*	Humans, mice, dogs
Metagonimus miyatai	Republic of Korea, Japan	**Snail**: *Semisulcospira globus, Semisulcospira libertina, Semisulcospira dolorosa*	**Fish**: *Morocco steindachneri, Zacco platypus, Zacco temmincki*	Humans, mice, rats, hamsters, dogs
Metagonimus minutus	Taiwan, China	**Snail**: *Semisulcospira libertina*	**Fish**: Mullet, *Mugil cephalus*	Humans, mice, cats

infections outside endemic areas have also been reported after consumption of imported fish or fish preparation ('Sushi'). Intestinal trematodiasis such as heterophyidae appears to be increasingly associated with fish culture in certain areas and fish-eating habits of the population.

Since a single fish can harbour several (>10,000) metacercariae, infection may occur even after consumption of one raw, undercooked, pickled, salted or fermented fish. Disease can also be transmitted through cutting knife and chopping board contaminated with the metacercariae.

Infection with *M. yokagawai* is endemic mostly around the large and small fresh water streams while *M. miyatai* and *M. takahashii* are more common along the big rivers. In Japan, the disease is most common in rural areas perhaps due to traditional habit of consuming preserved and raw freshwater fish. However, of late, these infections are also reported among people from high socio-economic classes in Hong Kong and Japan, owing to exotic food habits. Three cases of metagonimiasis have also been reported from India, two in 1994 (species not mentioned) and one case of *M. yokagawai* from New Delhi in 2005.

Diagnosis

Various diagnostic methods are available for diagnosis of *Metagonimus* infections in humans (Table 2).

Microscopy

Microscopy of stool to detect eggs or adult worms in stool is widely used for diagnosis of metagonimiasis. *Metagonimus* eggs are yellowish brown, operculated with inconspicuous shoulder. Heterophyidae eggs are similar in their morphology; therefore, species-specific identification of *Metagonimus* by microscopy is not possible. Microscopy is less sensitive and not suitable for diagnosis of ectopic *Metagonimus* infection.

Immunodiagnosis

Immunodiagnosis by antibody-based tests are frequently used for diagnosis of metagonimiasis. Excretory-secretory (ES) proteins and somatic antigen of adult flukes have been evaluated for use in many immunodiagnostic tests including the sELISA. Monoclonal antibodies produced against somatic antigen of *M. yokogawai,*100 kDa and 67 kDa proteins have also been evaluated in

Table 2 Diagnosis of human metagonimiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	1. Microscopic examination 2. Biopsy	Eggs and adult in stool and tissue	**Gold standard test** *Limitation*: Similarity of egg morphology with the other members of Heterophyidae makes it difficult for species identification. Less sensitive technique and cannot differentiate metacercariae in fish
Immunodiagnostics	ELISA using E/S proteins and adult somatic antigen	Circulating IgG against 100 kDa and 67 kDa proteins of *Metagonimus yokogawai*	Confirms active infection *Limitation:* Cross-reactivity with *other members of the genus*
Molecular assays	Polymerase chain reaction-based RFLP	18SrRNA, 5.8SrRNA gene and internal transcribed spacer 1 (ITS1)	High sensitivity and specificity for identification and speciation of *Metagonimus* in fish *Limitations:* Require skilled personnel
	Gene sequence	28SrDNA, ITS2 and mitochondrial *cox*1 gene	Can separate in two definite clades
	Multiplex real-time PCR	ITS 1, ITS 2	Eight intestinal parasites including *Metagonimus yokogawai* can be detected in a single platform without much cross-reactivity

the diagnosis of metagonimiasis. Cross-reactivity with other trematode of heterophyidae is major limitation of the serodiagnostic methods.

Molecular Diagnosis

Polymerase chain reaction-based restriction fragment length polymorphism of the 18SrRNA, 5.8SrRNA gene, and internal transcribed spacer 1 (ITS1) were used for molecular identification and speciation of *Metagonimus* in fish with success. Sequencing of 28S ribosomal DNA (rDNA, ITS2) and mitochondrial *cox*1 gene could separate six species of *Metagonimus* into two clades (*M. yokogawai*, *M. takahashii*, *M. miyatai*, *M. hakubaensis*) and (*M. otsurui* and *M. katsuradai)*.

Multiplex real-time PCR assay was developed and evaluated to detect eight intestinal parasites including *M. yokogawai* in a single platform, thus making it better than microscopy with high efficiency and accuracy in detection and identification of intestinal parasites including *Metagonimus* species.

Treatment

Praziquantel is recommended for treatment of metagonimiasis in both adults and children in a single dosage of 75 mg/kg/day in three divided doses. The WHO (2002) recommended its use in pregnant and lactating women as well. Praziquantel is administered along with liquids during meals. It is well tolerated and side effects are mild and transient. Praziquantel acts on the trematode by causing muscular paralysis and subsequent detachment of the flukes from the wall of the intestine. Bithionol, niclosamide and nicoflan have also been evaluated for treatment of metagonimiasis with good cure rate.

Prevention and Control

Prevention and control of metagonimiasis in humans depend on control of *Metagonimus* infections in all the three hosts (snail, fish and mammals including humans).

Control of snail populations by use of molluscicides prevents metagonimiasis to a great extent. Sanitization of fish nurseries is achieved by emptying the water body along with mud and cleaning and filtering the inlet water with mesh to prevent snail entry. Biological control of snails in nursery ponds by Indian carp (*Labeo rohita)* has also been evaluated with encouraging results.

Like most of the Heterophyidae, *Metagonimus* metacercariae in the fish can also survive about 2.5 years or throughout the lifespan of the fish; therefore, public awareness to avoid raw or undercooked fish consumption, handling during salting, drying, pickling, processing for food, is important. Implementation of water sanitation, and discouraging open field defecation can prevent release of eggs to contaminate water sources. Smoking at a temperature of 65 °C or more and storing the pickled fish for about 4 days can kill the metacercariae of *Metagonimus* in fish. Irradiation of fish to destroy metacercariae of *M. yokogawai* has also been recommended, but acceptability of irradiated fish for food and the cost of irradiation are the major barriers. Screening of fish in market and imported fish for *Metagonimus* and other fish-borne parasites is of importance. Therefore, mandatory surveillance of nurseries helps in controlling spread of fish-borne trematodes.

Mass chemotherapy and health education to change food habits are important to control infection in humans. Control of metagonimiasis in pet animals, birds, fish and snails is challenging. Metagonimiasis surveillance in wildlife and birds is difficult; however, pet animal screening in endemic areas and early treatment is important to control the reservoir of infection. Pet dogs and cats need to be screened regularly for fish-borne infection. All egg-positive persons including workers and residents and the domestic animals, including dogs, cats, and pigs in neighbouring households, need specific treatment by chemotherapy.

Case Study

During routine survey for estimating burden of intestinal parasites in rural areas of Assam, India,

during 1994, stool samples were examined. In one of the villages with a population of 1082, samples from 443 individuals were collected randomly. Two samples out of 443 (0.44%) showed presence of typical eggs of heterophyid in their stool. Both were husband (40 years) and wife (35 years), agriculture workers, residing in Dibrugarh district of Assam. On questioning, they gave history of abdominal pain and had few episodes of dysentery for the last 1 year. On examination, no significant clinical abnormality was detected. They have never moved outside Assam since birth. Routine blood examination and hepatic function tests were within normal limits. Stool samples, collected in 10% formol saline were examined after concentration. Microscopy revealed poly-parasitic infection including heterophyid eggs. Suspected heterophyid eggs were yellowish brown, operculated with thick walls and well-developed miracidium inside. Opercular shoulder was indistinct, mean length of 20 eggs was 31.5 μm ± 2 SD (29.3–36.0 μm) and breadth was 22.4 μm ± 1.9SD (21.3–27 μm). Egg morphology suggests that the eggs belong to the family Heterophyidiae. *Clonorchis* was not considered due to absence of abopercular knob. Majority of *Heterophyes* belonging to the family are detected in brackish water. There is no source of brackish water and area is far from sea. Further, people of the area consume only freshwater fish. The cases have not moved out of the area since birth. Hence, logically the eggs were thought to be of *Metagonimus* species. Infection with species belonging to heterophyid is an interesting and maiden finding and perhaps it indicates presence of indigenous transmission in fish. Further, it may be noted that people of Assam are fish eaters and many have a habit of eating smoked fish; therefore, they have potential risk of heterophyid infection. This finding may be the tip of an iceberg and further studies are needed.

1. What further studies can be undertaken based on the above findings to determine the prevalence of *Metagonimus* in the region?
2. How can the species of *Metagonimus* be determined?
3. What health education should be given to the community to prevent fish-transmitted parasitic infections?

Research Questions

1. What is the burden of metagonimiasis in India where fish is extensively consumed?
2. Why is it important to do research aiming to develop test system to detect infection in nurseries and in fish and snails?
3. How to go for screening of imported consignment of fish and fish product to prevent large-scale introduction of *Metagonimus* and other fish-borne parasites in the country?
4. Why there is an urgent need for development of immunodiagnostic and molecular diagnostic tool as an alternative to microscopy?

Further Readings

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Nanophyetus Infection

Parvangada Madappa Beena and Anushka Vaijnath Devnikar

Learning Objectives

1. To know about the limited geographic distribution of the parasite.
2. To understand Salmon Poisoning Disease in dogs and its pathogenesis.

Introduction

Nanophyetus spp. (*Troglotrema*), a parasite of carnivorous animals, particularly dogs, is an uncommon trematode that causes occasional zoonotic infection in humans. The trematode is known to infect 32 different species of piscivorous mammals and birds. Common definitive hosts include dogs, raccoons, minks, jackals and foxes. *N. salmincola* is endemic in parts of northern California, Oregon and Washington in the USA. Till date, only 20 human cases have been reported. Human infection is usually asymptomatic.

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History

In the early 1800s, a fatal disease among dogs following the ingestion of salmon was recorded in Oregon, USA. This gave rise to the name 'Salmon Poisoning Disease' (SPD). The condition was believed earlier to be caused by distemper (1859), amoebae (1911) or bacteria (1925), until 1925, when Donham demonstrated the presence of small trematodes in the intestine of infected dogs. Chapin (1926) described the trematode and suggested the name *Nanophyes salmincola* belonging to family Heterophyidae. In 1928, Chapin changed the name to *Nanophyetus salmincola* in the genus *Nanophyes*. Witenberg restudied the morphology, and based on his findings transferred the fluke to family Troglotrematidae and concluded that *Nanophyetus* is a synonym for *Troglotrema*. Later Wallace (1935) concurred that it belongs to family Troglotrematidae but disagreed to its assignment to the genus *Troglotrema*. He retained the genus *Nanophyetus* and created a new subfamily *Nanophyetinae* for it. A similar parasite was described in humans among eastern Siberian natives by Skrjabin and Podjapolskaja (1931) and was named as *Nanophyetus schikhobalowi*. Meanwhile, Simms (1931) hypothesized that SPD was probably due to a rickettsial or haemosporidian infection, Cordy and Gorham (1950) described intracytoplasmic rickettsial bodies in lymphatic aspirates from

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dogs dying of SPD. Philip (1953) named the agent *Neorickettsia helminthoeca*.

It was not known that *N. salmincola* was capable of causing human infection until a researcher experimentally infected himself in 1956. The first record of natural human infection in the USA was in 1987. The first record of human infection caused by *N. schikhobalowi*, the Siberian species, was described in the inhabitants of Siberia in 1931.

Taxonomy

Genus *Nanophyetus* belongs to family Troglotrematidae; superfamily Plagiorchioidea; order Plagiorchiida; and class Trematoda in the phylum Platyhelminthes. Genus *Nanophyetus* includes four species that cause infections in a wide variety of hosts. *Nanophyetus salmincola* (Chapin, 1927) is found in American Pacific northwest, *N. schikhobalowi* (Skrjabin and Podiapolskaia, 1931) in Siberia, *Nanophyetus asadai* (Yamaguti, 1971) and *Nanophyetus japonensis* (Saito, Yamashita, Watanabe and Sekikawa, 1982) in Japan.

Genomics and Proteomics

The genetic diversity of *Nanophyetus* spp. has been studied using mitochondrial and nuclear DNA markers. The genetic diversity of *Nanophyetus* spp. from Russia was compared with those from North America. The mtDNA *nad1* gene of *N. schikhobalowi* from Russia was sequenced and compared with that of *N. salmincola* from USA. The genetic difference between the Russian and the American samples was 15.5%. Studies involving variation in sequences of nuclear ribosomal genes (18S, ITS1-5·8S-ITS2 and 28S) showed a high level of divergence in each rDNA region, and it was concluded that *N. salmincola* and *N. schikhobalowi* are independent species, whereas *N. schikhobalowi* and *N. japonensis* are sister species.

The Parasite Morphology

Nanophyetus species has both adult and larval forms. The larval forms are miracidia, rediae, cercaria and metacercaria. Sporocysts are not found in this species.

Adult Worm

N. salmincola is a small intestinal trematode. It measures approximately 0.8–2.5 mm in length and 0.3–0.5 mm in width. It can take any shape ranging from a sphere to a blunt rod. The ventral sucker is slightly smaller than the oral sucker. It is a hermaphrodite with well-developed male and female reproductive systems. There are two testes, a single round ovary, a prominent cirrus sac but no cirrus, a seminal receptacle (fertilization chamber) and a uterus capable of holding 5–16 eggs.

Eggs

Eggs are ovoid and light brown in colour and are bile-stained. *N. salmincola* eggs measure 87–97 μm by 38–55 μm in size, and *N. schikhobalowi* eggs measure 52–82 μm by 32–56 μm in size. Eggs of *N. salmincola* are larger than the eggs of *N. schikhobalowi*. They are operculated at one end and have a small blunt point at the other end (Fig. 1). They are heavy, hence sink to the bottom in water. Eggs are seen in faeces of infected definitive host 5–8 days after eating infected fish. Freshly passed stools contain unembryonated eggs.

Metacercariae

These are oval in shape and their cyst wall is thin and transparent. As the metacercariae become older, they become larger and the cyst wall becomes tougher. This is the infective stage of the trematode.

Fig. 1 Egg of *Nanophyetus salmincola* (Source: DPDx/CDC)

Cultivation of Parasites

The miracidium from the eggs can be hatched using tap water. *N. salmincola* eggs can be hatched in water at pH 7.0 under continuous illumination at temperature range of 21–31 °C. The hatching rate increases with decreasing temperatures while death of the eggs increases with increasing temperature. *N. schikhobalowi* eggs can be hatched in standing water at 16–22 °C. For either species, the time required was about 3–5 months. If a surface layer of ice was present, then eggs hatched in 35–45 days for *N. schikhobalowi*.

Laboratory Animals

Domestic dog, domestic cat and white rat are the common experimental animals used to study the pathogenesis in definitive hosts. Young rainbow and brook trout and other salmonids can be experimentally infected by exposing them to the infected snails.

Life Cycle of *Nanophyetus* spp.

Host

The life cycle of *Nanophyetus* spp. is similar to those of other intestinal trematodes. It involves two intermediate hosts (a snail and a fish) and a piscivorous definitive host (Fig. 2).

Definitive Hosts

The trematode is known to infect 32 different species of piscivorous mammals and birds. Common definitive hosts include dogs, raccoons, minks, jackals and foxes.

Intermediate Hosts

The first intermediate host is a snail. *Oxytrema silicula* (also known as *Jugo plicifera* or *Juga silicula* or *Goniobasis plicifera)* is the snail species for *N. salmincola*, whereas *Oxytrema silicula* and pleurocerid stream snails *Semisulcospira laevigata* and *Semisulcospira cancellata* are the snail species for *N. schikhobalowi.*

Fish is the secondary intermediate host. *Nanophyetus* spp. is known to infect 34 species of fish, including salmonid fish, especially coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Oncorhynchus mykiss*).

Transmission of Infection

The infection is transmitted to the definitive host by consuming raw or undercooked infected fish or their eggs.

After ingestion, the metacercariae on reaching the gastrointestinal tract excyst and develop into adult worms within 6–7 days of infection. The sexually mature adult worms start producing unembryonated eggs which are excreted in the faeces and contaminate the water of ponds, rivers and other water bodies in which they develop further.

Eggs require nearly 31–200 days to hatch depending on various environmental factors. Still water and low temperatures favour hatching of the eggs. A fully developed egg contains an

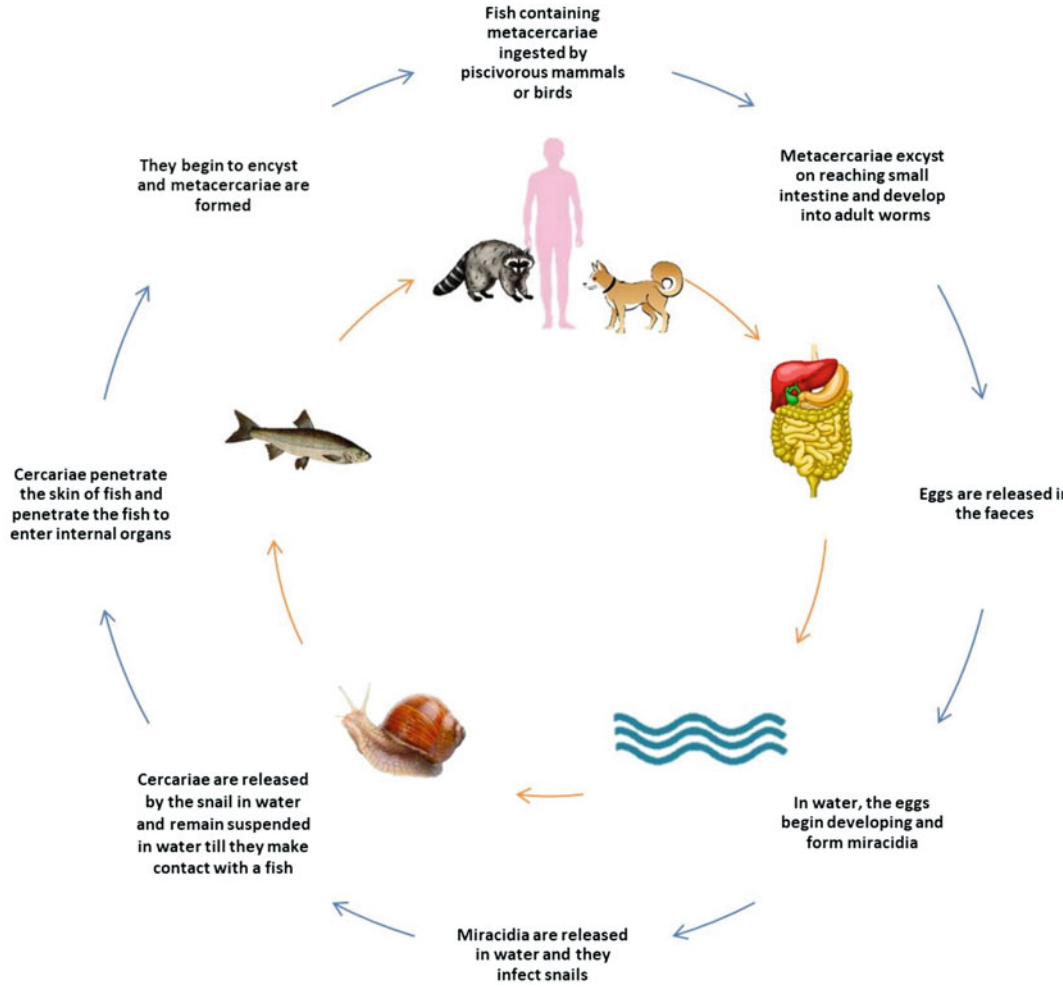

Fig. 2 Life cycle of *Nanophyetus* spp.

active miracidium. The miracidium measures 87–105 μm by 37 μm in size. The miracidium of Siberian species is relatively smaller and measures 63–105 μm by 21–42 μm. It has four rows of epithelial cells with one or more pores, and an apical gland surrounded by two multicellular glands, centrally located glands, germ cells and a pair of flame cells. On hatching, actively motile miracidia are released in water. In water, the miracidium swims in characteristic long, graceful curves.

These free miracidia infect freshwater snails (*Oxytrema silicula)* in which they develop into rediae. Rediae of various sizes ranging from 45 μm to 300 μm are found in the body of infected snails. They are distributed in all tissue of the host snail but are found more in numbers in the digestive glands. Smaller rediae are elongated, tapering posteriorly and are very active. Larger ones are more cylindrical and sluggish. Smaller rediae contain few cercariae while the mature rediae contain as many as 74–76 cercariae. Rediae mature and produce numerous cercariae which are released intermittently into water by the snail.

The xiphidiocercaria is microcercous. It measures 310–470 μm by 30–150 μm in size, with an average length of 390 μm, the tail is blunt and conical. It bears fine hair-like spines.

The cuticula is transparent and possesss fine backward-directed spines. They remain suspended in water until they make contact with fish, the second intermediate host. The cercariae penetrate the skin of fish within minutes of making contact. Once the cercariae penetrate the fish, they lose their tail and migrate towards the internal organs, where it begins to encyst. These cysts are known as metacercariae. Metacercariae are found in all tissues of the fish, but they are found in large numbers in the kidney, muscle and fins. When these fish laden with mature metacercariae are consumed by piscivorous mammals or fish, they get infected and the cycle continues. Raccoons, skunks and minks act as reservoir hosts.

Pathogenesis and Pathology

Nanophyetus spp. are parasites of the small intestine and are found primarily in the duodenum. The adult worm migrates actively on the mucosa of the host's intestine and buries itself partially or almost completely in the villi of the small intestine. The parasite causes damage in the intestinal tissue by its suckers.

Salmon poisoning disease in dogs caused by *N. helminthoeca* is associated with the extensive involvement of the lymphoreticular system resulting in lymphadenopathy and hyperplasia, haemorrhage and necrosis of other lymphoid organs and tissues.

Immunology

The dogs have shown lifelong immunity to SPD, but not to the trematode. Immunity to the disease in humans is yet to be studied.

Infection in Humans

Nanophyetus infection in humans is usually asymptomatic. In symptomatic cases, the clinical signs and symptoms are non-specific. These include diarrhoea, abdominal discomfort, bloating, nausea, vomiting, weight loss and fatigue, and are often indistinguishable from those of other gastrointestinal infections.

Siberian variant of *Nanophyetus* is a natural human parasite with a high incidence of infection (95–98%). Patients infected with the Siberian variant in symptomatic cases present diarrhoea or constipation, unpleasant sensations in the epigastric region on an empty stomach, copious nocturnal salivation and gastric pains. If left untreated, the infection may persist for 2 or more months and may resolve spontaneously.

Infection in Animals

The clinical manifestation of *Nanophyetus* infection in animals varies depending on the species of animal infected.

Nanophyetus causes Salmon Poisoning Disease, a disease with more than 90% mortality in dogs, foxes, coyotes and other canids. This fatal illness is due to an endosymbiont, *Neorickettsia helminthoeca*. Incubation period varies from 5 to 12 days. An infected dog initially presents with sudden onset of high fever (40 to 42 °C), sometimes with conjunctival exudate. This is accompanied by marked anorexia, weight loss, weakness and depression. The fever lasts for 4–7 days after which the body temperature returns to normal or may fall below normal (hypothermia). Later, they develop vomiting and diarrhoea or bloody diarrhoea. If left untreated, the animal succumbs to the illness within 2 weeks.

Raccoons develop mild fever and recover spontaneously.

Epidemiology and Public Health

Nanophyetus spp. are capable of infecting a wide range of definitive hosts and intermediate hosts. However, they are host-specific when it comes to the first intermediate host. Therefore, the geographical distribution of first intermediate host is the main factor in determining the enzootic area

Table 1 Epidemiological aspects of *Nanophyetus* spp.

Species	Definitive hosts	First intermediate hosts	Second intermediate hosts	Geographic distribution
Nanophyetus salmincola	Dogs, raccoons, minks, jackals and foxes	Snails: *Oxytrema silicula*	Fish including salmonids	Parts of USA and Canada
Nanophyetus schikhobalowi	Dogs, raccoons, minks, jackals and foxes	Snails: *Oxytrema silicula, Semisulcospira laevigata* and *Semisulcospira cancellata*	Fish including salmonids	Eastern Russia
Nanophyetus japonensis	Dogs, Japanese badger and Japanese water shrew	Snails	Japanese common char	Japan

of the fluke (Table 1). Human infections due to *N. salmincola* in the northern Pacific region are uncommon. To date, around 20 cases have been reported.

In animals, *N. salmincola* infection is endemic in parts of northern California, Oregon and Washington in the USA. It is also endemic in British Columbia, Canada. *N. schikhobalowi* is endemic in eastern parts of Russia within Amur and Ussuri valleys of Khabarovsk territory and the Sakhalin Island (Fig. 3). Raccoons are numerous in number in regions where this trematode is endemic. They eat fish from water and defecate in the vicinity. Hence, it is suggested that they are responsible for maintaining the life cycle in nature. The ecology of both *N. salmincola* and *N. schikhobalowi* is similar. Nevertheless, in contrast to the Pacific northwest species, the prevalence of infection is high among locals residing in endemic zones in Siberia. Although local Siberian dogs eat raw fish and are infected by the fluke, they do not develop SPD. This is either because *N. schikhobalowi* does not carry *Neorickettsia helminthoeca* or may be they carry a different species that is probably non-pathogenic.

Diagnosis

Diagnosis in Humans

A presumptive diagnosis of *Nanophyetus* spp. infection in humans is made with a history of ingestion of raw or uncooked fish or fish eggs, coming from an area endemic for the disease and presenting with typical signs and symptoms. Most patients infected with the trematode have eosinophilia.

Microscopy

Demonstration of characteristic light brown and operculated egg of *Nanophyetus* spp. in the faeces confirms the diagnosis of the condition. Eggs are usually demonstrated in the faeces of patients 5–8 days after consumption of infected fish (Table 2).

Serodiagnosis

No serological tests are available for diagnostic purpose.

Molecular Diagnosis

It may be used for species differentiation using rDNA as target.

Diagnosis in Animals

Nanophyetus infection in animals is diagnosed by demonstrating eggs of *Nanophyetus* in their faeces.

Diagnosis of salmon poisoning disease in dogs is made by demonstration of characteristic intracytoplasmic rickettsial bodies in lymph node aspirate or necropsy specimens stained with Giemsa or Macchiavello's stain. Other diagnostic tests include culture of *Nanophyetus helminthoeca* in DH82 cells, serological tests by immunofluorescence or immunoblot assays using *N. helminthoeca* specific antigens and polymerase chain reaction (PCR) to detect DNA of *N. helminthoeca*.

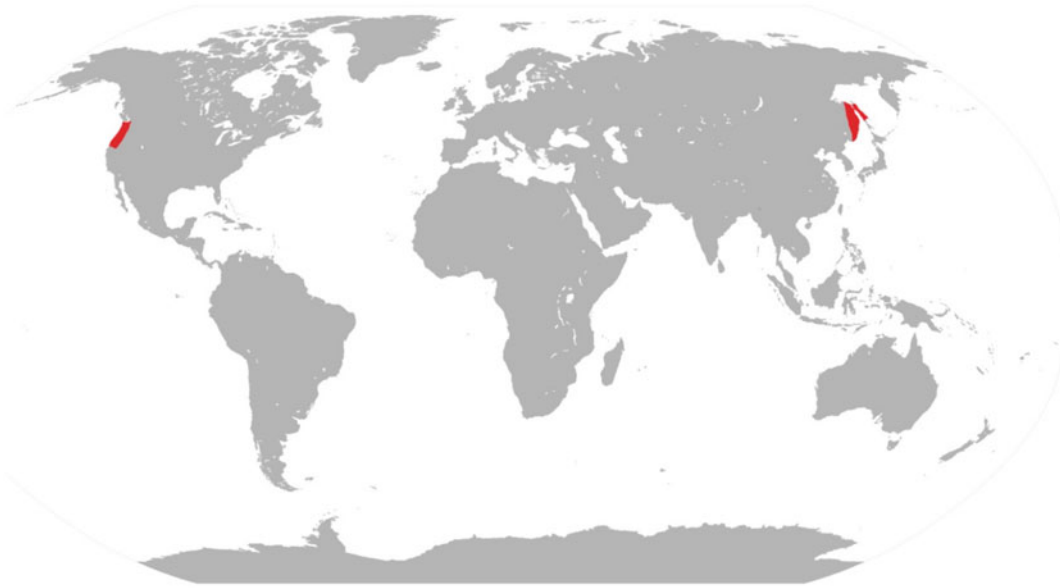

Fig. 3 Geographical distribution of human infections due to *Nanophyetus* spp. (Adaptation of world map blank from Wikimedia commons by San Jose, used under CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=1483026)

Treatment

In endemic areas in Siberia, fern extract or quinacrine was used earlier to treat the infection. Praziquantel is the drug of choice. The recommended dose is 20 mg/kg body weight divided in three divided doses for 1 day. Alternately, oral bithionol (50 mg/kg) and niclosamide (2 g) have also proved effective in some patients. Combined use of praziquantel and doxycycline is effective for treatment of SPD in dogs and other canids caused by *N. helminthoeca*.

Prevention and Control

Prevention of *Nanophyetus* infection in humans is based on avoiding consumption of raw, improperly cooked fish and fish eggs. Thorough cooking of fish or freezing the fish at −20 °C for more than 24 h can kill the metacercariae. Since infection can occur even while handling infected fish, adequate precautions must be taken by fish handlers. No vaccines are available.

Case Study

A middle-aged businessman presented with complaints of abdominal pain and diarrhoea. He also complained of feeling bloated. His travel history showed that he had travelled to Oregon, USA, last month on business. On further questioning, he revealed that during his visit, he ate 'some' fish, most likely salmon at a local restaurant. Blood investigations revealed eosinophilia. Stool sample was collected from the patient and sent for microbiological examination.

Table 2 Diagnostic methods for *Nanophyetus* infection

Diagnostic approach	Methods	Target	Remark
Microscopy	Examination of stool	Eggs	Not a sensitive method. Species identification not possible
Molecular methods	PCR	rDNA	Species identification

Direct microscopy revealed oval, brownish, operculated eggs.

1. Why do dogs suffer from severe fatal infection but the disease is mild in humans?
2. What are the preventive measures one should take to avoid this infection?
3. What are the other trematode eggs that can cause confusion with *Nanophyetus* eggs?

Research Questions

1. Why epidemiological studies should be done not only in endemic but also in non-endemic areas given the finding of eggs of *Nanophyetus* spp. in the wild animals of some non-endemic regions?
2. Whether it is correct to assume that salmon poisoning disease does not occur in humans or is there misdiagnosis due to lack of knowledge about this organism?

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Part IV

Zoonotic Helminth Infection: Cestode

Diphyllobothriasis

Aradhana Singh and Tuhina Banerjee

Learning Objectives

1. To understand the significance of the parasite as one of the causative agents of megaloblastic anaemia.
2. To review the worldwide distribution of the parasite and its association with the common practice of freshwater fish consumption.

Introduction

Diphyllobothriasis is caused by infection with pseudophyllidean tapeworm of the genus *Diphyllobothrium*. For a long period of time, *Diphyllobothrium latum* was identified as the only causative agent of diphyllobothriasis. However, differences in the biology and morphology of the adult worm were also evident. Eventually, cases of diphyllobothriasis were attributed to other species also. The most commonly reported other secondary species include *Diphyllobothrium dendriticum, Diphyllobothrium ursi, Diphyllobothrium dalhae, D. nihonkaiense, Diphyllobothrium pacificum* and *Diphyllobothrium klebanovski*. Molecular studies reveal that till date 14 species of the *Diphyllobothrium* genus have been identified. Freshwater fish like perch, trout, salmon, char and pike serve as the reservoir for the *D. latum*. Other *Diphyllobothrium* species have been found to be associated with the marine fishes.

History

The habit of fish foraging from the ancient times has allowed the broad tapeworms to find their way to the human intestine. The archaeological surveys have indicated the evidence of *Diphyllobothrium* spp. in the excavated materials from very ancient times. The reports of diphyllobothriasis date as back as pre-historic era. The first evidence of *Diphyllobothrium* was dated to fifth century A.D. in Prussia. The parasite was first described by Dunus and Wolpius in 1592, and confirmed by Plater in 1602. The transmission of the parasite by fish was described by Braun in 1883 in experimental studies where he fed the larva to medical students, cats, and dogs and collected the eggs from the faeces. Janicki and Rosen explained the role of crustaceans as intermediate host in 1917.

Taxonomy

The genus *Diphyllobothrium* belongs to the family Diphyllobothriidae, order Pseudophyllidea,

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https://doi.org/10.1007/978-981-16-7204-0_31

Class Cestoda and sub-class Eucestoda. Molecular studies have identified 14 species of the genus *Diphyllobothrium* that cause infection in humans and in marine animals. *D. latum* is the most important species that cause infection in humans. *Diphyllobothrium* species of importance to humans have been listed in Table 1.

Genomics and Proteomics

Despite the medical relevance of the *Diphyllobothrium* species, only its mitochondrial genome has been sequenced. Very little is known about its nuclear genome. The mitochondrial DNA sequences of *D. latum* and *D. nihonkaiense* have been found to be covalently closed circular molecules containing genes for 12 proteins, two rRNA and 22 tRNA. The overall A + T content of the genome is higher (68.3% and 67.8% in *D. latum* and in *D. nihonkaiense* respectively). The complete mitochondrial sequence of *D. latum* is 13,608 base pairs in length. The gene order of the mitochondrial DNA of *Diphyllobothrium* is identical to mitochondrial DNA of *Taenia* and *Echinococcus*. A total of 18 intergenic non-coding region, representing a length of 484 bp has been detected in *D. latum* mitochondrial genome. In an attempt to study the genomic DNA of *D. latum*, the *Pst*I restriction products were analysed through which a cluster of repetitive element DL1 was discovered.

A better understanding of a parasite's adaptations and host-parasite interaction is facilitated by proteomics studies. However, data regarding the proteome analysis of *Diphyllobothrium* is very scanty. The secretory-excretory protein analysis of *D. dendriticum* showed appearance of new high molecular fractions associated with plerocercoids during the incubation of the parasite with the medium containing host blood serum. Another study demonstrated protein profile analysis of the four *Diphyllobothrium* species using isoelectric focussing that it can be a useful aid in the taxonomic study.

The Parasite Morphology

Diphyllobothrium tapeworms are among the largest human parasites. They can grow at a rate of 1 cm/h or 22 cm/day. They are capable of living inside the host for up to 20 years or more.

Adult Worm

D. latum has been known to be the longest human tapeworm measuring 4–15 m in length, even in human intestine it has been reported to grow up to 25 m in length. In the adult worms, the proglottid is wider as compared to length, thus the name 'Broad tapeworm'. The adult worm has three separate morphological segments: head, neck and the lower body. Each side of the head, also called *scolex*, has slit-like groove called *bothrium.* These are responsible for the attachment to the host intestine. Neck is usually present posterior to the *scolex* and the remaining body (*strobila*)has many proglottid segments which contain the reproductive organs of the worm of both sexes. The genital pores open midventrally as in all pseudophyllid cestodes. Testes are oval to spherical in shape and numerous in number. Posterior one-third of each segment contains the bilobed ovary. Abundant follicles spread throughout the segment forming the vitellarium. The uterus is present far anterior to the ovary and tubular in structure.

Infective Larvae

The plerocercoid larvae are the infective stage. The infective larvae plerocercoid can occur freely within skeleton muscle or peritoneum or it can be encapsulated by a host response. The plerocercoids measure 1 mm to several cm in length and have a scolex with 2 bothria.

Table 1 Distribution of *Diphyllobothrium* spp. of human importance

Species	Distribution	Second intermediate host	Definitive host
Diphyllobothrium latum	Europe, North America, Asia	Mainly pike, burbot, perch, char	Humans, dogs, bears
Diphyllobothrium dendriticum	Circumpolar	Salmonids, coregonid fish	Fish-eating birds, mammals including humans
Diphyllobothrium dalliae	North America (Alaska)	Alaska blackfish, dolly varden	Dog, gulls, occasionally humans
Diphyllobothrium alascense	North America (Alaska)	Burbot	Dog, occasionally humans
Diphyllobothrium ursi	North America (Alaska)	Salmonines	Bear, occasionally humans
Diphyllobothrium nihonkaiense	North Pacific Ocean	Pacific salmons, Japanese huchen	Brown bear, humans
Diphyllobothrium pacificum	South America, Japan	Marine fish	Sea lions, occasionally humans
Diphyllobothrium lanceolatum	Circumpolar	Sardine cisco	Hair seals, occasionally dogs and humans

Eggs

Eggs are unembryonated when laid and measure 35–80 cm in length and 26–65 cm in width. The size of the egg varies depending upon the species. The different stages of development of *Diphyllobothrium* larva has been shown in Fig. 1.

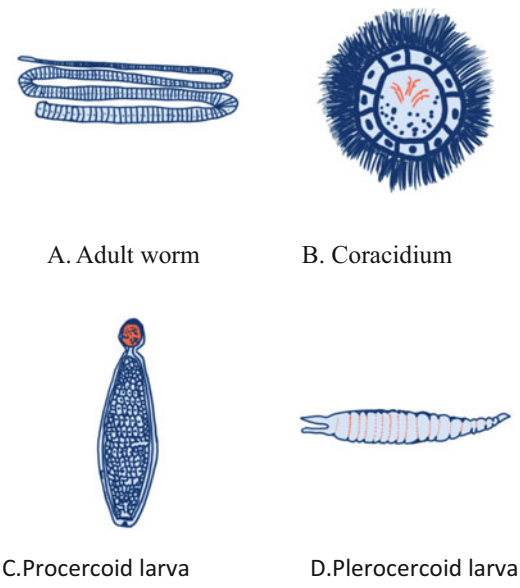

Fig. 1 Morphological forms of *Diphyllobothrium* spp. (**a**) Adult worm (**b**) Coracidium. (**c**) Procercoid larva. (**d**) Plerocercoid larva

Cultivation of Parasite

The cultivation of the *Diphyllobothrium* sp. has been attempted with the plerocercoids in suitable medium such as medium 199. The optimum incubation temperature has been reported to be 38.5 °C. Addition of sodium bicarbonate salt and procaine penicillin enriches the in-vitro cultivation of the worms. Coagulated new-born calf serum provides a proper semi-solid base for the worms to grow. The cultivation has been carried out in Carrel bottles and Petri-dishes, with a maximum of 5 plerocercoids in each culture bottle. The culture medium is changed after 16 h and 28 h. The in-vivo maintenance of the worms has been done by introducing them into animal model such as golden hamster.

Laboratory Animals

Animal models have been used to study about the immunological and pathological response of host against *Diphyllobothrium* infection. Artificially infected hamsters have been used to study about the efficiency of the praziquantel against *D. latum*. Rainbow trout infected with *D. dentriticum* has been used to study about the immunological parameters during the worm infection.

Life Cycle of *Diphyllobothrium* Species

Host

Diphyllobothrium spp. have three characteristics that make them suitable for a parasite. These are high biotic potential, producing millions of eggs from a single adult worm; longevity; and the ability of the plerocercoid to be transferred laterally from one fish host to another. *Diphyllobothrium* has been reported from old as well as new worlds and in fishes from freshwater as well as marine water which implies that they have highly successful transmission mechanisms.

Definitive Hosts

Humans, and other piscivorous animals (dogs, cats, bears, fish-eating birds, gull, fur seals and sea lion).

Intermediate Hosts

Aquatic animals.

First Intermediate Host: Fresh water crustaceans like Cyclops, Mesocyclops or diaptomus (*Acanthodiaptomus*, *Diaptomus*, *Arctodiaptomus*, *Eudiaptomus*, *Boeckella*, *Eurytemora*).

Second Intermediate Host: Freshwater fish like pike, trout, salmon perch etc., anadromous or marine fish.

Infective Stage for Humans

Plerocercoid larva.

Transmission of Infection

Transmission to humans or other definitive hosts occurs when they consume raw or inadequately cooked fish and the plerocercoid enters its small intestine and matures in 5–6 weeks (Fig. 2). Humans appear to be a good and optimum host for majority of *Diphyllobothrium* species. Adult worms of *Diphyllobothrium* are found in ileum and jejunum of humans consisting of 3000–4000 proglottids. Their reproductive capacity is quite high producing almost 1 million eggs each day. Unembryonated eggs are passed in the human faeces, that contaminate water bodies such as liver, lakes, ponds, etc.

In the water**,** under appropriate conditions the egg becomes embryonated within 18–20 days. During this period, oncospheres, the first larval form develops within the eggs. This is followed by the development of a spherical ciliated embryo called coracidium containing three pairs of hooklets within the egg shell. The mature coracidium measuring 40–50 μm in size escapes into the water. It must be ingested within 12 h by a suitable intermediate host such as freshwater crustaceans like Cyclops, Mesocyclops or diaptomus. On ingestion by these hosts, the coracidium loses its cilia in the intestine and penetrates the intestinal wall to come to lie in the body cavity (hemocoele) of Cyclops. It then differentiates into a ~0.5-mm procercoid, the second larval stage, in 2–3 weeks. Procercoid lacks the differentiated anterior end for attachments but possesses posterior appendages containing embryonic hooks. A maximum of two procercoids are present in each crustacean.

The procercoid, present in the infected copepod, on ingestion by a second intermediate fish host, comes out in the intestinal lumen. The procercoid subsequently penetrates the gut wall and enters the body cavity where it encysts or it enters the fish muscle and differentiates into the third larval stage, plerocercoid in 1–3 weeks. It is white in colour, somewhat flattened and marked by irregular unsegmented wrinkles. There is a depression at the anterior end representing the inverted future head of the worm. The sites for development may differ according to the species. Plerocercoid is the infective stage of the larva and further, when large piscivorous fish eats a second intermediate host, the plerocercoid reinvades the muscle of the fish.

Pathogenesis and Pathology

The adult worms remain attached in loops to the wall of small intestine. Attachment occurs mostly at the level of ileum. Despite the large size of this parasite, the majority of cases remain asymptomatic. Prolonged or untreated infections can cause

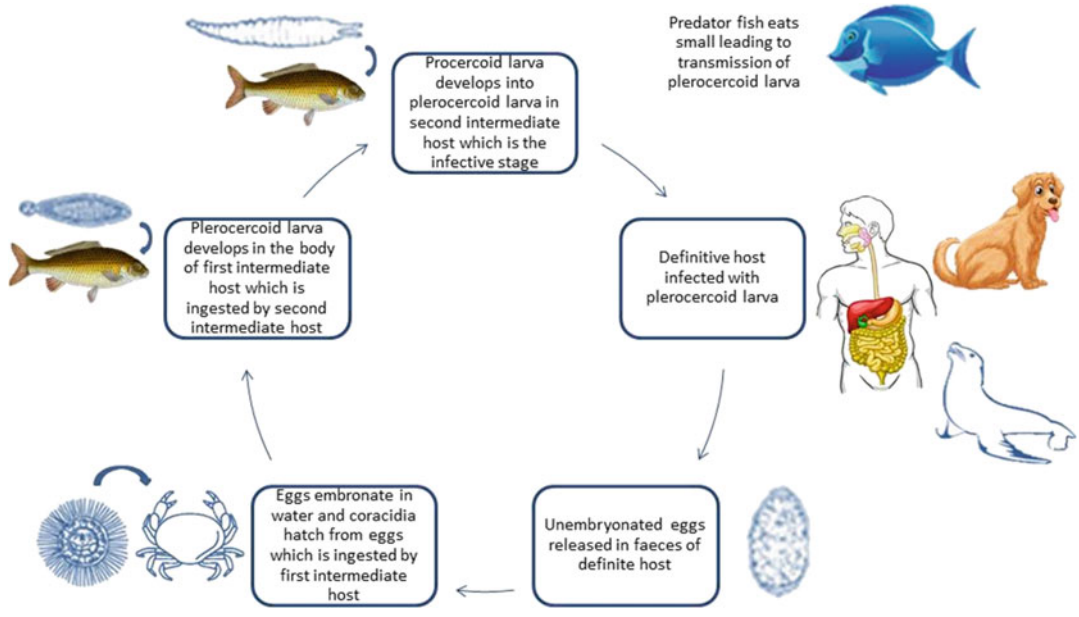

Fig. 2 Life cycle of *Diphyllobothrium* spp.

megaloblastic anaemia. These parasites can cause dissociation of vitamin B_{12} intrinsic factor complex in the host's gut lumen causing deficiency. The severity of the infection is directly related to the worm burden. The infected person begins to pass the eggs in the stool after approximately 15–45 days of ingestion of the plerocercoid larvae.

Immunology

The parasitic worms survive in the host body for long durations because of their ability to regulate the host immune response. The basic mechanisms of evasion of the host immune system are antigen mimicry, immunomodulation and immunosuppression. It has also been suggested that to escape the host immune response the parasitic worms are most likely to produce soluble modulators that may bind and destroy the immunocompetent cells that will otherwise interact with them. However, much data on the immunoregulators in the fish parasite is not available but a study proposed that *D. dentriticum* produces Prostagladins E2 as a regulator of the host immunity. Plerocercoids of the *D. dentriticum* have been known to evoke a significant inflammatory and humoral response in the animal models. Granulocytes and macrophages have been known to be the main leucocytes involved in these responses. Neutrophils have been demonstrated as a major granulocyte observed in response to developing plerocercoids. Specific antibodies are induced in response to the *D. dentriticum* infection. Components of complement system and the receptors of the complement fragments on macrophages have been characterized in salmonids.

Infection in Humans

Diphyllobothriasis is asymptomatic in majority of the cases in humans. However, if left unattended the adult worm can reside in the host for more than 20 years.

The main clinical presentation of diphyllobothriasis is gastrointestinal manifestations. The classical symptoms of gastrointestinal diphyllobothriasis include vomiting, diarrhoea, abdominal pain and anaemia. Besides, bowel obstruction occurs in limited number of cases and sometimes pieces of the worms are also vomited. The

symptoms also include dizziness, myalgia, fatigue, dyspepsia, sudden nausea, epigastric pain and mild abdominal cramping. Rarely, but in case of massive infections it may also result in intestinal obstructions.

The infection with *D. latum* has shown to be associated with vitamin B_{12} deficiency. This deficiency is the result of higher rates of absorption of vitamin B_{12} by the parasites as compared to the human gut. *Diphyllobothrium* can affect different organ systems with varied manifestations including central nervous system (paraesthesia, demyelinating symptoms, headaches and encephalopathy), ocular manifestations (optic neuritis), gastrointestinal manifestations (constipation, intestinal obstruction, sub-acute appendicitis, cholecystitis and cholangitis), haematological manifestations (pancytopenia, eosinophilia as well as pernicious anaemia), respiratory manifestations (dyspnoea) and dermatological manifestations (glossitis, allergic symptoms and pallor).

The human infection of *D. nihonkaiense* is generally very mild but it has been reported that it can lead to emotional and economic impact on the patients and families as the segments take a long time to evacuate.

Infection in Animals

Diphyllobothrium spp. require two intermediate hosts to complete the life cycle before reaching the definitive host. The definitive hosts for the different species of the *Diphyllobothrium* include dogs, cats, bears, fish-eating birds, gull, fur seals and sea lions. Fish species serve as transport and play a major role in the life cycle of *Diphyllobothrium* species. The definitive hosts become infected by consumption of the plerocercoid in the infected fish. Infection with the *Diphyllobothrium* species in dogs and cats has been associated with gastrointestinal symptoms. Clinical signs include diarrhoea, vomiting and weight loss. But these animals do not create an immediate zoonotic risk as the coracidium stage that is shredded in their stool is only infective to the first intermediate host.

Epidemiology and Public Health

Diphyllobothriasis affects any gender and age, though majority of the identified reports were of middle-aged humans. Diphyllobothriasis has been increasing worldwide from 9 million in 1970s to 20 million currently.

Diphyllobothrium has a worldwide distribution being endemic in North America, Eurasia and now in South America. The presence of *D. latum* in South America has been quite controversial as some researchers believe that *D. latum* was introduced in South America by European immigrants while others strongly argue that *D. latum* was already present in South America quite before the immigration. During last years, studies have shown a decline of diphyllobothriasis in most of Europe and particularly North America. The most endemic countries such as Finland and Alaska also have shown a decrease in the prevalence of diphyllobothriasis in the last few decades. However, increased reports of the disease have been documented from South America. Moreover, new cases of diphyllobothriasis have been reported from different parts of the world including Japan, Siberia, Europe and Korea. Among sub-tropical and tropical Asia, the presence of *Diphyllobothrium* has been reported from China, Taiwan, Malaysia, India and Pakistan. Also, recently some part of the world has shown re-emergence of diphyllobothriasis which can be a result of changing food habits and globalization (Fig. 3).

Diagnosis

Laboratory diagnosis of diphyllobothriasis includes microscopy, in-vitro culture, serodiagnosis and molecular diagnosis (Table 2).

Microscopy

Morphological identification of human broad tapeworm is mostly based on finding the egg in stool of the patients of typical ovoid shape with operculum on narrow end (Fig. 4). The size of the egg varies from 35 to 80 by 25 to 60 μm. This is

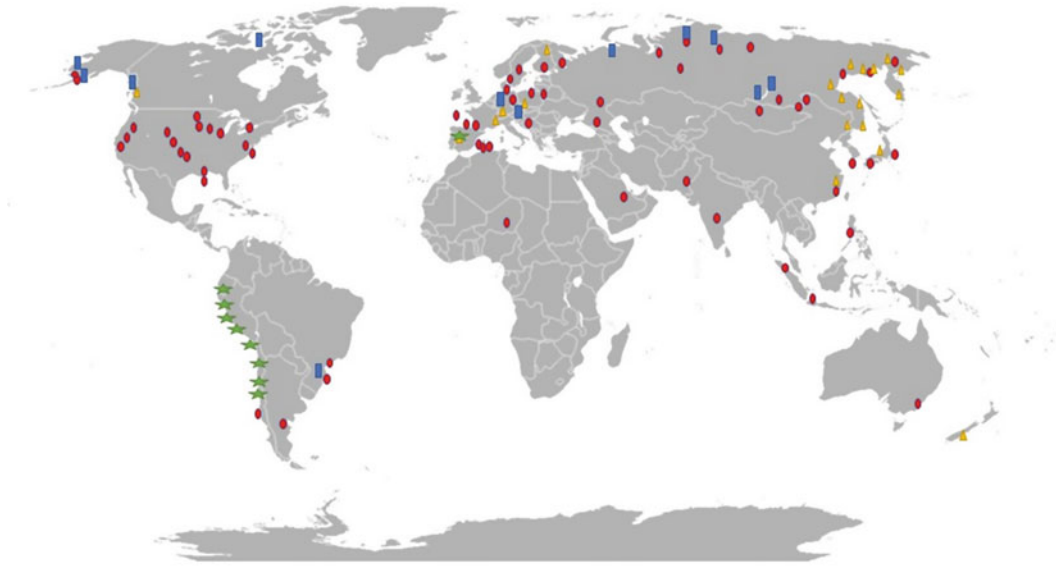

Fig. 3 World map showing distribution of human cases of *Diphyllobothrium latum* (indicated by red circles), *Diphyllobothrium nihonkaiense* (indicated by yellow triangle), *Diphyllobothrium dendriticum* (indicated by blue square) and *Diphyllobothrium pacificum* (indicated by green star)

an easier and most economical way of diagnosis However, the identification of exact species based on the morphological features of the eggs is a difficult task. Additionally, the misdiagnosis of *Diphyllobothrium* eggs as those of flukes is also possible. The morphological identification of plerocercoids is difficult. Identification of *plerocercoid* for the three main parasitic species i.e. *D. latum, D. dendriticum* and *D. nihonkaiense* is usually based on their body surface, retraction of scolex, length of microtriches and number of subtegumental longitudinal muscles.

In-vitro Culture

Plerocercoids of medium to large size (1.5–2.5 cm) are generally used for the in-vitro culture of *Diphyllobothrium* species. The plerocercoids are transferred to Petri dishes and culture bottles containing the cell culture medium

Table 2 Laboratory diagnosis of diphyllobothriasis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Stool examination	Eggs	Typical ovoid shape with operculum on narrow end. Size varies from 35 to 80 by 25 to 60 μm
	Morphology	*Plerocercoid*	Identification of plerocercoid can be done based on their body surface, retraction of scolex, length of microtriches and number of subfegumental longitudinal muscles
Cultivation	In vitro culture	Culture of plerocercoid	Medium 199 most commonly used for culture of plerocercoids. Optimum incubation temperature: 38.5 °C
Molecular diagnosis	PCR RFLP	Genes such as *cox1*, tRNA, NADH dehydrogenase subunit 3 and cytochrome b	Identification to the species level possible. *SmaI*, *HinfI* and *HhaI* has been used as a species-specific marker

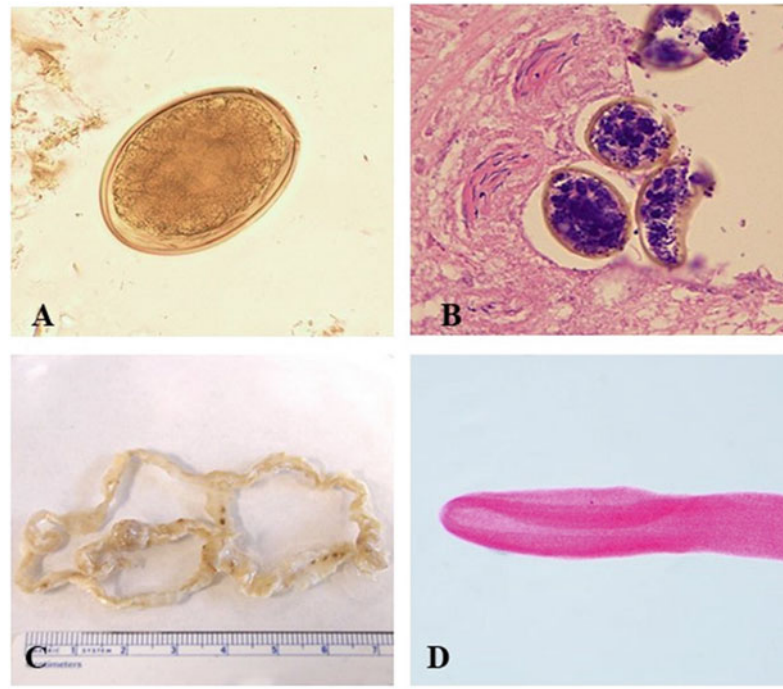

Fig. 4 (**a**) Unstained wet mount of *Diphyllobothrium* sp. egg (**b**) Egg of *Diphyllobothrium* sp. with Haematoxylin & Eosin staining (500×) (**c**) Section of adult worm containing many proglottids (**d**) Elongated scolex and neck region (Adapted from DpDx, CDC)

and incubated at 38.5 °C for the growth and development of the trematode. The worms are fixed using Susa fixative or Bouin's fixative, embedded and then stained with paraldehyde fuchsin (PAF) for the diagnosis purposes.

Serodiagnosis

Serodiagnosis is not frequently used in the diagnosis of diphyllobothriasis.

Molecular Diagnosis

Till date, molecular identification remains the most reliable method for specific identification of *Diphyllobothrium* species. Restriction fragment length polymorphism (RFLP) with SmaI, HinfI and HhaI has been used as a species-specific marker to distinguish between *D. latum* and *D. nihonkaiense*. The analysis of phylogenetic tree using ribosomal genes and inter-transcriber sequences (ITS) has been very useful in confirming the relationship among the different taxa. The *cox1* gene has been frequently used as a target for the identification of human broad tapeworm. The concentration of the eggs and use of sonication to disrupt the egg shell for releasing the content can be useful before the molecular analysis. The most appropriate targets for the identification include *cox1*, tRNA, NADH dehydrogenase subunit 3 and cytochrome b genes. The molecular detection of the *D. nihonkaiense* by RFLP of the PCR product of *cox1* gene has been shown in Fig. 5.

Treatment

Praziquantel is the drug of choice for treatment of diphyllobothriasis, which is safe even in pregnancy. Patients infected with *Diphyllobothrium* has been treated with first oral dose of praziquantel (10 mg/kg) and a second dose (8.5 mg/kg) a week later. A dose of 25 mg/kg has been found to be very effective against *D. latum* in the animal model, however, in case of other species lower doses can be administered. Other treatment options included administration of 200 mL of gastrografin in combination with vagostigmin. Also, administration of 300 mL of amidotrizoic acid through an intraduodenal tube has been evaluated for treatment of

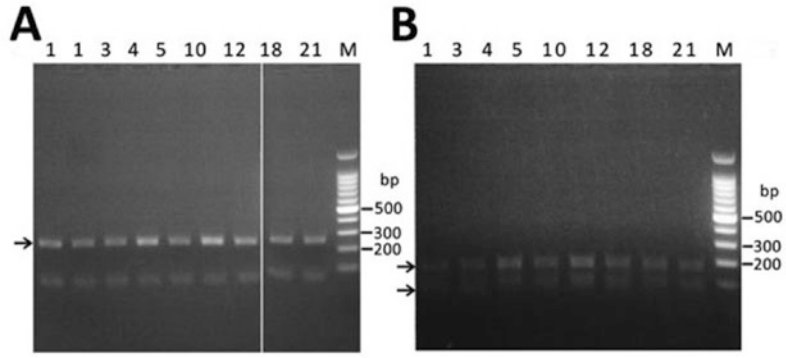

Fig. 5 Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified *cox1* gene fragments of *Diphyllobothrium*. The count above each lane shows the number of proglottids in the given sample. (**a**) Restriction digestion of *cox1* gene fragments with *Age*I. (**b**) Restriction digestion of *cox1* gene fragments with *BspH*I, M: marker. (Adapted from Epidemiology of *Diphyllobothrium nihonkaiense* Diphyllobothriasis, Japan, 2001–2016)

diphyllobothriasis. Another alternative drug for *D. latum* infection is niclosamide, administered in a single dose of 2 g for adults and 1 g for children.

Prevention and Control

The best way to prevent diphyllobothriasis is to avoid consumption of raw fish. Other preventive measures include (1) prevention of contamination of faecal matter in drinking water sources, (2) inspection of food markets providing raw fishes, (3) creating awareness on potential infections through consumption of raw fishes, (4) proper treatment of fish fillets to eliminate any chances of infectivity, (5) temperature treatment of simultaneous freezing and heating to kill the plerocercoids and (6) drug therapy.

Case Study

A 10-year-old-boy presented to paediatric department with history of light-coloured segments in stool. Similar episodes had occurred since last 1 year and each episode was accompanied by abdominal pain. No complaint of vomiting or diarrhoea was found. He gives history of handling and consumption of raw fish. The peripheral blood smear examination revealed anaemia with moderate eosinophilia. The macroscopic examination of the segment released in the faeces was creamy white in colour with the measurements of 5 cm by 1.5 cm. No identifiable scolex was present but histopathological examination of the adult worm showed gravid segments filled with characteristic oval operculated eggs. Stool examination revealed operculated oval eggs with knob on both sides.

Questions

1. What is the diagnosis and what parasite was demonstrated in stool microscopy?
2. Mention intermediate host and zoonotic association of the parasite.

Research Questions

1. Identification of newly emergent zoonotic *Diphyllobothrium* species of public health importance for humans.
2. Molecular methods for early detection and identification of the source of *Diphyllobothrium* infection in humans.

Further Readings

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Taeniasis

Subhash Chandra Parija

Learning Objectives

1. To have a knowledge about the importance of *Taenia asiatica* as a new species and its clinical manifestation.
2. To explain the importance of the extraneural manifestations of cysticercosis, particularly cardiac and ocular involvement.
3. To review the various serological tests including EITB in the diagnosis of neurocysticercosis.

Introduction

Taeniasis and cysticercosis are defined as the parasitic infection of humans and animals caused by adult and larval stages of tapeworms (*Taenia solium, Taenia saginata* and *Taenia asiatica*) respectively. The disease has worldwide distribution especially in areas where cattle and pigs rearing are carried out extensively. Though the distribution of the disease varies across the continents, the prevalence rate is found to be high in developing countries. Effective control and prevention of the condition is achieved by measures that include prevention of cattle or pigs from grazing on faeces or sewage polluted grass, avoiding the use of untreated human faeces as manure for land and avoiding the eating of raw or undercooked meat and meat products.

History

T. saginata has been identified as an intestinal parasite infecting man since ancient times and has even been depicted in Charaka Samhita, an ancient Indian medical book. In the third century BC, Aristophanes and Aristotle first described the cysts in pigs, and later in 1550, Parunoli noticed this infection in humans. In 1782, Goez differentiated it from other species *T. solium.* The role of cattle as an intermediate host and the complete life cycle of *T. saginata* was described by Leuckart, in 1861. A case of neurocysticercosis was reported from a coolie in Madras, who died due to a seizure (Armstrong 1888). In 1912, Krishnaswamy was the first to report the cases of muscle pains and subcutaneous nodules with abundant cysticerci in muscles, heart and brain through autopsy.

T. asiatica was first identified in Taiwan and later in Korea and other Asian countries; hence it was named Asian *T. saginata*. In 1966, suspicion of aetiology other than conventional *T. saginata* was put forth by S.W. Huang, as the Taiwan aborigines hardly eat beef. The naming of the parasite as *T. saginata asiatica* was by a group

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of Taiwanese parasitologists namely P.C. Fan, C. Y. Lin, C.C. Chen and W.C. Chung.

Taxonomy

The genus *Taenia* belongs to the Phylum, Platyhelminthes; Class, Cestoda; Order, Cyclophyllidea; and Family, Taeniidae. *T. saginata, T. solium* and *T. asiatica* are three important species that cause infections in humans.

Genomics and Proteomics

The genome size of *T. solium* is 122,393,951 bp with 12,467 coding genes. The complete nucleotide sequence of the tapeworm *T. solium* mitochondrial DNA (mtDNA) has been determined. The sequence is 13,709 base pairs in length and contains 36 genes (12 for proteins involved in oxidative phosphorylation, 2 for ribosomal RNAs and 22 for transfer RNAs). The gene content and organization of the *T. solium* mtDNA are identical to the mtDNAs of the other two species. The size of the protein-coding genes of the three human *Taenia* tapeworms did not vary, except for *T. solium* nad1 (891 amino acids) and nad4 (1212 amino acids) and *T. asiatica* cox2 (576 amino acids).

Genomic analysis shows much larger assembly sizes of *T. saginata* and *T. asiatica* than *T. solium* (169 Mb and 168 Mb vs 131 Mb), though GC content remains similar (43.2% vs 43.5%). Coding genes in *T. solium* were estimated to be 11,902 only with gene density 90.9/Mb, and in *T. saginata*, 13,161 and 77.9/Mb, respectively. While mean exon length remains similar (237 bp) across the three species, introns are longer in *T. saginata* (864 bp) than *T. solium* (775 bp) and *T. asiatica* (831 bp). The mitochondrial genome shows comparable assembly sizes (13,700, 13,670 and 13,703 kb) with 70% genes coding for proteins like other cestodes. Nucleotide composition of the mitochondrial genome is asymmetrical with a positive GC-skew. *T. solium* differs by 12.3% and 12% from *T. saginata* and *T. asiatica*, respectively in cox1 nucleotide composition. The latter two differ only by 4.6%; in fact, 18S rRNA genes are 99.2% identical. Genomic features strongly revoke the sister relationship between *T. saginata* and *T. asiatica* with a higher evolutionary drive in mutation rate, heterozygosity and expansion of genes related to ion transporters and tegumental components in the latter. However, the absence of a significant intra-species genetic variation in *T. asiatica* suggests that it could be an endangered status. *T. saginata*, in comparison, shows high genetic polymorphism (0.2–0.8%). A striking discordance has been discovered between mitochondrial and nuclear DNA among a few isolates of *T. saginata* and *T. asiatica* from Taiwan and China, raising the possibility of hybrids.

Proteomic analysis of metacestodes, oncosphere, vesicular fluids and excretory-secretory products redefines host-parasite relationship, immunological responses, identification of the diagnostic antigens and vaccine candidates. Tandem mass spectrometry and BLAST studies identified important proteins including microtubule-based movement/tegumental proteins (paramyosin, H17g), chaperones (HSP90), metabolic proteins (elongation factor 1 alpha, GAPDH, malate dehydrogenase) and detoxification molecules (ferritin, glutathione S-transferase). GP50 and T24 are diagnostically important proteins.

The Parasite Morphology

The three morphological forms of the parasites are adult worm, egg and larva.

Adult Worm

T. saginata: The worm is long, flattened and ribbon-like (Fig. 1). The adult worm consists of a head (*scolex*), neck and strobila. The *scolex* has four cup-like muscular suckers (or acetabula) which help in attachment. Situated next to scolex is neck, which is the narrow growing region from which the proglottids arise. Neck is longer in

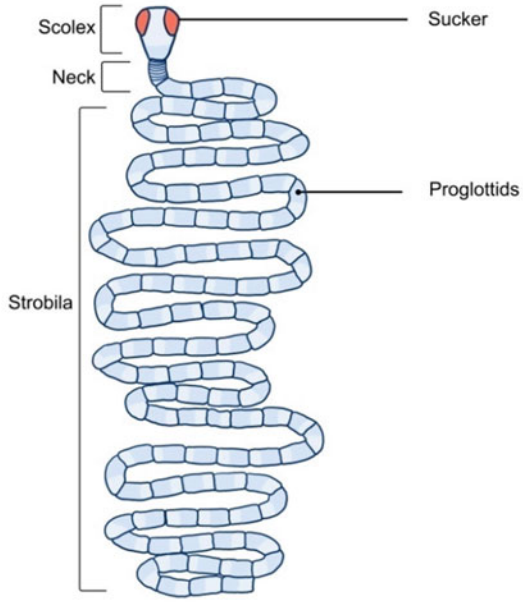

Fig. 1 Schematic diagram of *Taenia saginata* adult worm (5–10 m in length)

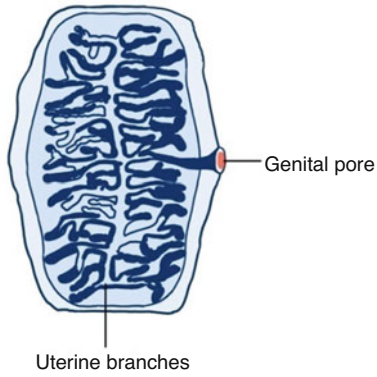

Fig. 2 Schematic diagram of *Taenia saginata* gravid proglottid

T. saginata than *T. solium*. The trunk or body is the *strobila* which consists of many segments or *proglottids*. Proglottids are further divided into immature, mature and gravid segments. The mature proglottids have both male and female reproductive organs. The female reproductive system includes ovary, a closed uterus with branches, ootype, single mass of vitelline gland and a genital pore which is laterally situated. Male organs consist of testes (follicles), vas deferens

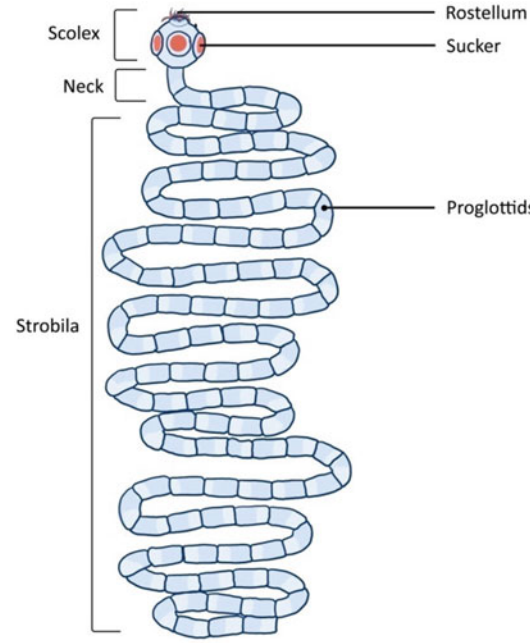

Fig. 3 Schematic diagram of *Taenia solium* adult worm (2–3 m in length)

and cirrus. The uterus in the gravid segment of *T. saginata* has 15–30 lateral branches as compared to *T. solium* (7–13 branches) (Fig. 2). The presence of a prominent vaginal sphincter and lack of accessory ovarian lobe differentiate it further from *T. solium*. The lateral wall of the segments has a common genital pore opening. Since there are no separate uterine openings, the gravid segments escape through the anal sphincter following which the eggs are released by the rupture of the uterine wall.

T. solium: The adult worm measures around 2–3 m in length. The shape of the scolex of *T. solium* appears globular, which has four large cup-like suckers and a rounded rostellum, armed with a double row of alternating round and small dagger-shaped hooks (Fig. 3). The neck is short and thick. The strobila is made up of proglottids numbering less than a thousand. Each gravid segment measures 12 mm by 6 mm, with a length twice that of breadth. There are around 150–200 follicles in the testes. The female reproductive system consists of a uterus which has thick lateral branches of about 5–10 (under 13) and an

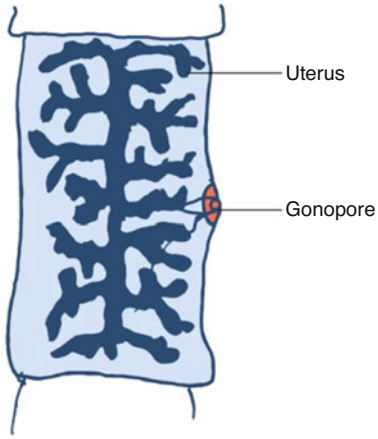

Fig. 4 Schematic diagram of *Taenia solium* gravid proglottid

accessory ovarian lobe. There is no vaginal sphincter. The genital pore is situated laterally and is alternate in position, appearing on the right and left sides of the adjoining segments (Fig. 4). In contrast to *T. saginata,* there is passive expulsion of short chains of the gravid proglottids. Rupture of the uterine wall releases the eggs.

T. asiatica: The worm measures about 350 cm in length and 1 cm in breadth and is divided into the anterior scolex and a short neck followed by strobila. The body cavity or digestive system is absent. The scolex bears four simple suckers and a distinct rostellum, which bears two rows of rudimentary hooklets, which distinguishes it from *T. saginata*. The strobila has more than 700 proglottids, but less than 1000, in comparison to *T. saginata* which have more than 1000 proglottids in the strobila. Similar to *T. saginata* the uterus has 13 lateral branches. The most defining and differentiating features of *T. asiatica* include a large number of uterine twigs in gravid proglottids and the presence of posterior protuberance.

Eggs

The eggs of the three *Taenia* species are morphologically similar to each other. Eggs are enclosed in the gravid proglottids. The spherical egg

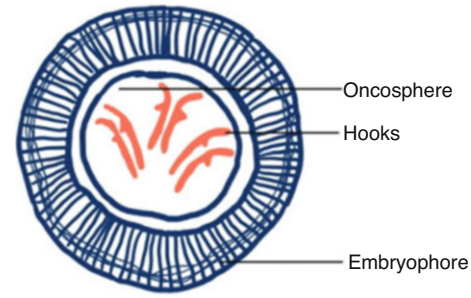

Fig. 5 Schematic diagram of *Taenia* egg

measures 30–40 μm in diameter with a thin hyaline embryonic membrane surrounding it. The radially striated inner embryophore is yellow-brown in colour due to bile staining. The fully developed embryo (oncosphere) consists of three pairs of hooklets (hexacanth embryo) in the centre (Fig. 5). *T. saginata* eggs are infective to cattle, while *T. solium* eggs are infective to both pigs and humans, but *T. asiatica* eggs are infective to pigs only.

Larva

T. saginata: *Cysticercus bovis* is the larval stage of *T. saginata*, which is infective to humans. It is small, measuring 6–9 mm size, round, greyish-white bladder-like worm containing opaque invaginated scolex without hooklets (bladder worm). The larva is commonly situated in the muscles of mastication, cardiac muscles, diaphragm and tongue of infected animals.

T. solium: Cysticercus cellulosae, the larval form of *T. solium*, is located in various organs of pigs as well as in humans. It is an oval-shaped milky-white structure which is about 5 mm by 10 mm in size. The invaginated scolex within the bladder appears as the larva along with its suckers which is visible as a thick white spot. It remains viable for several months.

T. asiatica: Cysticerci of *T. asiatica* appear morphologically similar to *T. saginata* except that they are smaller in size and are located primarily in the liver. They possess two rows of rudimentary hooks which are absent in *T. saginata.*

Cultivation of Parasites

In vitro culture of metacestodes of *T. saginata* is carried out in a biphasic medium consisting of solid phase made up of coagulated calf serum and fluid phase, consisting of buffered RPMI-1640 medium enriched with sodium pyruvate and foetal calf serum. The growing tapeworms show sexual organelles in the early developmental stage. In vitro culture of *T. solium* is done in a system of cell monolayer (HCT-8) without gas phase which induces most of the oncospheres of *T. solium* to enter post-oncospheral (PO) development. The larvae usually survive for up to 16 days. Experimental studies have shown that HCT−8 cell lines favour the formation of PO up to 32%, compared to other cell lines. The developmental forms can be visualized by an ordinary light microscope or electron microscope.

The cultivation of the parasite is useful since the changes occurring in the PO forms can explain the protection of the parasite from the host immune system and the changes observed in protein expression will aid in the development of new targets for vaccine production.

Laboratory Animals

Experimentally, immunosuppressed mice can be used to grow early larval stage and hamsters to grow the immature adult stage of *T. saginata*.

Experimental animal models for *T. solium* include hamsters, gerbils and chinchillas. Of these, chinchillas are the most successful experimental definitive model for adult *T. solium. Mesocestoides corti*, a cestode organism related to *T. solium,* was used for intracranial infection of mouse models to study pathogenesis and immune response associated with neurocysticercosis. There is also a report of a natural progression of innate, early induced and adaptive immune responses in infected mice.

Mice with severe combined immunodeficiency (SCID) when injected subcutaneously into the back with in-vitro-hatched oncospheres of *T. asiatica* developed into fully matured cysticerci. The morphology of the cyst was more advanced and bigger in size which suggested that SCID mice be valuable experimental animal models for studying human taeniid cestode infections.

Life Cycle of *Taenia saginata, Taenia solium and Taenia asiatica*

Hosts

Definitive Host

Man.

Intermediate Hosts

Cattle (*T. saginata*), pigs (*T. solium, T. asiatica*), wild boars and cattle (*T. asiatica*).

In the case of *T. solium,* humans can act as both definitive and intermediate hosts.

Infective Stage

Cysticercus (*Cysticercus cellulosae* and *Cysticercus saginata*), the larval stages of *T. solium* and *T. saginata*, respectively, are infective to humans, while eggs are infective to cattle or pigs and also to humans.

Transmission of Infection

Humans acquire the infection by (a) ingestion of undercooked/raw beef containing encysted larval stage (*T. saginata*), (b) ingestion of undercooked/raw pork containing encysted larval stage (*T. solium, T. asiatica*), and (c) ingesting food (mainly vegetables) or water contaminated with *Taenia* eggs (Fig. 6).

Following ingestion, the encysted larva is digested by the stomach gastric juice. The scolex evaginates out of the cysticercus in the small intestine and gets itself attached to the mucosa of the intestine and develops into the adult worm in a period of about two to three months by strobilization. Adult worms become sexually mature in about 10–14 weeks, fertilization occurs and eggs are formed and later released to the faeces. These eggs are infective to cattle, pigs and other animals.

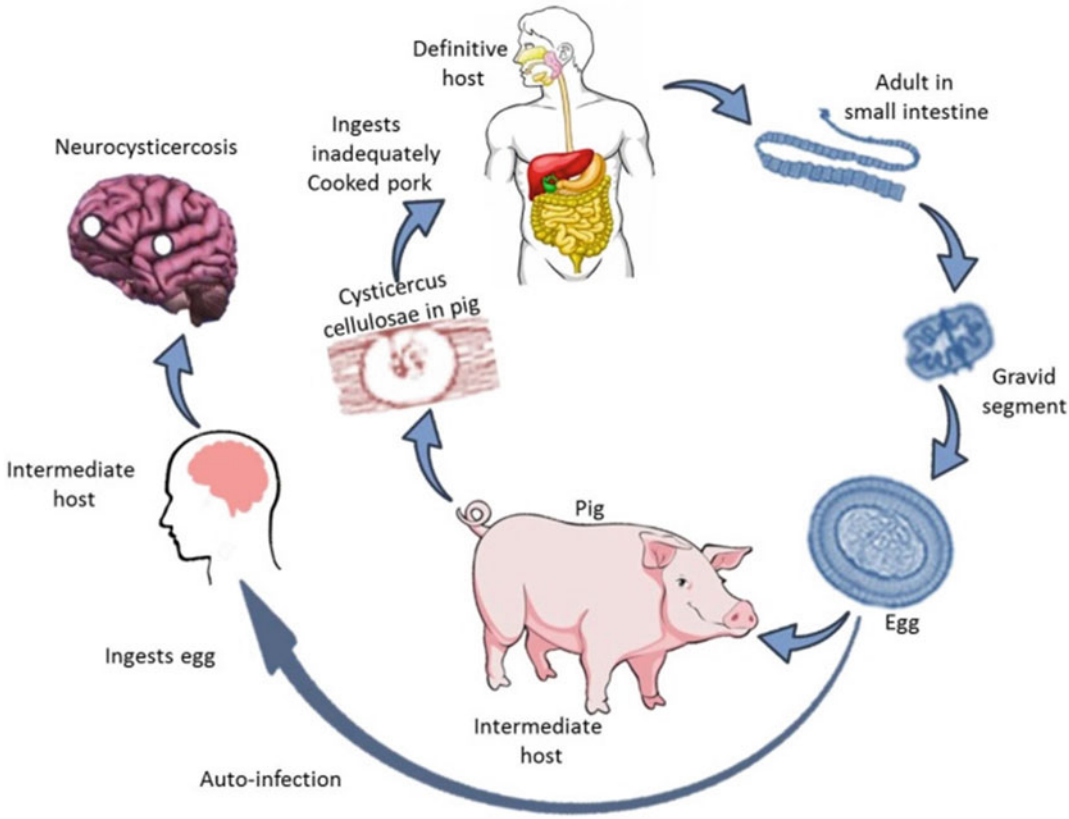

Fig. 6 Life cycle of *Taenia solium*

The animals are infected by the ingestion of eggs while grazing the field. In the small intestine, the oncosphere is released by rupture of the embryophore surrounding the egg. The oncospheres penetrate the intestine, with the help of hooklets and reach the skeletal muscle through the circulations, where they get transformed into bladder-like larvae. The larvae get encysted and deposited as cysts and this process is completed in 10–15 weeks of time. This larval stage of *T. saginata* is infective to man and causes intestinal taeniasis.

Humans also frequently act as intermediate hosts by ingesting eggs with contaminated food and water. In humans, the eggs develop in the same manner as in pigs. The onchosphere is released from the eggs in the first or second part of the small intestine and it penetrates the wall of the intestine to lie in the mesenteric venules or lymphatics. From here, they are carried to various tissues of the body by systemic circulation. These are mostly filtered in the muscle tissue where larval development takes place. Humans are dead-end hosts and the larvae die after variable periods of time.

Pathogenesis and Pathology

Due to aberrant migration of the segments of adult *Taenia* worms in the intestine, obstructive appendicitis or cholangitis can result. During the episodes of vomiting, the proglottids can obstruct the respiratory tract, enter the middle ear via the eustachian tube or tend to localize in adenoid tissues. The irritative action of the worm leads to inflammatory reactions. Moderate eosinophilia

is also frequently seen. In the intermediate host, *T. asiatica* causes hepatocyte degeneration and spotty necrosis of pig liver tissues. Following two months of infection, major changes noted around cysticercus include granulomatous reactions and focal liver fibrosis.

The migration of the *C. cellulosae* to extraintestinal sites such as bone muscle, skin, eye and bone presents a variety of pathology depending on the location of the cysts. The migration of the larva and its presence in the brain causes neurocysticercosis (NCC). The pathology in NCC depends upon the number, location, size and evolutionary stage of the parasites, as well as the presence and degree of the inflammatory response of the host. Parasitic larvae in the parenchyma of the brain tissue commonly present with seizures. The cyst which is viable in the due course of time goes into the involution process due to host immune response. Studies have proven the viability of the cyst for months even after treatment with antiparasitic agents. Initially the viable cysts appear as round vesicles of a membrane filled with clear fluid, containing a scolex. Following the attack by the host immune system, the cyst fluid becomes turbid accompanied by the degeneration of the parasitic membrane and scolex. The cysts gradually shrink and are replaced by hyaline and fibrotic tissue which later disappear or leave a residual calcified scar. It has been observed that cysts in the subarachnoid space tend to grow and infiltrate, which manifest as a space-occupying lesions and blocking the circulation of the cerebrospinal fluid with subsequent hydrocephalus. Unlike intraparenchymal NCC, subarachnoid disease is progressive and associated with significant mortality.

Immunology

Adult *Taenia* spp. are weakly immunogenic. They produce moderate eosinophilia with increased IgE levels. Acquired immunity against *Taenia* infections in humans following the elimination of infection has not been documented, whereas concomitant immunity plays a significant role. Animals once infected usually develop resistance to infection.

In contrast, the larval form of the parasite, *Cysticercus cellulosae* in tissues elicits an active immune response in humans with evasion and suppression of immunity. Viable *cysticerci* do not manifest any symptoms, but in contrast, the immune-mediated inflammatory response around degenerating cysts may precipitate symptomatic diseases. The mechanism behind the evasion of the host immune response by the parasite could be due to masking of *Cysticercus* antigens by host immunoglobulins, concomitant immunity, molecular mimicry and suppression or deviation of host responses. Predominant cellular components activated in the inflammatory response include plasma cells, lymphocytes, eosinophils and macrophages.

Among several immunoglobulin classes secreted against this parasite, IgG is found to be more frequent and are detected in the serum, cerebrospinal fluid and saliva. Antibodies are most frequent among cases with live or dying parasites and rarely in cases with calcified cysts. They engulf parasite remnants, leaving behind a gliotic scar with calcification. There is always a correlation between the presence of antibodies and the intensity of infection, as well as the viability of the parasite.

Increased levels of interleukins IL1, IL-6, IL-5 and Tumour necrosis factor-alpha have also been recorded in the CSF of patients with inflammatory neurocysticercosis, suggesting acute phase response. All these factors suggest a mixture of Th1 and Th2 responses in human brain granulomas caused by cysticerci.

Infection in Humans

The infections in humans are broadly of two types: intestinal taeniasis and cysticercosis.

Intestinal Taeniasis

Most patients with adult *Taenia* cause intestinal taeniasis which is mostly asymptomatic.

Clinical manifestations include mild abdominal pain, nausea, loss of appetite, weight loss, headache and change in bowel habits. In a few cases, proglottids may appear in the stools and even protrude from the anus. Patients may experience perianal discomfort or pruritus, when proglottids, which are often motile, are discharged. This may cause psychological disturbances in patients. Rarely, obstruction by the migrating proglottids can result in appendicitis or cholangitis.

Cysticercosis

Cysticercosis is defined as the tissue infection caused by larval stage of the tapeworm *T. solium*. This larval cyst has the tendency to develop in any part of the body such as CNS, skeletal and heart muscle, skin, subcutaneous tissues, the lungs, liver and other tissues. They can be classified based on the location into extra neural cysticercosis and neural cysticercosis (NCC).

Neurocysticercosis: T. solium cysticerci have a greater predilection to develop in the brain. The infection of the CNS by this parasite is termed neurocysticercosis. The nature and severity of infection depend upon the site, size and number of larvae in tissues and also on the immune response of the host. The symptoms of NCC include headaches, dizziness and seizures. Among these, seizures are the most common clinical manifestation and contribute to the prevalence of epilepsy in around 30% of cases in endemic regions. Other CNS manifestations include sensory deficits, involuntary movements and dysfunction of the brain stem.

NCC based on its location can be further classified into parenchymal and extraparenchymal disease. Parenchymal NCC is characterized by the development of cysticerci within the brain tissue, while the extraparenchymal NCC is characterized by cysts in subarachnoid space, meninges, ventricles and so on. *Racemose cysticercosis* is a rare and severe variant of cysticercosis, caused by an unusually large, multilobular, clustered *Cysticercus* that lacks scolex. The *Cysticercus* is usually located in extraparenchymal sites but mixed parenchymal and extraparenchymal infections can also occur. The condition poorly responds to treatment and is associated with increased morbidity and mortality.

Extraneural cysticercosis: Subcutaneous cysticercosis usually appears in the arms or chest as painless, small, mobile nodules. Muscular cysticercosis is an accidental finding in radiology which can appear dot-shaped or ellipsoidal calcifications in the thighs or arms muscle. About 5% of patients can have asymptomatic manifestations of cardiac cysticercosis.

Ocular cysticercosis: It is most commonly found in the vitreous humour or in the subretinal space as a free-floating cyst. Clinically, based on the degree of retinal tissue damage and development of chronic uveitis, it can present as a visual disturbance. Other sites of locations include the anterior chamber of the eye, conjunctiva or extraocular muscles.

Infection in Animals

Animals harbouring cysticerci usually are asymptomatic, but in massive and severe infections stiffness of muscles has been reported. The larval form, *C. bovis,* is not pathogenic for cattle, unless a vital organ such as the heart is massively infected. In order to prevent humans from acquiring infections from animals, whole carcasses need to be condemned at slaughterhouses, as there is always a risk of consuming improperly cooked meat. *C. cellulosae* infections in swine are usually asymptomatic except in cases when vital organs like the heart are massively infected. Muscle stiffness has also been documented in case of massive infections.

Epidemiology and Public Health

Incidence of taeniasis has been reported worldwide, especially in countries where raw or undercooked beef or pork is consumed. There is a dearth of knowledge on taeniasis and

cysticercosis in both developed and developing countries. Though some prevalence data are available, their accuracy remains questionable partly due to imperfect diagnostic tests. This has led to gross underestimation of the true prevalence of the disease. The prevalence of taeniasis ranges between 0.1% and 15%. Bovine, porcine and human cysticercosis prevalence varies from 0.03% to 80%, from 0.6% to 60% and from 1.3% to 40%, respectively.

An estimated 60–70 million people are considered to be carriers of *T. saginata* globally. In regions like Eastern Europe, eastern Africa, Latin America, Southeast Asia and Russia, where raw beef is consumed, *T. saginata* taeniasis has been reported. *T. saginata* infections are rare in Northern America, except in instances where cattle and humans live in close proximity and poor sanitary conditions prevail. A study on the prevalence of *T. saginata* in Asia indicated the highest incidence in the Philippines (33.7%), followed by Pakistan (7%), Vietnam (5.85%), Indonesia (4.68%), Nepal (4.37%) and India (3.84%) (Fig. 8). This may be in part due to traditional dishes containing raw beef, improper cooking of beef before consumption, co-habitation and poor sanitary conditions.

T. solium infections are more prevalent in communities with poor sanitation and where people eat raw or undercooked pork, like in Latin America, Eastern Europe, sub-Saharan Africa, India and Asia (Fig. 7). *T. solium* infections are on the rise in the United States largely due to immigrants from endemic areas like Latin America. A similar rise has also been witnessed in Europe due to immigration and increased travel to endemic areas. Neurocysticercosis had been described as endemic in north Portugal and the western provinces of Spain. According to World Health Organization (WHO), 30% of all epilepsy cases in endemic countries and 3% globally may be due to neurocysticercosis. In India, the disease is prevalent throughout the country but varies between the states. In the northern states of India, where pig rearing is common, the prevalence of taeniasis as high as 18.6% has been recorded.

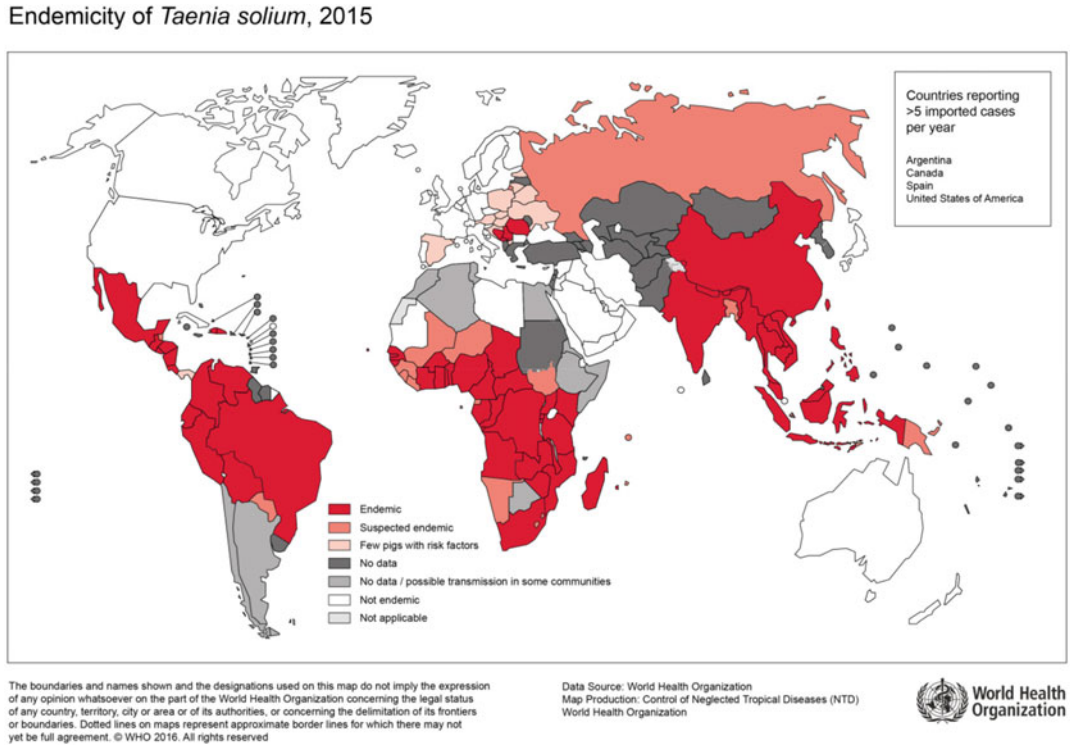

Fig. 7 Epidemiology of *Taenia solium* (Courtesy-WHO)

Table 1 Epidemiological features of *Taenia* spp.

Species	Definitive host	Intermediate host	Geographic distributions
Taenia saginata	Humans	Cattles	Africa, Latin America and Asia as well as in some Mediterranean countries
Taenia solium	Humans	Pigs, humans	Asia, Africa, Latin America
Taenia asiatica	Humans	Pigs, cattle, goats	Korea, China, Taiwan, Indonesia, Thailand, Japan, the Philippines, Vietnam, Nepal

T. asiatica is limited to Asia and cases have been reported from the Republic of Korea, China, Japan, Taiwan, Indonesia, Thailand and Nepal (Table 1). There is a lack of study on *T. asiatica* since it is difficult to identify carriers and needs expensive molecular diagnostic methods for species identification. Due to this, the true prevalence of *T. asiatica* taeniasis remains unknown.

In many countries of East, Southeast and South Asia, which are rich in cultural, ethnic and religious diversity, all three different human *Taenia* species have been shown to circulate in the region. A high prevalence of taeniasis and cysticercosis are reflective of deficient sanitation measures, below par health standards and poor food safety measures. Therefore, there is a need to improve local surveillance, sanitation, diagnosis and overall regulatory systems.

Diagnosis of Taeniasis

A wide variety of diagnostic tests are available, but there is wide variation in the detection levels and discriminating powers between the various *Taenia* species (Table 2).

Table 2 Laboratory diagnosis of taeniasis and cysticercosis

Diagnostic approach	Method	Target	Remarks
Microscopy	Stool examination	Eggs	Cannot distinguish between the species
		Proglottids	Can differentiate *Taenia solium* from *Taenia saginata. Taenia asiatica* has the same morphology as *Taenia saginata*
Antigen detection	CSF, Blood ELISA	HP10 (excretory/secretory glycoprotein of *Taenia saginata*), 87 kDa and 100 kDa of antigen from somatic extracts of adult *Taenia saginata,* 65 kDa antigen from excretory-secretory antigens of *Taenia saginata* cysticerci	Works better with CSF samples compared to serum in neurocysticercosis. Higher the number of viable cysts, the higher the antigen level
	Stool (Coproantigen) ELISA	Somatic antigens of the adult worm or excretory-secretory products	Genus specific. Some test specific for *Taenia solium*. Can detect immature tapeworm stages, before egg shedding
Antibody detection	ELISA	Antibody against Recombinant 50 kDa, Recombinant 24 kDa, Synthetic 8 kDa, Cathepsine L-like 53/25 kDa antigens	Cannot distinguish between active and inactive infection. Have a low positive predictive value in cases with viable cysticercosis
	EITB	GP50, GP42–39, GP24, GP21, GP18, GP14, and GP13 antigens	The presence of any one of the seven antibody bands is taken as positive. Test of choice
Molecular diagnosis	PCR, RFLP, RT-PCR, LAMP	pTsol9, HDP2, 12S rDNA	Highly specific and sensitive. Species specific diagnosis

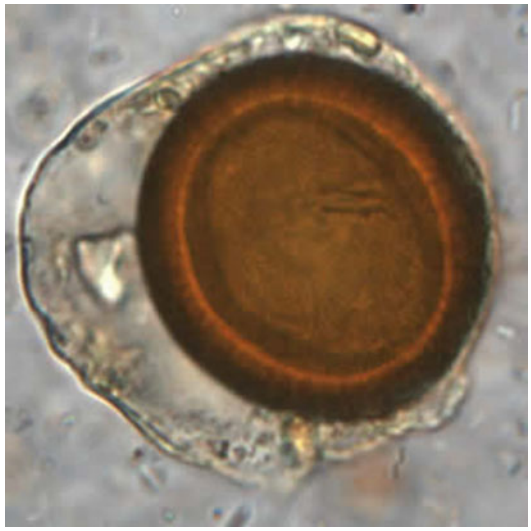

Fig. 8 Egg of *Taenia* spp. (wet mount, Iodine stained). (Courtesy: Oregon State Public Health Laboratory/CDC)

Microscopy

Direct microscopy of stool is performed to demonstrate *Taenia* eggs and proglottids in the diagnosis of intestinal taeniasis. *Taenia* eggs are round, 30–40 μm in size, bile-stained, with thick, brown, radially striated shell, and are embryonated, with a six-hooked oncosphere (Fig. 8). *Taenia* species eggs are morphologically similar to each other. Repeat stool examination and concentration methods such as formalin-ether sedimentation are frequently used to increase the detection rate.

Differentiation between *T. saginata* and *T. solium* is carried out by detection and identification of gravid proglottids containing uterine branches. In order to facilitate the counting of uterine branches, dyes such as carmine or Chinese ink are injected using a fine needle. Longitudinal histological sections stained with haematoxylin-eosin also allow more accurate counting of the branches.

T. asiatica is identified by the demonstration of rostellum on the scolex, presence of more than 57 uterine branches in the gravid proglottids, prominent protuberances in the posterior part of gravid proglottids and wart-like formation on the surface of the larvae.

Serodiagnosis

Demonstration of *Taenia* coproantigens in the stool is diagnostic of intestinal taeniasis and is carried out by a polyclonal antibody-based sandwich ELISA or dipstick ELISA. The tests have the advantages of increased sensitivity, no cross-reactions with other intestinal helminth infections such as *Ascaris*, *Trichuris* and *Hymenolepis* spp. and the ability to detect *Taenia* carriers However, this test is only genus specific and cannot differentiate between intestinal *T. solium* and *T. saginata* infections.

Molecular Diagnosis

The advantages of molecular methods include large-scale screening to detect worm carriers, to diagnose human intestinal taeniasis in animals and to differentiate between the three species.

CoproPCR is highly specific and sensitive to detect both mature and immature *Taenia* worms in stool, although the DNA extraction procedure is costly.

Several formats and targets have been utilized in the detection and differentiation of *Taenia* sp. in worm extracts. PCR coupled with the nucleotide sequencing of the amplified product is the most common approach. The markers used are mitochondrial (cox1, cob, nad1, 12S rRNA), nuclear (18S rRNA, 5.8S rRNA, 28S rRNA and ITS2), elongation factor-1-alpha (ef1) and ezrin/radixin/moesin-like protein (elp). Various approaches include (a) restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR), (b) random amplified polymorphic DNA-PCR (RAPD-PCR), (c) single strand conformation polymorphism (SSCP), (d) multiplex PCR and (e) loop mediated isothermal amplification (LAMP).

The RFLP-PCR, based on studying the restriction fragment length polymorphism (RFLP) of the nuclear ribosomal DNA (rDNA) or other genomic regions, including mitochondrial DNA, is carried out for differentiation of *Taenia* species. The target sequence studied is internal transcribed spacer 1 (ITS1) containing the 5.8S gene, mitochondrial cytochrome c oxidase subunit 1 (cox 1), mitochondrial 12S rDNA and so on.

RAPD-PCR is a relatively simple, rapid technique in which genomic DNA is amplified by PCR using a single oligonucleotide primer of arbitrary nucleotide sequence. However, the test is run along with other available DNA techniques to get a reliable result.

SSCP is a mutation scanning method with the potential to discriminate DNA sequences differing by a single nucleotide. The genes targeted to differentiate *Taenia* spp. in SSCP include mitochondrial cox 1 and NADH dehydrogenase subunit (nad1) genes. DNA sequencing of mitochondrial cox 1 and nad1, cytochrome b, 12S rDNA, nuclear 28S rDNA and ITS1/ITS2 rDNA genes are also valuable in differentiating mainly *T. saginata* from *T. solium*. The test has the advantage of the analysis of large numbers of samples in a short period.

Multiplex PCR employs genus-specific and species-specific primers used to differentiate *Taenia* species. Based on mitochondrial cox 1 as the target gene, specific amplicon size of 827 bp for *T. saginata* and 269 bp for *T. asiatica* have been observed. Specific amplicon size of 720 and 984 bp have been reported for *T. solium* American/African and Asian genotypes respectively. This technique is relatively easy and time saving, as it does not require DNA sequencing, hence most extensively used in the diagnosis of various forms of cysticercosis including neurocysticercosis.

The isothermal amplification methods such as LAMP is increasingly used for the diagnosis and differentiation of *Taenia* species and is found to be more sensitive and specific than the multiplex PCR. Among the two target genes for LAMP, primers targeting the *cox 1* differentiate the three species of *Taenia,* whereas the primers targeting *clp gene* failed to differentiate between *T. saginata* and *T. asiatica.*

Diagnosis of Cysticercosis

Microscopy

Microscopic examination of surgically resected lesions shows typical parasitic tegumental cytology in association with cholesterol crystals and calcareous corpuscles. Demonstration of invaginated scolex with the hooklets is diagnostic of *cysticerci*. Cysts can be in various stages of degeneration with higher intensity of inflammatory reactions in the later unviable stages. Fine-needle aspiration provides a cheaper alternative to open biopsy.

Serodiagnosis

Antibody-based serological tests such as ELISA and enzyme-linked immunoelectrotransfer blot (EITB) (Fig. 9) are most frequently used in the diagnosis of cysticercosis caused by *C. cellulosae*.

The ELISA platforms with the newer FAST-ELISA format use oncospheral peptides and crude antigen extracts to detect antibodies with suboptimal sensitivities and cross-reactions with hydatid disease and hymenolepiasis. EITB assays using lentil lectin purified glycoproteins (LLGP) like GP50, T24, 8 kDa proteins provide robust sensitivity but cannot distinguish the recent from a past infection. A newer EITB format using a recombinant antigen rT24H is showing promise with 94% sensitivity and 98% specificity. Western blot using the LLGPs provides highly specific results with 95% sensitivity.

The EITB, utilizing highly specific protein bands, helps in *Taenia* spp. differentiation. Immunoblot band of 21.5 kDa has been found to be highly specific for *T. asiatica*. In addition to it, there occur two other immunodominant candidate antigens which have been identified and expressed as recombinant molecules: *T. asiatica* Lactate dehydrogenase (rTaLDH) and the recombinant *T. asiatica* enolase (rTaENO). These proteins are found to be located in the tegument of adult *T. asiatica* and the embryonic membrane of the oncosphere.

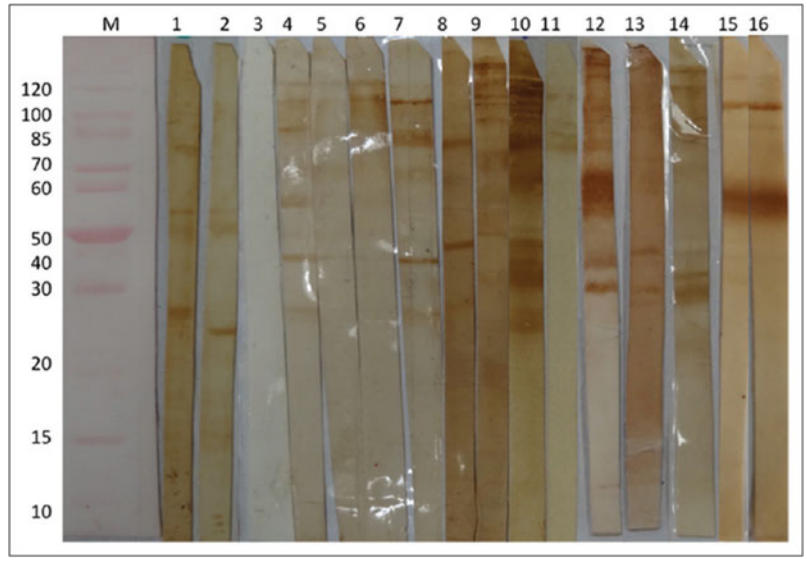

Fig. 9 Enzyme-linked immunoelectrotransfer blot for anticysticercal antibodies. Lane M: Ponceau stained protein ladder; lanes 1, 2, 4, 7–10, 12–16: positive serology for neurocysticercosis; lanes 3, 5, 6, 11: negative serology for neurocysticercosis. (Author's Collection)

The major drawback of the antibody-based serological tests including EITB is that these cannot differentiate between the present and past infection and antibodies persist even after.

Antigen-based tests are frequently used for demonstration of circulating *Cysticercus* antigens in the serum, saliva and urine for diagnosis of cysticercosis. The ELISA using monoclonal antibodies against HP10 antigen and excretory/secretory antigen B158/B60 is increasingly used for detection of *cysticercus* antigen in the serum as well as in the CSF for the diagnosis of neurocysticercosis. The detection of antigen in the serum and other body fluids is suggestive of recent and active cases of cysticercosis and is of prognostic value as antigen disappears from the circulation after clinical and parasitological cure of the cysticercosis.

Other Methods

Imaging methods such as X-ray, CT and MRI are used for the diagnosis of neurocysticercosis. Plain X-ray of skull and soft-tissue radiography is used to demonstrate calcified or dead cyst. CT and MRI are the currently used techniques to demonstrate the number, size, site and stage of cysticerci. CT is the method used for detecting dead, calcified and multiple cysts. MRI appears superior to CT in identifying non-calcified cysts and the cysts located in parenchymal and extraparenchymal tissues. The brain lesions are confused with tuberculoma and tumour on CT/MRI scans.

Any one of the absolute criteria (histological parasitic demonstration in the brain or spinal cord biopsy, CT/MRI showing scolex ("hole-with-dot") or fundoscopic demonstration of the subretinal parasite) provides a definitive diagnosis. Major criteria include highly suggestive lesions on neuroimaging, positive serum EITB assay, spontaneously resolving small single enhancing granuloma or resolution of an intracranial cystic lesion with albendazole or praziquantel therapy. Minor criteria consist of lesions compatible with NCC on neuroimaging, clinical manifestations suggestive of NCC, positive CSF ELISA for antigen or antibody and cysticercosis outside the central nervous system. One major criterion with two minor criteria constitutes a probable diagnosis of NCC. In unfulfillment of absolute criteria, meeting two major criteria and one minor criterion can provide a definitive diagnosis in conjunction with epidemiological criteria (endemic residence, travel history). Epidemiological criteria help in establishing a probable

diagnosis if combined with three minor criteria or one each from the major and the minor criteria.

Diagnosis of Ocular/Orbital Cysticercosis (OCC)

Ocular/orbital cysticercosis (OCC) is a common form of cysticercosis accounting for 75–80% of cysticercosis cases worldwide. Localization of the cysts can be extraocular (orbital tissues, muscles, lacrimal or subconjunctival) or intraocular. OCC can manifest in different ways in a patient depending upon the site of the cyst.

Depending on the cyst location, the outcome of an 8-point ophthalmological exam will vary. Visual acuity, pupils, external evaluation (proptosis, nodular mass, lid swelling, ophthalmoplegia), slit-lamp examination and funduscopic examinations are common in diagnosis.

CBC and anterior chamber paracentesis may reveal eosinophilia. FNAC can be used to aspirate the cyst for a confirmatory diagnosis. Histology can identify scolexes with hooklets within the cyst. The dying cyst presents with a fibrous cyst wall and granuloma with giant cell formation. ELISA can aid in the diagnosis, but negative test results do not rule out OCC (about 50% confirmed OCC patients test negative on ELISA).

MRI, CT and USG are far more reliable in diagnosing OCC than routine laboratory diagnostic methods. A B-scan ocular USG can reveal a well-defined cyst in the orbit with a hyperechoic scolex while an A-scan shows high amplitude spikes corresponding to calcification of cyst walls and scolex. CT may show a characteristic hypodense mass (non-enhancing lesion) with a central hyperdense scolex and adjacent soft-tissue inflammation. However, the scolex may not be identifiable if the cyst is dead and is occluded by surrounding oedema. MRI reveals a hypointense cyst and hyperintense scolex. Inflammation due to the cyst enhances CT and MRI signals.

PCR-based methods and DNA probes are also used to detect parasitic genomic materials in the tissue samples with high sensitivity and specificity.

Treatment

Praziquantel as a single oral dose of 5 or 10 mg/kg is recommended for the treatment of intestinal taeniasis caused by *T. saginata*. Niclosamide, administered in a single oral 2-g dose (50 mg/kg), is also effective. When no *Taenia* eggs are identified in faeces sample 1 and 3 months following treatment, the treatment can be considered successful.

Corticosteroids, antiseizure drugs and therapy by albendazole or praziquantel are recommended for neurocysticercosis. Albendazole is considered superior to praziquantel for NCC. The combination of albendazole plus praziquantel has been reported to result in a higher rate of radiologic resolution. Surgery may be necessary for obstructive hydrocephalus. Orbital cysticercosis is treated with albendazole and corticosteroids.

The prognosis of the disease largely depends on the site, stage of infection, patient's immunity status and surgical capabilities.

Prevention and Control

Intestinal taeniasis is prevented by adequate cooking of beef or pork viscera either by exposure to temperatures between 63 and 71 °C or by refrigeration or salting for long periods or freezing at −10 °C for 9 days. Effective disposal of faecal matter should be done to prevent cattle and pigs from getting infected. The various methods which can be adopted include the following.

Preventive measures require adequate sanitation, sewer treatment and pig corralling to prevent intermediate hosts from the exposure of the parasitic ova. Additionally, food hygiene like avoiding the use of the same chopping board for uncooked and cooked meat, hand hygiene and personal hygiene is necessary.

Meat hygiene is extremely important to prevent disease in humans; irradiation, salt pickling (12–24 h) and freezing at minus 24 °C for 24 h provide excellent results. Meat brought to a temperature between 60 and 65 °C until it loses its pink colour is effective. A strict market control of

infected pigs and cattle is difficult due to economic factors. Meat inspection detects heavy infections. Though the regulations vary globally, pigs are inspected for cysticerci in thigh muscles, diaphragm, heart, intercostals muscles and tongue. Masseters, ventricles of heart, liver and diaphragm of the cattle are the sites to look for. Targeted preventive therapy in specific human risk groups results in a sustained reduction in cases.

Vaccination and anthelmintic therapy of the cattle and pigs help reduce the burden. For pigs, SP3VAC and TSOL18 vaccines show high efficiency in combination with oxfendazole. The TSA9/TSA18 vaccine against *T. saginata* is promising in cattle. However, these vaccines cannot destroy the existing cysts.

Case Study

A 56-year-old man presented at a hospital with a history of passing segments of some worm in his stool of one-month duration. No other abdominal symptoms were present. Stool examination showed proglottides but no eggs. To ascertain the species, PCR amplification of mitochondrial cytochrome *c* oxidase subunit I gene and elongation factor-1 alpha was done with the segment. A 100% match with *T. saginata* was found with both markers. Repeat stool examination after treatment with praziquantel for 3 days showed the absence of any segments or ova.

Questions

1. What relevant dietary history would have been helpful in this case?
2. What tests other than PCR can be done here to arrive at a diagnosis?
3. What precautions should be taken to prevent the infection?

Research Questions

1. A simple diagnostic tool for species identification in taeniasis other than molecular methods in resource constraint settings has to be developed for effective management.
2. There exists a lacuna in defining the role of the immune mechanism during the course of this parasitic infection in humans and animals.
3. There is a lack of an appropriate animal model to study the pathogenesis and host-parasite relationship of these parasites.

Further Readings

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Echinococcosis

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Learning Objectives

1. To identify the two important manifestations of echinococcosis (cystic and alveolar) caused by two different species and the differentiating features.
2. To know about the various methods for laboratory diagnosis and their limitations.
3. To have a knowledge about the important role of surgery in the treatment of echinococcosis.

Introduction

Echinococcosis denotes an infection caused by both the adult and larval forms of the tapeworms belonging to the genus *Echinococcus.* The cestode inhabits the small intestine of carnivores, which are the definitive hosts. *Echinococcus granulosus*, *Echinococcus multilocularis, Echinococcus oligarthus* and *Echinococcus vogeli* are the four recognized species that cause infection in humans. Unilocular/cystic echinococcosis and multilocular/alveolar echinococcosis are caused by *E. granulosus* and *E. multilocularis* respectively. *E. vogeli* causes polycystic echinococcosis, while rare infections have been attributed to *E. oligarthrus*.

History

Hippocrates, Aretaeus, Galen and Rhazes have described echinococcosis since ancient days. In the seventeenth century, Francesco Redi suggested its animal origin. Subsequently, Pierre Simon Pallas in 1766 suggested that the hydatid cysts of infected humans were the larval forms of tapeworms. Goeze and Batsch described the accurate morphology of cyst and head of the tapeworm. In 1863, Rudolf Leuckart identified *E. multilocularis.* Subsequent research and experimental studies in the early to mid-1900s demonstrated the life cycle, pathogenesis and clinical spectrum of human disease caused by *E. granulosus* and *E. multilocularis*. *E. oligarthrus* and *E. vogeli* were the two other species identified to be the causes of human echinococcosis.

Taxonomy

The genus *Echinococcus* belongs to Family, Taeniidae; Order, Cyclophyllidea; Class, Cestoda; and the Phylum, Platyheminthes. The

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genus currently includes nine recognized species. The species of human importance includes *Echinococcus* sensu lato causing unilocular echinococcosis, namely *Echinococcus granulosus* sensu stricto (G1, G2 and G3 strains), *Echinococcus equinus* (G4 strain), *Echinococcus ortleppi* (G5 strain), *Echinococcus canadensis* (G6, G7, G8, G9 and G10 strains) and *Echinococcus felidis* (the lion strain) along with *E. multilocularis* causing multilocular echinococcosis, *E. vogeli* and *E. oligarthrus* causing neotropical echinococcosis and *Echinococcus shiquicus*. Table 32.1 summarizes the *Echinococcus* species causing infections in humans.

Genomics and Proteomics

Zoonotic genotypes within the *E. granulosus* sensu lato complex have been classified into ten genotypes, G1–G10 based on their host specificity. Sheep strain consists of G1–G3, camel strain G6, pig strain G7, cervid strain G8 and G10 and so on. Genome sequence of *E. granulosus* G1 genotype shows a 151.6 Mb genome with a GC content of 42.1%. Similar GC content was noted in *E. canadensis* (G7) with a smaller genomic size (115 Mb). However, *E. canadensis* has a significant lesser gene density than the G1 genotype (13 vs 75 per Mb). On the other hand, *E. multilocularis* has genome size ranging from 115 to 141 Mb. Number of predicted genes in G7 and G1 genotypes were similar (11,449 and 11,325, respectively). Average exon and intron lengths were comparable between G7 and G1 genotypes (219 bp and 714 bp vs 214 bp and 726 bp, respectively). Maldonado et al. (2017) compared inter-species echinococcal genomes using the Circos plot software to reveal high degrees of genetic conservation between *E. canadensis* and *E. multilocularis* (average 94.6%) and between *E. canadensis* and G1 genotype (average 98.3%). The SNP frequency of G1 strain is high, at least ten-fold higher than *E. canadensis*. Detailed analysis shows the loss of genes for synthesis of most amino acids in *E. granulosus* and *E. multilocularis*. *E. granulosus* also lost genes for purine, pyrimidine and fatty acid oxidation. Instead, several genes encoding multiple proteases and solute carrier family proteins were up-regulated. BLAST analysis shows 3903 uniquely *Echinococcus*-specific genes in *E. granulosus* which are the key genes for its biological traits. Acquisition of proteases and carrier proteins point towards the ideal parasitic life to derive nutrition from the host as well as resisting immune attacks. Lack of cholesterol synthesis machinery mandates scavenging the essential fats from the host using fatty acid transporters and lipid elongation enzymes. It is crucial for the metacestodal expression of antigen B (AgB). Specialized detoxification mechanism and homeobox gene loss are characteristic.

Kyoto Encyclopedia for Genes and Genomes (KEGG) analysis suggests *E. granulosus* has the complete pathways for glycolysis, tricarboxylic acid cycle and pentose phosphate. Recently, *E. canadensis* proteome analysis showed a length of about 49,000 amino acids. Similar to tapeworm, echinococcal proteomics show many novel domains related to cell-to-cell adhesion and tegument formation like cadherins and tetraspanins. The unique trait of carbohydrate-rich laminated layer is characterized by the presence of apomucin family and the diversion of the galactosyltransferases. An expansion of the heat-shock proteins (HSP), particularly of HSP110 and HSP70 clades, is striking. Stage-specific proteomic studies document predominance of antigen 5 (Ag5) in the early cystic stage but scarcity in the later stages. Proteomic studies have revolutionized the echinococcal diagnostics. Studies on proteome of hydatid fluid of *E. granulosus* and *E. multilocularis* have revealed very close relation in phylogeny and evolution of both the species. Each species exhibits distinct protein-protein interaction (PPI) networks. While in *E. granulosus*, proteins involved in carbohydrate metabolism are the major PPI, in *E. multilocularis*, extracellular matrix proteins (ECM) are the major PPI to maintain the highly complex multilocular structure of the cysts.

The Parasite Morphology

Adult Worm

E. granulosus adult worm measures nearly 3–6 mm in length. It consists of a scolex, a short neck and strobila (Fig. 1). The pyriform scolex bears four suckers with a rostellum having two rows of hooklets in a circular manner. The length of the neck is around 3 mm × 6 mm. The strobila consists of three proglottids: immature, mature and gravid, respectively, distal to the neck. The terminal gravid proglottid bearing the egg-laden branched uterus is the largest in dimension. The lifespan of an adult worm is around 6–30 months.

Adult worms of other species are similar to each other but with variations in their length. Usually, the length of *E. multilocularis* adult worm is 1.2–4.5 mm. *E. vogeli* and *E. oligarthrus* adult worm reaches up to 5.5 mm and 2.9 mm respectively. The structure of the scolex, neck and strobila is comparable in all the species. Among the species, *E. vogeli* has the largest strobila often reaching a length of 12 mm.

Egg

The eggs of *Echinococcus* are brown coloured and ovoid in shape which contains an embryo with three pairs of hooklets (Fig. 2). The average diameter of the eggs is around 30 μm. The most central layer of the eggshell is called the embryophore which like a typical taeniid egg is radially striated.

Eggs of other species are similar to that described above and they cannot be distinguished microscopically.

Larval Form

Hydatid cyst is the larval form of *E. granulosus* found in the intermediate host. The larva has a

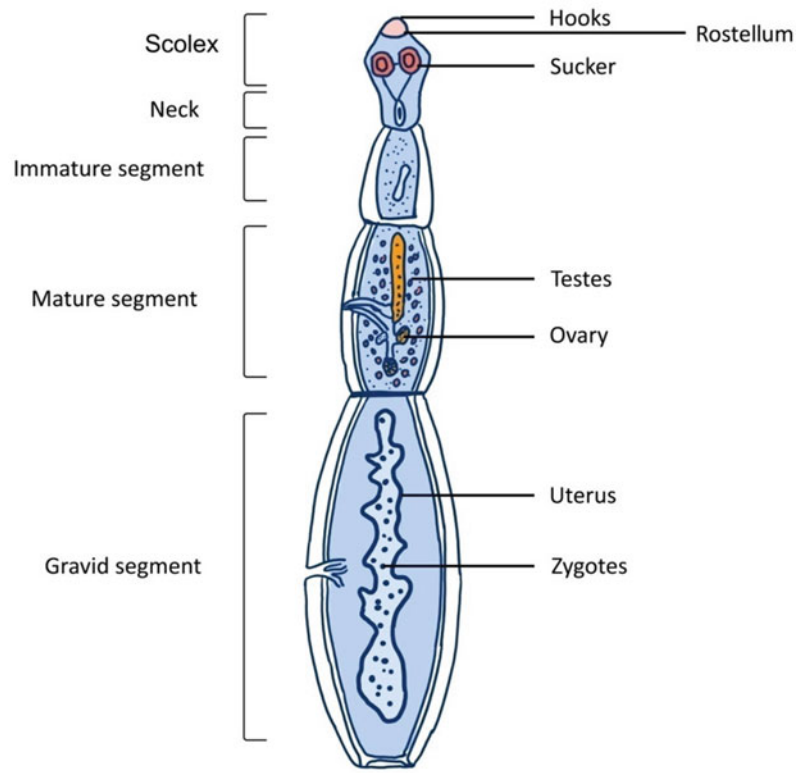

Fig. 1 Schematic diagram of adult *Echinococcus granulosus*

Fig. 2 Schematic diagram of egg of *Echinococcus granulosus*

vesicular body bearing the invaginated protoscolices, akin to the scolex in the adults. Soon after entering into the definitive host, there occurs evagination of the scolex with suckers and rostellar hooklets, which later develops into an adult worm. A fully developed hydatid cyst is unilocular and bladder-like in appearance. It is spherical and varies in size from 2–3 mm to more than 30 cm. The cyst wall consists of three distinct layers, namely the pericyst made up of fibrous tissue produced by host interactions, an ectocyst made of tough, elastic hyaline and an endocyst which is the germinal layer responsible for the secretion of hydatid fluid in the cyst (Fig. 3).

The structure of cyst of other species is similar to the hydatid cyst. However, unlike in hydatid cyst, *E. multilocularis* and *E. vogeli* forms multiple small cysts called locules distributed widely in organs. Cysts of *E. oligarthrus* are unicystic like that of hydatid cyst.

Cultivation of Parasites

Basic culture medium for in-vitro cultivation of the oncospheres of *E. granulosus* consists of medium 858 containing glucose and potassium supplemented with the appropriate host sera or sera obtained from young animals. Cultivation is

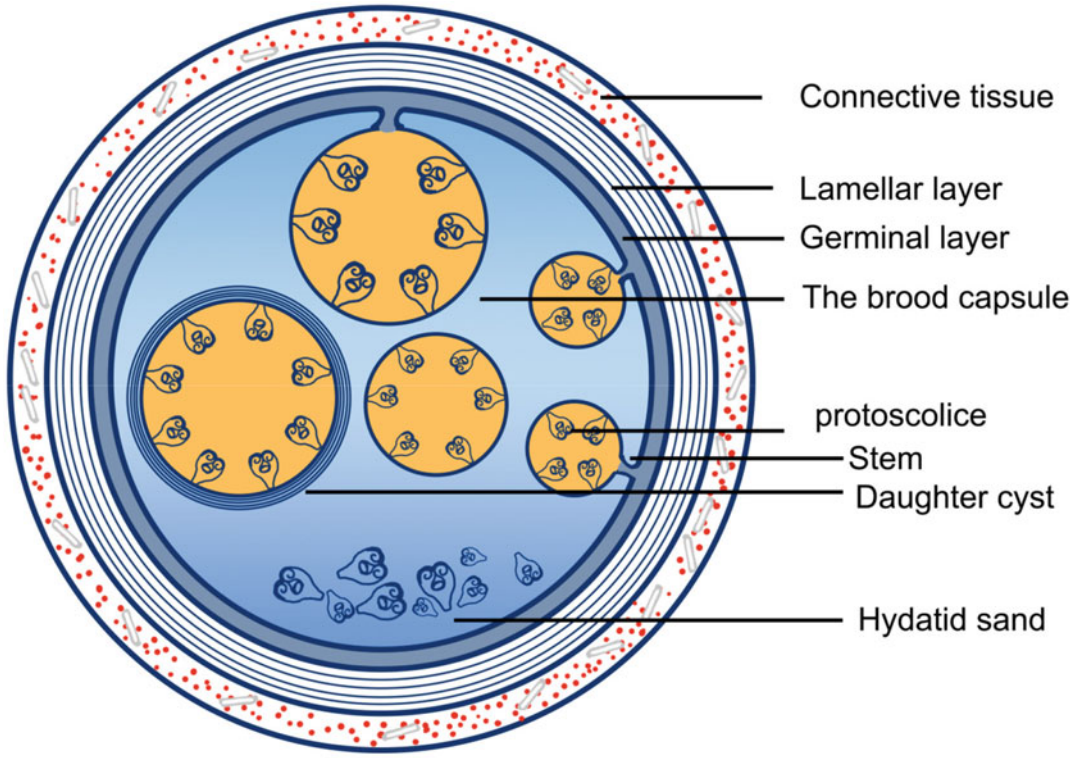

Fig. 3 Schematic diagram of hydatid cyst

carried out at 37 °C in roller tubes with a gas phase of 10% O_2 + 5% CO_2 in nitrogen. This technique of hatching and subsequent cultivation of larva is done to study cytology and histology of developing larvae.

The protoscoleces of *E. multilocularis* have been successfully cultured in a modified RPMI1640-based medium containing 25% (v/v) fetal bovine serum (FBS). *E. vogeli* metacestodes have also been cultivated in the above media along with metacestodes of *E. multilocularis.*

Laboratory Animals

Swiss-albino mouse was developed for *E. granulosus* and was subsequently used to find in vivo efficacy of mebendazole. Experimental animals are infected in various ways such as intraperitoneal, subcutaneous, chest and brain injection by protoscolices. The animal model plays an important role in the translational study for novel drugs, surgical approaches and vaccine development. Gerbils have been intraperitoneally infected with *E. multilocularis*, while mice are commonly used as experimental animals for *E. vogeli.*

The Life Cycle of *Echinococcus* spp.

Hosts

Definitive Hosts

Wild and domestic canids (*E. granulosus*); fox, dog, raccoon (*E. multilocularis*); bush dog (*E. vogeli*); wild neotropical felids (*E. oligarthrus*).

Intermediate Hosts

Sheep, cattle, camel, cervid (*E. granulosus*); rodents (*E. multilocularis*); paca (*E. vogeli*); rodents, lagomorphs (*E. oligarthrus*). Humans are the accidental intermediate hosts.

Infective Stage

Echinococcus eggs are the infective stage of the parasite for humans and other intermediate hosts.

Transmission of Infection

Ingestion occurs through faeco-oral contamination.

Cystic Echinococcosis

Dog, the definitive host, becomes infected by consumption of raw offals such as liver or lungs of slaughtered sheep, goats etc. containing the infective hydatid cysts. On ingestion, in the small intestine, protoscolices evaginate and get attached to the mucosa and develop into a sexually mature adult worm containing gravid proglottids (Fig. 4). Eggs released from the gravid proglottids are passed in the faeces.

Humans are the accidental intermediate hosts. Other intermediate hosts and humans acquire infection by ingesting vegetables, food and water contaminated with eggs. Upon entry inside the intermediate host's gastrointestinal tract, the eggs hatch to release the six-hooked oncospheres. These oncospheres disseminate to different organs through the circulation after directly penetrating the gut wall. Main locations include the liver, lungs and muscle. On entering these organs, the oncosphere slowly transforms into a thick-walled hydatid cyst. The cyst increases in size gradually, with the development of protoscolices and daughter cysts which fill the interior of the cyst. Spontaneous resolution of cysts may sometimes occur in a few cysts, whereas others may grow in size finally to rupture. Following rupture, secondary echinococcosis may be the course or there can be more severe anaphylactic reaction to the hydatid fluid components. The life cycle is a dead end in the infected humans.

Alveolar Echinococcosis

Fox, the definitive host, acquires the infection by ingestion of cyst-containing organs in the rodents. Following ingestion, in the intestine, the protoscolices evaginate and attach themselves to the intestinal mucosa. The larvae develop into sexually mature adult worms, and gravid proglottids liberate eggs that are excreted in the faeces.

The freshly passed egg in the faeces is infectious immediately. The humans and other intermediate hosts acquire infection by the ingestion

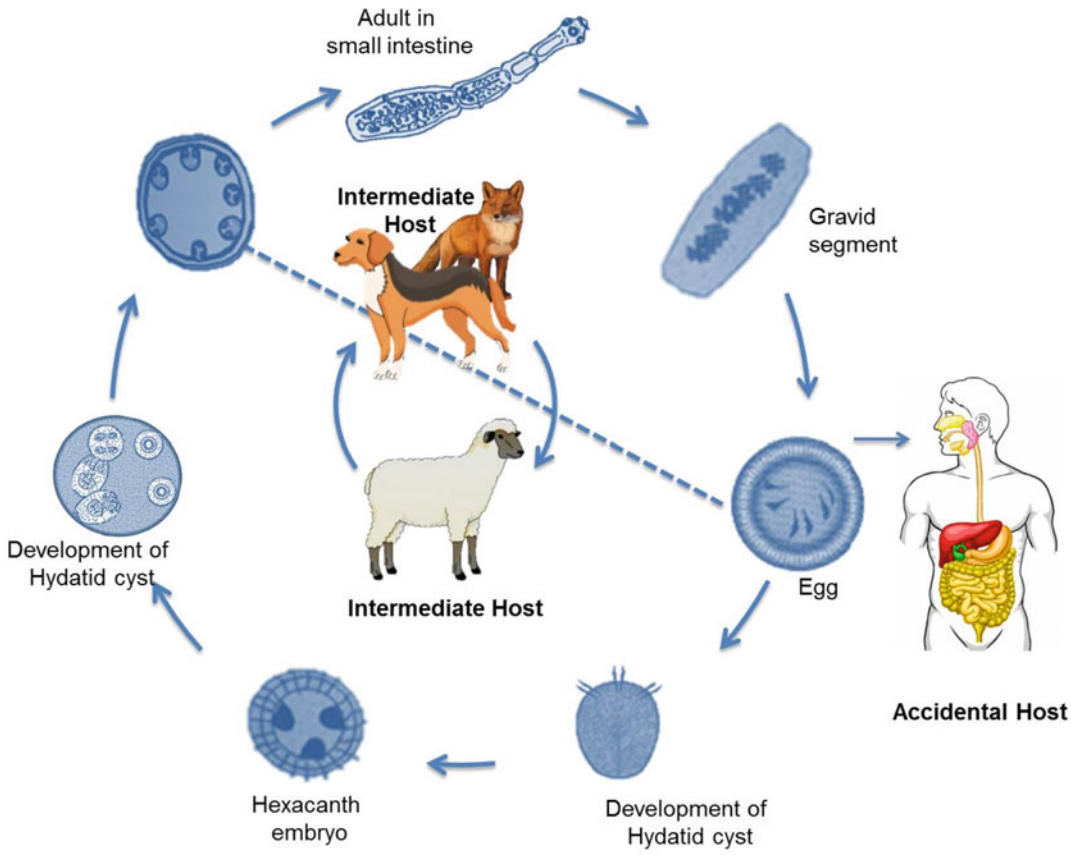

Fig. 4 Life cycle of *Echinococcus granulosus*

of the food and vegetables contaminated by the faeces of the fox that are eaten raw or undercooked. The ingested eggs hatch and release a six-hooked oncosphere which penetrates the mucosal wall of the small intestine. The oncosphere then travels through the circulatory system into various organs, most commonly in the liver. At these sites, they further develop into a thin-walled, multi-chambered ("multilocular") hydatid cyst. The cysts proliferate further by outward budding and multiple protoscolices develop within these cysts.

Humans are considered to be an aberrant intermediate host (Fig. 5). The primary cyst develops in the liver, but in due course metastasis or dissemination by the release of protoscolices from the cyst to the other organs of the body such as lungs, brain, heart and bone can occur.

Polycystic Echinococcosis

The life cycle of *E. oligarthrus* and *E. vogeli* causing polycystic echinococcosis is same except for their differences in hosts, morphology and cyst structure. Wild cats (Felidae) are the definitive hosts of *E. oligarthrus*, while bush dogs are the final hosts of *E. vogeli*.

Pathogenesis and Pathology

Cystic Echinococcosis

Humans, the intermediate hosts, acquire the infection by eating foods contaminated with *E. granulosus* eggs or by drinking contaminated water. In the gut, the eggs hatch and release oncospheres. The oncospheres then penetrate the intestinal wall, migrate via the circulation and

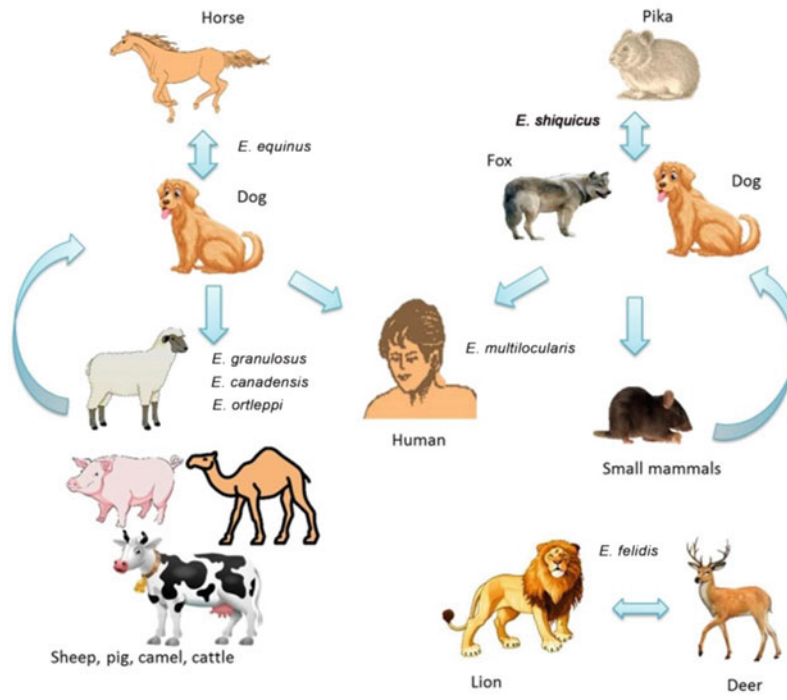

Fig. 5 Life cycle of different species of *Echinococcus*

lodge in the liver or lungs or, less frequently, in the brain, bone or other organs.

In tissue, oncospheres grow and progress into cysts, which grow slowly into large unilocular, fluid-filled lesions popularly known as hydatid cysts. Brood capsules consisting of plenty of infective protoscolices are formed within the cysts. The well-formed hydatid cyst consists of highly antigenic hydatid fluid and thousands of protoscolices. Daughter cysts tend to form either inside or outside the primary cysts. The primary cysts are found in the liver, lungs, other viscera, muscle and tissues of infected hosts. The pathology of the disease is caused by the pressure effect of the cysts. Adult worms are not found in the gastrointestinal tract of humans.

Alveolar Echinococcosis

The chronic hydatid lesion is typically found in the liver. Typically, a tiny fibrous covering contains whitish amorphous materials inside a necrotic cavity. Extensive infiltrative growth by outward budding damages the surrounding tissues. Larval masses entangled within the inflammatory reaction have the metastatic potential to lung and brain. Disseminated echinococcosis often presents as metastatic malignancies. Cancer-like infiltrative growth in the liver and other organs is seen.

Polycystic Echinococcosis

The laminated hyaline layer of cyst found in various organs of intermediate hosts, in polycystic echinococcosis, remains well-preserved. In human, the larval development occurs by invagination invading the whole organ resembling malignancy. The invading and invaginating larval growth gives rise to multiple folds and pockets inside the primary vesicle.

Immunology

Various factors contribute to the evasion of host immune system by *Echinococcus* spp. These include antigenic variation, shedding of surface protein, protease production, active modulation including immunosuppression, skewing of the

Th1/Th2 cytokine profile, molecular masking and mimicry. The cestode the larvae and their products released during growth and development are highly immunogenic. They stimulate pro-inflammatory cellular responses, stimulate antibody production and increased cell-mediated responses in their human and intermediate hosts.

Serum IgG4 and IgE are elevated in patients with progressive cystic echinococcosis in humans. IgG4 is considered as the immunological marker of cystic echinococcosis (CE) and higher IgG1 and IgG3 in patients with stable disease. These findings suggest that the human immune response to echinococcosis is primarily controlled by Th2 cell activation. A strong Th2 response primarily indicates the presence of active cyst while Th1 correlates with the presence of an inactive cyst.

Cystic fluid antigen leakage in cases of both CE and AE in humans leads to an elevated level of specific IgE antibodies in the serum. Associated high level of IL-5 is characteristic. However, eosinophilia and allergic reactions are scarce in alveolar echinococcosis. The host's genetic makeup could be important in progression of the disease; severe disease associated with HLA-DR3DQ2 haplotype has been documented.

The immunology of polycystic echinococcosis is largely unexplored. The host immune response is blunted, possibly Th2-type as observed in cystic echinococcosis, in the presence of multi-chambered cysts compared to the unicystic lesions in cystic echinococcosis. Surgical intervention causing a breach in the laminated layer results in a shift to a weak Th1 response.

Infection in Humans

The locations, number and size of the cysts or the metacestode mass determine the signs and symptoms in humans in echinococcosis.

Cystic Echinococcosis

E. granulosus infection in humans causes cystic echinococcosis with a long incubation period. The condition remains asymptomatic for years until the cysts enlarge to cause dysfunction in the affected organs.

Primary echinococcosis consists of a single cyst and the most common site is the liver, followed by the lungs and other organs. In addition to the liver and lungs, other organs involved are the heart, spleen, bone, kidneys, brain and eyes. A palpable hepatomegaly with dyspepsia-like symptoms is common. In some cases, spontaneous or trauma-induced rupture of the cyst releases viable cystic fluid proteins, scoleces, allergens and so on to the circulations. This results in the dissemination of infection to various other sites in the human host, and the condition is called secondary echinococcosis. Cysts rupture with the release of cystic fluid cause symptoms of fever, urticaria, eosinophilia and potentially anaphylactic shock and even fatal.

Alveolar Echinococcosis

Alveolar echinococcosis (AE) primarily affects the liver as a slow-growing and destructive tumour-like lesion. In humans, the larval forms do not mature fully into cysts, but these vesicles tend to invade and destroy surrounding tissues which cause abdominal discomfort or pain, weight loss and malaise. AE can ultimately end with liver failure and death. Metastasis to the lungs, spleen and brain can occur rarely. The lesion left untreated may turn out to be highly fatal. In infected people living in remote area, the fatality rate has been recorded between 50% and 75%.

Polycystic Echinococcosis

E. vogeli which presents as a slow-growing tumour predominantly affects the liver, with secondary development of a cyst. Subsequently in a subset of patients, the cysts compress upon portal veins and biliary system causing jaundice and portal hypertension. Pleural cavity and mesenteric involvement have been reported. Clinical cases of *E. oligarthrus* have been rarely reported involving the orbit and myocardium.

Infection in Animals

Adult worms in habit the small intestine of the definitive hosts, usually cause mild symptoms. Reduced growth, decreased production of milk,

meat and wool, reduced birth rate and losses due to condemnation of organs have been observed in the infected livestock. Many infected animals are usually slaughtered before they show manifestations of the symptoms. Multiple cysts of *E. granulosus* have been noted in the brain, kidneys, bones or testes with the manifestation of severe illness.

E. multilocularis and other species rarely infect cattle, sheep and pigs. Infections in foxes, definitive hosts, are largely asymptomatic. Interestingly, domestic dogs can harbour both the cestode and the metacestode stages. Thus, akin to the human alveolar echinococcosis cases, internal organ involvements are well described in dogs.

Infections in animals by the other two species are largely asymptomatic and often go unnoticed. One outbreak in a Los Angeles zoo affected several gorillas and other juvenile primates with affection of the liver and pleural cavities. However, limited evidences suggest that proliferation in the liver is rare in natural animal hosts.

Epidemiology and Public Health

Echinococcosis is prevalent worldwide (Table 1).

Cystic echinococcosis is widely distributed in every continent except Antarctica, whereas the geographic distribution of individual *E. granulosus* genotypes appears to be variable (Fig. 6). At present, the most commonly distributed genotypes of *E. granulosus* are G1 and G3 (associated with sheep). Some genotypes like G6 and G7 ("*E. canadensis*") are commonly found in the Middle East, Africa and North and South America, while northern Holarctic distribution is common in genotypes G8 and G10. In endemic regions, human incidence rates for cystic echinococcosis can reach more than 50 per 100,000 person-years and prevalence levels as high as 5–10%, and in hyperendemic areas, the prevalence rate is from 20% to 95% of slaughtered animals.

Alveolar echinococcosis is predominantly found in the regions of the northern hemisphere.

Table 1 Distribution of *Echinococcus* spp.

Species	Major distribution	Intermediate host	Definitive host	Human infection
Echinococcus granulosus sensu stricto	Cosmopolitan	Sheep	Wild and domestic canines	Commonest cause of cystic echinococcosis cases
Echinococcus equinus	Europe, Asia, Africa	Horse, cervids	Domestic dog	Not reported
Echinococcus ortleppi	Europe, Asia, Africa	Cattle	Domestic dog	Cystic echinococcosis
Echinococcus canadensis	Europe, Asia, Africa, America	Camel, pig, cervids	Domestic dog, wolf	Second most common cause of cystic echinococcosis cases
Echinococcus felidis	Africa	Lion	Hyena, zebra, giraffe, deer and so on	Not reported
Echinococcus multilocularis	Europe, Asia, Africa, North America	Rodents and small herbivorous mammals	Fox, wolf, racoon	Multilocular echinococcosis
Echinococcus vogeli	Central and South America	Paca	Bush dog	Neotropical echinococcosis
Echinococcus oligarthrus	Central and South America	Opossum, agouti	Wild felids	Neotropical echinococcosis
Echinococcus shiquicus	Tibetan plateau	Pika	Tibetan fox	Not reported

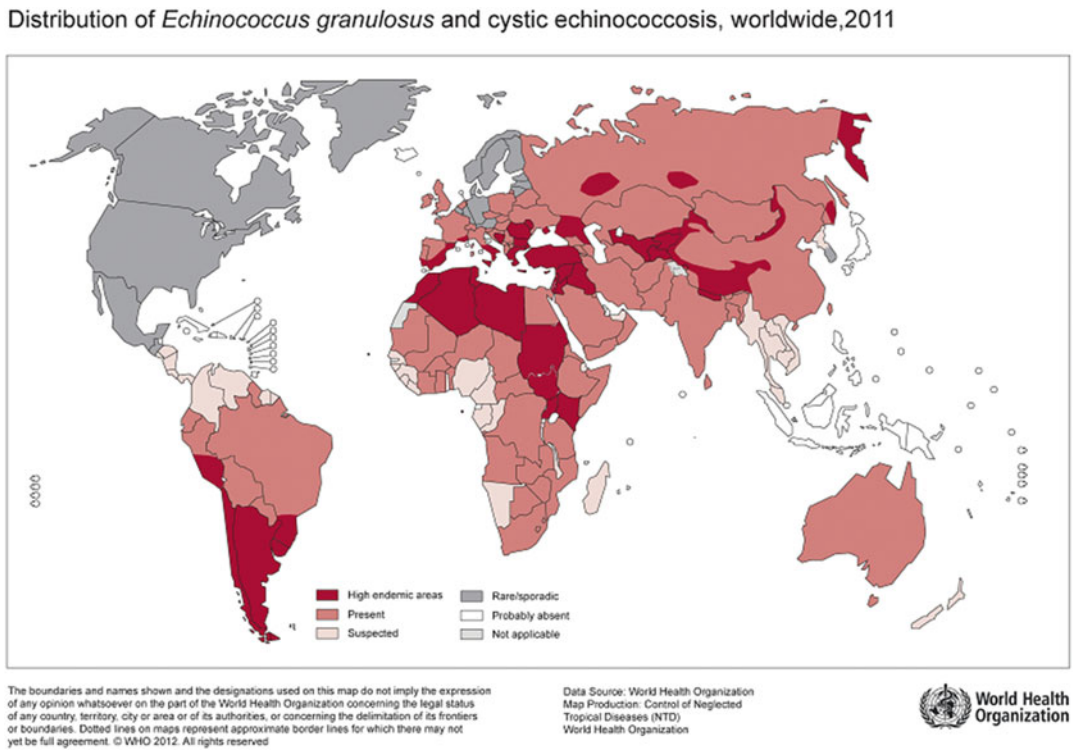

Fig. 6 Global prevalence of *Echinococcus granulosus*

Japan and the Tibetan plateau are increasingly reporting alveolar echinococcosis cases. The vast majority of cases are found in China. Human cases are reportedly being recognized from new areas like Germany and Switzerland. The disease is often mistaken for malignancy and also it behaves like a malignant tumour. On average, it demands more than 6 lakh Disease Adjusted Life Years (DALYs) per year.

Human polycystic echinococcosis is very rarely reported and restricted to Central and South America. *E. vogeli* cases have been reported from Nicaragua, Costa Rica, Argentina, Uruguay and Chile. *E. oligarthrus* cases additionally have been found in Mexico. Neotropical echinococcosis has invaded Peru too. Brazil is becoming the country with the largest number of polycystic echinococcosis cases. However, human neotropical cases outside the ranges traversed by the bush dog are possibly due to *E. oligarthrus*, where the coyotes could establish the transmission.

Diagnosis

Laboratory diagnosis plays an important role in confirming the diagnosis of echinococcosis in both humans and animals (Table 2).

Casoni's Skin Test

In 1912, Tomaso Casoni performed the skin test for the diagnosis of hydatid disease in humans. The procedure involves intradermal injection of 0.25 ml of hydatid fluid which is sterilized by Seitz filtration on one forearm and an equal volume of saline injected on the other forearm. A positive reaction is observed as a wheal which is produced within 15 minutes, surrounded by a concentric red zone, which later disappears with the wheal (immediate hypersensitivity). The test, nevertheless, is nearly being abandoned for use in many laboratories.

Table 2 Diagnostic methods for human echinococcosis

Diagnostic approaches	Methods	Targets in cystic echinococcosis	Targets in alveolar echinococcosis	Targets in polycystic echinococcosis
Microscopy	Hydatid fluid examination Histological examination	Protoscolex, brood capsule and cyst wall demonstration	Biopsy provides exclusion of neoplasm and careful detection of laminated layer remnant	Protoscolex, brood capsule and cyst wall demonstration. Size of rostellar hooks provides differentiation.
Antibody detection (mainly serum IgG)	ELISA Dot-ELISA Immunoblot	Crude hydatid fluid: Sensitivity 95%	Em2: 77–92% sensitive Em2+ recombinant II/3–10 multiplex: 97% sensitive and > 90% specific	*Echinococcus granulosus* metacestodes are used as antigen. Purified antigens of *Echinococcus vogeli* are available. Ev2 antigen cross-reacts with *Echinococcus multilocularis* but differentiates from *Echinococcus granulosus*. Arc5immunoelectrophoresis detects *Echinococcus oligarthrus* (cross-reactivity with *Echinococcus granulosus*).
		Purified Antigen B: • ELISA: 60–85% sensitive • Immunoblot: 60–92% sensitive		
		Purified Antigen 5: 50–87.5% sensitive		
		Purified other proteins: 45–100% sensitive		
		Recombinant AgB • rAgB8/2 and 2B2t: 93.1% sensitive and 99.5% specific • rAgB8/1: 55–84% sensitive		
		Recombinant Ag5 (rAg-38 s): 21% sensitive		
		P176 peptide: poor sensitivity		
	Dipstick	Camel hydatid fluid: highly sensitive and specific	Not available	Not available
Antigen detection (mainly serum)	ELISA, CIEP, co-agglutination, RPHA, Latex agglutination	Low sensitivity (25%) but high specificity (up to 98%)	Not available	Not available
Molecular (DNA detection)	PCR, LAMP	Highly sensitive and specific; genotyping and speciation	Confirms diagnosis and assesses post-treatment viability	Highly specific for both the species but are available in research settings.

Microscopy

Microscopy of the hydatid fluid aspirated by exploratory cyst puncture is the simplest parasitic method of diagnosis. Microscopy of Wet mount of hydatid fluid or staining the centifuged deposit of the hydatid fluid by an acid-fast stain is carried out to demonstrate the brood capsules and protoscolices (Fig. 5). However, the diagnostic aspiration is usually carried out because of the risk of anaphylaxis due to leakage of cyst fluid and the risk of secondary echinococcosis due to spillage of protoscolices from the cyst.

Serodiagnosis

Serology is widely used in the diagnosis of cystic echinococcosis. These tests detect specific antibodies in the serum as well as hydatid antigen in the serum, urine and other body fluids. Serum antibody detection is more sensitive than detection of the circulating antigen but suffers from lower specificity due to cross-reaction and difficult standardization.

Antibody Detection

Current practice mostly relies upon the detection of IgG class of antibodies against native or recombinant echinococcal antigens in the diagnosis of cystic echinococcosis. The IHA, CIEP, ELISA, immunoblot and so on are frequently used with variable sensitivity and specificity.

Crude hydatid fluid, extracts of protoscolices cyst wall, adult worm and purified native antigens from hydatid fluid are various sources of antigens used in these tests. Crude hydatid fluid contains germinal layer-derived protein mixture and provides good sensitivity, around 95% but with low specificity. Crude extracts of protoscolices, cyst wall, adult worm as antigens perform with low or sensitivity 69.4–96.9% and non-reproducibility due to of extract heterogeneities. Purified native antigens such as Antigen B, a 120 kDa polymeric lipoprotein from hydatid fluid, Antigen 5 and a thermolabile 400 kDa also show cross-reactivity with *E. multilocularis* due to >90% homology. Recombinant antigens (recombinant antigen B, recombinant antigen5, ap176 peptide synthesized from antigen B1, etc.) used in immunoblot or ELISA make the test more specific but low sensitivity. Immunoblotting format increases the specificity at the cost of sensitivity. A rapid dipstick assay with camel hydatid fluid reported 100% sensitivity with 91.4% specificity.

Antibody-based diagnosis has many limitations. First, a subset of cystic echinococcosis patients shows poor variable antibody responses in extra-hepatic lesions, in single and small cysts. Second, substantial false negative results, reaching up to 50% in population surveys, may occur due to low levels of specific IgG, variant Ig expression and formation of antigen-antibody complex. Third, false positive results can occur due to cross-reactivity with *E. multilocularis*, *Taenia*, *Onchocerca*, *Schistosoma* and *Toxocara* sp. Fourth, these tests can not differentiate between old and recent infections, as the hydatid antibodies persist longer on the circulation even after parasitological cure. Nevertheless, recent studies have demonstrated that IgG subclass assays might help as early cysts mount IgG4 response while the inactive stages are associated with IgG1, IgG2 and IgG3 types of antibodies. Specific antibodies against HSP20 and p29 antigens provide useful information. IgE or IgM classes of antibody can be of value post-intervention.

Compared to cystic echinococcosis, serology is more useful in the diagnosis of alveolar echinococcosis. ELISA or its modifications are the most frequently used tests. Specific antigens used include the excretory/secretory constituents (Em2 and Em492), alkaline phosphatase (EmAP), EmP2 and Em10 derivatives (II/3–10, Em18) for ELISA. Em2 ELISA provides 77–92% sensitivity. A commercialized $Em2^{plus}$ ELISA combining Em2 and recombinant II/3–10 showed a promising sensitivity (97%) and high specificity (>90%), though cross-reacted with cystic echinococcosis (25.8%). Interestingly Em10 distinguishes alveolar echinococcosis from cystic ones in humans despite the near-total identity with Eg10 and Eg11.3 proteins of *E. granulosus*.

Since the infection with *E. vogeli* is considered to be very rare, not much study has been conducted on their diagnosis of polycystic echinococcosis. The antigen Ev2 has been described to distinguish *E. vogeli* from *E. granulosus*, but cannot differentiate from *E. multilocularis.*

Antigen Detection

Detection of hydatid antigen in serum or urine indicates recent infection and is of prognostic value, as the antigen disappears from the

circulation after the cure from the disease. Several formats are in use including the more common ELISA, counter current-immunoelectrophoresis (CIEP), reverse passive haemagglutination (RPHA), latex agglutination and co-agglutination tests. Serum remains the primary sample; other body fluids like urine and hydatid fluid also are used in different formats of testing. However, the sensitivities and specificities vary widely.

Antigen detection in other forms of echinocccosis is currently not available.

Molecular Diagnosis

Molecular diagnostic methods in echinococcosis include the conventional PCR for genus/species identifications, nested PCR to identify the genome of the parasite from faecal samples, and multiplex PCR for *Echinococcus* sp. differentiation.

Conventional PCR, based on sequencing of the mitochondrial gene *cox1* (460 bp), is used for species or genotypes identification of strains belonging to *Echinococcus*. Further studies on the genetic diversity of strains belonging to *E. granulosus* sensu stricto has been attempted by sequencing of the mitochondrial gene cox1 (880 bp). Multiplex PCR is performed for rapid identification of the majority of species/genotypes belonging to *Echinococcus* and also for the differentiation of eggs belonging to *E. granulosus, E. multilocularis* and *Taenia* spp.

Real-time PCR (qPCR) is a quantitative PCR and has added advantages compared to conventional PCR in detecting parasitic infections with increased sensitivity and specificity and quicker results and quantifying the amount of DNA in the given sample. Some newer approaches like DNA fishing/magnetic capture which is followed by qPCR result in high sensitivity and high specificity, in conditions with worm burdens >100 worms. In alveolar echinococcosis, nucleic acid detection by real-time PCR from biopsy samples is the most rewarding in terms of diagnosis and post-treatment viability assessment.

The loop-mediated isothermal amplification method (LAMP) is utilized in areas of low resource setting endemic for alveolar and cystic echinococcosis but has a disadvantage of false positives due to its high sensitivity.

Other Methods

CT scan, MRI and even chest skiagram can detect the presence of pulmonary hydatid cysts. Radiological sensitivity increases with calcified lesions in the other organs. Ultrasonography is also used as a screening methodology which often typically demonstrates the *water lily* sign. Ultrasound also helps in monitoring of treatment.

An informal working group on echinococcosis of the World Health Organization (WHO-IWGE) has classified hepatic echinococcal cysts in five types: CE1 to CE5 based upon ultrasonography. CE1 and CE2 indicate biologically active stages; CE4 and CE5 represent biologically inactive stages while CE3 is in transitional stage. Similarly, alveolar echinococcosis lesions have been assigned a PNM staging incorporating the parasite lesion, neighbour organs and metastases. Fluorodeoxyglucose–positron emission tomography (FDG-PET) has gained more popularity in AE. MRI has provided better imaging for the CE than CT scan; T2-weighted MRI can reveal the pathognomonic micro cysts of AE.

Ultrasound-guided fine-needle biopsy is used for confirmation of diagnosis. Periodic acid-Schiff (PAS) stain of the cyst wall removed at surgery or by fine-needle biopsy demonstrates the characteristic laminated membrane of the cyst wall and brood capsules (Fig. 3). The procedure has an inherent risk of allergic reactions or leakage of hydatid fluid and protoscolices facilitating secondary recurrence.

Treatment

Indications for treatment of echinococcosis depend on the location of the cyst, size, type and associated complications.

Surgery is the treatment of choice for cystic echinococcosis. It is specially recommended for secondarily infected liver cysts, size of the cyst larger than 7.5 cm with biliary communication or cysts located in other sites such as brain, lungs or kidney. Other formats of surgery include cyst puncture and aspiration of contents. Percutaneous aspiration of the contents followed by protoscolicidal chemical injection and re-aspiration (PAIR) often achieves better outcome particularly with the hepatic cysts. Chemotherapy alone or in combination with surgical interventions provides good results in hydatid cysts. In some cases, preoperative use of chemotherapy is found to have additional advantages of safe handling of the cysts during surgery, inactivating protoscolices and altering the integrity of the cysts membranes. PAIR is turning out to be promisingly indicated for the patients who are unfit for surgery as well as in the relapse cases. Failed chemotherapy cases also become eligible for this technique.

Albendazole is the preferred chemotherapeutic agent with disease regression rates varying from 30% to 50%. This is particularly beneficial for the small cysts and cysts scattered onto different locations. It is given in a dosage of 10–15 mg/kg body weight per day for a period of 1–6 months. Mebendazole is another option with a higher dosage (daily 40–50 mg/kg body weight) administered over several months.

Radical surgery is the preferred treatment in alveolar echinococcosis, supplemented with or without chemotherapy. Since there is an increased risk of recurrence, treatment with chemotherapy involves administration of benzimidazoles for a period of at least two years and monitoring for ten years. These approaches inhibit the progression of the disease and also the size of the mass appears drastically reduced.

Prevention and Control

Most human infections are prevented by the strict compliance of personal hygiene by washing hands with soap and water after handling dogs. Hand hygiene prior to food intake is also important.

Public health remains a critical yardstick in the control of echinococcosis. Health education coupled with improved access to healthcare is the unmet need. Improvement of slaughter hygiene and meat inspection, dog registration and sanitation measures are various strategies followed to control cystic echinococcosis.

Animal handlers, veterinarians and dog owners are all at higher risk of infection since the eggs are shed with faeces in the environment. Ingestion of contaminated fruits, vegetables or water and direct contact with the fur of an animal containing eggs increases the risks of infection through transmission of the eggs of the parasite. Cystic echinococcosis causes a significant economic loss by affecting human and animal health. Therefore, public awareness creation about the transmission and control of the disease is of utmost importance. Safe animal slaughtering practices including offal destruction and prevention of feeding on infected organs are recommended. Canine praziquantel administration adds to the success. Vaccination of sheep using Eg95-based vaccine trials has shown encouraging results in China and South America.

Controlling alveolar echinococcosis is difficult due to the involvement of wild animals in the cycle. Promising results with praziquantel-impregnated baits has prompted using a bait-delivered vaccine for the wild foxes. A coordinated surveillance programme based on coproantigen testing, necropsy and quantification of proglottids in stool samples from foxes can provide a reasonable control strategy.

Very little is known about the control of polycystic echinococcosis. The focal existence seems favourable in terms of control. However, the asymptomatic nature largely prohibits the case detection. *E. vogeli* cases can possibly be contained by restricting the human access to its eggs by following food and personal hygienic measures. Currently, *E. oligarthrus* has a negligible public health impact. Restriction of bush dog access areas and of importing felids from endemic loci are important measures to control.

Case Study

A 43-year-old woman presented with acute onset of severe right upper quadrant abdominal pain of 7 days duration. The patient complained of severe cough associated with haemoptysis, wheezing, fever and malaise. History of loose stools and weight loss was also elicited. She gave a history of close contact with dogs. Her serological assay showed positivity for hydatid cyst antibody (IgG). On chest X-ray, a lesion was noted in the inferior right lobe of the lung with calcified shell and her abdominal CT demonstrated multiple cysts in the liver, which confirms the presence of multi-organ echinococcosis.

Questions

1. What are the suggestive clues from the clinical history?
2. What are the serological assays available?
3. What could be the species involved?
4. How would you proceed for the definitive diagnosis?

Research Questions

- Preventive measures in the control of infection transmission are the major hindrance due to the role of wild animals in the life cycle of this parasite which is more often influenced by climatic changes and landscape.
- Another major challenging issue in near future is developing a safe and effective vaccine by defining an optimum target in the definitive host to interrupt the transmission cycle of *Echinococcus*.
- Newer drug discovery to overcome the limited efficacy of current drugs.
- Transcriptomic and genetic deciphering of the complex life cycle events.

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Sparganosis

K. Vanathy

Learning Objectives

1. To emphasise the various modes of transmission of infection to the human beings.
2. To understand danger of ocular and central nervous system involvement.
3. To know about the challenges in the clinical and laboratory diagnosis of this condition.

Introduction

Sparganosis is a zoonotic infection in humans caused by plerocercoid larvae of Pseudophyllidean cestodes of the genus *Spirometra.* The plerocercoid larval form (L3) *Spargana* causes the infection. Humans are the accidental hosts. *Spargana* in infected humans can invade the eyes, brain, subcutaneous tissue, breast or spinal cord and can cause a threat to human health. Many species of the genus *Spirometra* including *Spirometra mansoni, Spirometra ranarum, Spirometra erinaceieuropaei* and *Spirometra proliferum* and recently described *Spirometra decipiens* cause sparganosis.

History

The parasite was first described by Patrick Manson in 1882 from China. He identified the most common species of Asia, *S. mansoni* during the post-mortem examination. The first case of human sparganosis was reported by Stiles from Florida, USA, in 1908. In 1959, *S. erinaceieuropaei* was identified and considered as a single species which was previously considered as separate species like *S. mansoni* and *S. erinacei.* This is one of the most common species worldwide. Morphologically, *S. erinaceieuropaei* and *S. decipiens* were differentiated by the presence of a number of coils in the uterus which was 5–7 and 4.5, respectively. *S. mansonoides* was reported by Mueller in 1935. It was found that *S. erinaceieuropaei* was distributed in the Asian region and *S. mansonoides* in North America. A few cases of *S. theileri* was reported in 1974, among the Masai tribe of East Africa. It was identified from excised nodule which contains sparganum.

The first case of *S. proliferum* was reported by Ijima in 1905. The parasite was found to multiply inside the host and hence it was also known as proliferating sparganum. It tends to proliferate with one plerocercoid in one lesion and thus disseminated throughout the body involving all

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visceral organs and subcutaneous tissue. The parasite causes infection in immunocompromised hosts, such as AIDS patients, is fatal and can be identified during autopsy. The first case of *S. proliferum* was reported from the USA by Stiles in 1908, but the most common species identified later in 1935 was *S. mansonoides*. *S. decipiens* was another species reported in humans in the year 2015.

Taxonomy

The genus *Spirometra* belongs to Family, Diphyllobothriidae; Order, Pseudophyllidea; Sub-class, Eucestoda; Class, Cestoidea and Phylum, Platyhelminths. Species of two genera, *Diphyllobothrium* and *Spirometra,* are closely related. Phylogenetic studies based on ribosomal internal transcribed spacer 2 sequences are carried out to know the relationship between different species of the genus *Spirometra* with that of *Diphyllobothrium.*

Genomics and Proteomics

S. erinaceieuropaei possesses the largest genome among tapeworms of 1.26 Gb. The beta tubulin gene analysis demonstrated that this particular species may not respond to treatment with albendazole. The nucleotide variations were very minimal among strains found in Australia, Asia and New Zealand. DNA sequence analysis of mitochondrial dehydrogenase, cytochrome oxidase, iron sulphur protein analysis has shown that although *S. proliferum* is closely related to *S. erinaceieuropaei,* both the species are quite distinct.

The Parasite Morphology

Adult Worm

Spirometra are long, segmented and flattened dorso-ventrally. The worm is 60–110 cm long and 0.5–0.8 cm in width. Adult worm consists of head or scolex, neck and body or strobila (Fig. 1). The scolex is elongated and spoon-shaped, does not have any suckers and possesses a pair of longitudinal grooves called bothria. This groove is helpful for their attachment to the intestinal tissue. The neck is followed by body or *strobila* which contains many, nearly 1000, proglottids that may be immature, mature and gravid. The worm is hermaphrodite, both well developed male and female reproductive organs are found in the worm.

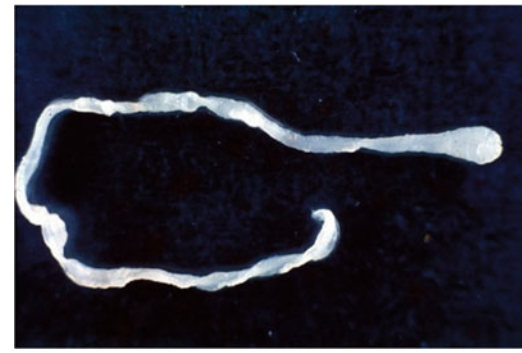

Fig. 1 Adult worm of *Spirometra* (Courtesy: CDC)

Eggs

Eggs are ovoid in shape and measure about 65 × 35 μm in size (Fig. 2). Each egg contains an embryo inside which has three pairs of hooklets. The egg is surrounded by a thin membrane or capsule and it has an operculum at one end. The egg is not embryonated when hatched. Embryonation occurs in water.

Larva

Sparganum is the larval stage of the parasite. It is wrinkled, white, ribbon-shaped measuring 3 mm in breadth and 30 cm in length. The larva does not have any suckers, but instead have two longitudinal grooves at the anterior end called bothrids. It has a solid body with no bladder. The larva has unsegmented strobila of 20–30 cm in length. The strobili consist of the scattered longitudinal

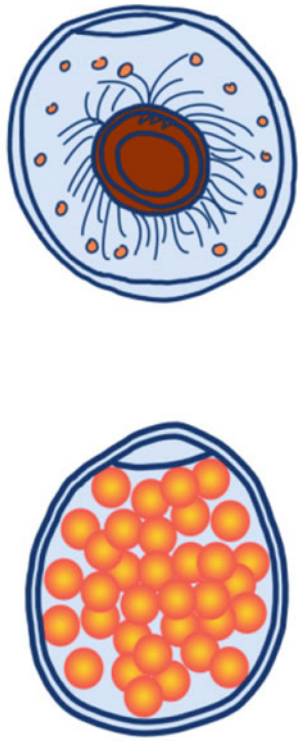

Fig. 2 Embryonated and unembryonated egg of *Spirometra*

muscle fibres in the mesenchyme and a thick tegument.

Cultivation of Parasite

S. mansonoides has been cultivated in vitro, in primary cell culture or cell lines. Those monolayer cell cultures include human amnion, rhesus monkey kidney, rat or hamster embryo. WI-38 and L-cells are the cell lines used in vitro culture. They are maintained in eagle's medium or medium 199 containing 10% calf serum.

Laboratory Animals

The plerocercoids can be maintained by serial passage in BALB/c mice every 10–12 months. The mice can be infected orally with sparganum infection. This model has been used for immunological and other studies.

Life Cycle of *Spirometra* spp. Hosts

Host

Spirometra spp. completes its life cycle in a definitive host and in first and second intermediate hosts (Fig. 3).

Definitive Hosts

Dogs, cats, birds and wild carnivores. Humans are the accidental hosts.

First Intermediate Hosts

Cyclops and fresh-water crustaceans.

Second Intermediate Hosts

Frogs, snakes, birds, mammals and other amphibians.

Infective Stage

Plerocercoid larva, L3 larva, (Sparganum) is the infective stage.

Transmission of Infection

Humans, the accidental hosts, acquire infection by (a) ingestion of contaminated water with Cyclops harbouring procercoid larva (L2) which develops into sparganum in human intestine and (b) ingestion of raw or undercooked reptiles and birds infected with plerocercoid larva (L3), the *sparganum*. (c) The infection is also acquired by application of the infected flesh of the second intermediate host over the human skin, conjunctiva or vagina as a poultice on the infected wound. In either of the above modes of transmission, man acts as a definitive or second intermediate host. Man acts as a dead-end host.

Dogs and cats acquire the infection by eating frogs, snakes, amphibians or mammals which contain sparganum larva. *Spirometra* adults live in the small intestine of mammals and other definitive hosts. The nutritive materials absorbed through their tegument are transferred to internal tissues and they get metabolised. They are

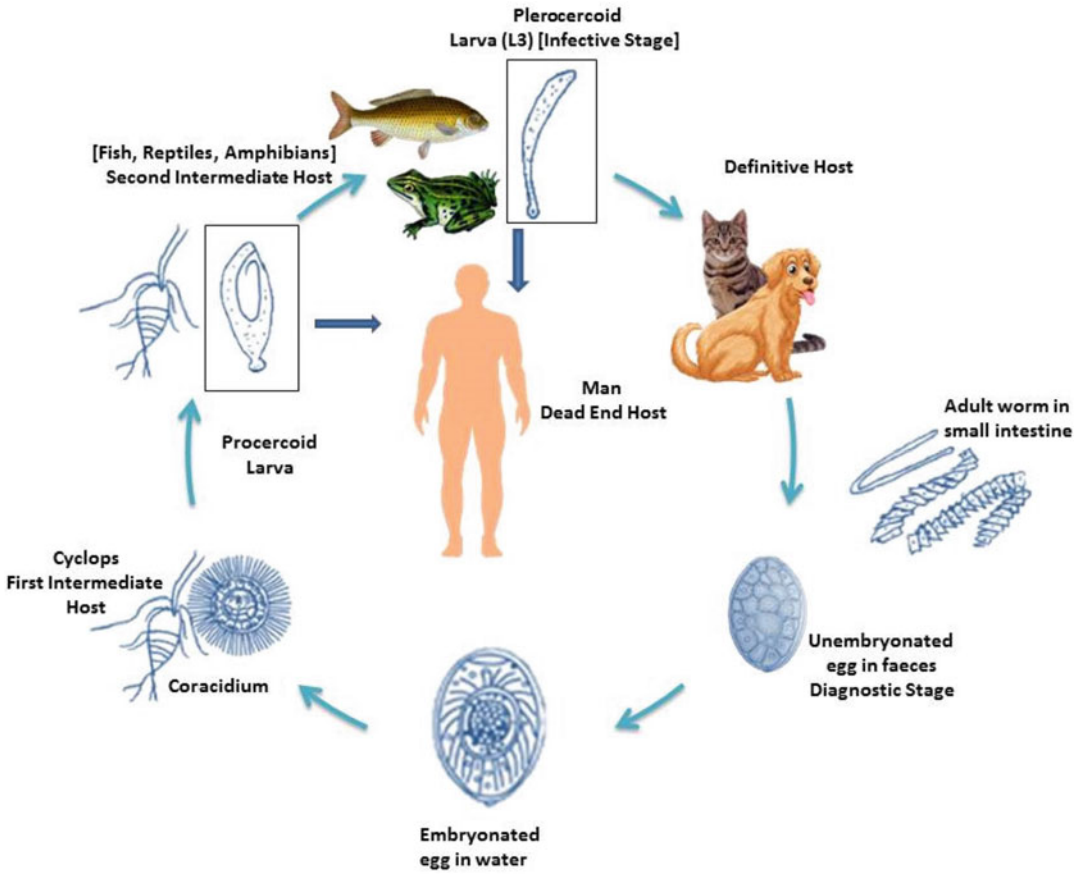

Fig. 3 Life cycle of *Sparganum*

hermaphroditic. The adult worms live for many years inside the host and release the eggs in faeces.

The eggs hatch in fresh water as coracidia. Coracidium is around 80–90 μm in diameter and is covered by the ciliated membrane. They are ingested by Cyclops, the copepods in water, in which they develop into the procercoid larvae (L2) in 3–11 days. Procercoid larvae are oval in shape and vary from 260 ×44–100 in size.

Frogs, snakes, mammals and amphibians, among others, acquire the infection by ingesting the infected cyclops. On ingestion, the larvae which are released in the intestine penetrate the intestinal wall, migrate to the tissues and develop into sparganum larvae. The life cycle is repeated by ingestion of these infected hosts.

Pathogenesis and Pathology

Pathogenesis of sparganosis depends on the migration of the larva (Sparganum) and its subcutaneous location. The migratory larvae, during the course of infection, are found in the muscle or tissue in the extremities, the chest and abdominal wall. Eyes, pleura, pericardium, brain, spinal cord, lymph nodes, intestinal wall, urinary tract and scrotum are the other sites. Sparganum at these sites typically causes an inflammatory reaction in the tissues surrounding the subcutaneous site, subsequently resulting in nodules. These discrete subcutaneous nodules are the typical pathology of sparganosis that may appear and disappear during the course of infection. These nodules cause various clinical manifestations depending on their presence on various sites.

Table 1 Distribution of *Spirometra* species

Spirometra species	Distribution	Intermediate hosts	Definitive hosts
Spirometra erinaceieuropaei	Far East, Europe, Asia	*First intermediate hosts*: Cyclops and other crustaceans *Second intermediate hosts*: Frogs, snakes, birds, mammals and other amphibians	Dogs, cats, foxes, birds and wild carnivores. Humans are the accidental hosts
Spirometra decipiens	Korea, China		
Spirometra mansonoides	North America		
Spirometra theileri	East Africa — among Masai tribe in Kenya and northern Tanzania		
Sparganum proliferum	Far East and America		

Immunology

Limited immunological studies in experimental mice have revealed the important role played by T-regulatory cells which are initially up-regulated, followed by down regulation and final up-regulation. Regarding the cytokines, Interleukin-6 first increases and then returns to normal levels. There is a decrease in the levels of Interleukin-2, interferon-γ and IL-17α production but an increase in IL-4 and IL-10 levels.

Infection in Humans

There are two forms of sparganosis in humans: proliferative and non-proliferative.

In humans, the plerocercoid larva migrates to various organs, subcutaneous tissue and forms tender nodular mass. They are slow growing. Pleural cavity, brain, lung, CNS, eyes, subcutaneous tissue, breast, abdominal viscera and urogenital viscera, among others, are frequent sites of infection by the larva. Depending on the site of the location of the larva, the symptoms vary from non-specific discomfort, pruritus, elephantiasis and brain abscess to peritonitis and so on. Ocular sparganosis may involve conjunctiva and orbit of the eye causing periorbital oedema, lacrimation, orbital cellulitis, ptosis and movement disorder. Involvement of anterior chamber can cause hypopyon, synechia and secondary glaucoma. The nodules resemble a tumour mass in genital sparganosis involving labia, testes, scrotum, vagina, ureter and urinary bladder. Cerebrospinal involvement causes symptoms such as limb weakness, hemiparesis, paresthesias, headache and confusion. It mainly involves cerebral hemisphere mainly the fronto-parietal lobe extending to cerebellum. The lesion over the skin is a clear, rubbery, cystic swelling which remains painless for many years and may result in sudden pain.

Aberrant sparganosis caused by *S. proliferum* is called proliferative sparganosis in which the parasite continues to grow by branching and budding. Proliferating sparganosis involves subcutaneous tissue, bone and spinal cord. The larva may undergo continuous branching and budding to form many plerocercoids at single site. They begin as small tumour-like mass in the subcutaneous tissue of thigh and neck and then extent to involve internal organs like brain, lungs, abdomen, skin and muscles. They form cutaneous nodules and their adult form is unknown.

Epidemiology and Public Health

After the first reported case of sparganosis in China in 1882, many cases of sparganosis have been reported worldwide with a large number of cases in China, Thailand, Korea and the USA. In Thailand, the reported cases were seen involving the eyes, cerebrospinal region and viscera. The cutaneous and ocular sparganosis was due to the application of frog flesh as poultice for the treatment of sore eyes and also drinking contaminated water. Reports of human sparganosis have also been documented in Japan, India and Sri Lanka (Table 1). The liver sparganosis was first reported in India and was cured by aspiration of the worm from abscess followed by treatment with metronidazole.

Diagnosis

Human sparganosis is often misdiagnosed since the clinical features are not specific. Proper history taking and examination serves as a guide to some extent. Patients from endemic regions, eating undercooked frog and snakes and drinking contaminated water add to the suspicion of sparganosis. Also, they may present with migratory, painful, subcutaneous nodule. The diagnosis can be made by surgical excision of the nodule and removal of the worms. The absence of suckers and hooklets differentiates sparganum from cysticercus and coenurus. Definitive diagnosis can be made by inoculation of adult worm into the definitive host and collecting the worm or faeces specimen from the intestine (Table 2). But this is time consuming and cumbersome procedure.

Microscopy

The tissue section of the organ involved shows proliferating sparganum in H & E stain. The morphology of the larva removed at surgical resection reveals white, ribbon-shaped structures with a wrinkled surface ranging in size from a few millimetres to a few centimetres.

Serodiagnosis

The diagnosis can be made by antigen-specific IgG antibodies by ELISA from peripheral blood or crude somatic antigen. Antigenic polypeptide 28.7 kDa (SmAP) that is expressed by the sparganum stage increases the sensitivity and specificity of the test. Serological diagnosis may be used to confirm a suspected radiological finding.

Molecular Methods

Molecular techniques are considered to be superior to other procedures. PCR is used for the *Spirometra* species identification and the gene targets are small subunit (18S) and large subunit (28S) ribosomal RNA, ribosomal internal-transcribed spacer 1 and ribosomal internal transcribed spacer 2, *cox1*, *nad3* and nuclear *sdhB* genes.

Other Diagnostic Modalities

These include CT, MRI and USG. It is useful for cerebral and ocular sparganosis where CT shows hypodensity, ventricular dilatation and calcifications. It has to be differentiated from brain mass, cysticercosis and paragonimiasis.

Treatment

The main modality of treatment of sparganosis is surgical removal. It is necessary to remove the entire body of sparganum without leaving scolex which may lead to recurrence. The complete removal can be confirmed by repeated serological tests. A decreasing titre of anti-sparganum IgG

Table 2 Diagnostic methods of human sparganosis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Biopsy	Tissue section-H & E stain	Primary method of diagnosis limitation: invasive
Serology	IgG antibodies by ELISA	Antigenic polypepetide 28.7 kDa (SmAP)	*Limitation*: Cross-reactivity with *Clonorchis* and *Paragonimus*
Molecular technique	PCR	Small subunit (18S) and large subunit (28S) ribosomal RNA, ribosomal internal transcribed spacer 1 and ribosomal internal transcribed spacer 2, *cox1*, *nad3* and nuclear *sdhB* genes	High sensitivity and specificity *Limitation*: Require skilled personnel

antibody confirms the complete removal of the worm. Localised chemotherapy is preferred if surgical removal cannot be done. The drug that may be used is praziquantel 120 mg/kg in divided doses. Mebendazole can also be used. For proliferating sparganosis, surgical removal is the only choice of treatment.

Prevention and Control

Preventive measures include avoidance of undesirable cultural practices such as consumption of raw or undercooked flesh of frog or snakes or application of fresh frog flesh as poultice over skin or sore eye or drinking contaminated water with infected copepods. It is also controlled by treatment of cestodiasis. Preventing hunting and sale of wildlife especially frogs and snakes, and increased public awareness on mode of the transmission, clinical presentation, treatment and prevention mainly for people travelling to endemic region, also contributes to control of the infection.

Case Study

A 33-year-old woman came with a history of painful migratory swelling over the left thigh for the past 6 months. Erythema was seen around the swelling. Initially it was diagnosed as a soft tissue tumour. Later MRI revealed several elongated tubular tracts in the medial aspect of the left thigh from which a long, wrinkled, whitish worm was removed. The diagnosis was made by histopathological examination as *Spirometra* Spp.

1. What are the modes of transmission of the infection in humans?
2. What are the measures necessary to prevent infection?
3. How to make a pre-operative diagnosis of sparganosis?

Research Questions

1. How to elucidate the pathogenesis and virulence factors of *Spirometra* Spp. which remains largely unknown?
2. What medical treatment may be useful which can obviate the need for surgery?

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Dipylidiasis

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Learning Objectives

1. To have knowledge about the acquisition of the infection in children having dogs or cats as pets.
2. To understand the innocuous nature of the infection which normally does not cause any serious illness.

Introduction

Dipylidium caninum, the causative agent of dipylidiasis, is a common tapeworm of canines and cats, occasionally found in humans. It has a few common names like "insect tapeworm", "cucumber tapeworm", and "two-fold pored tapeworm". The parasite is transmitted by the ingestion of fleas infected with the cysticercoids larva. The adult helminth develops in three to four weeks after infection and parasite load is directly related to the number of cysticercoid larvae present in the fleas and the number of ingested insects. Dipylidiasis is a zoonosis with worldwide distribution and the condition has been reported on all the continents. Human *D. caninum* infection has been reported mostly in children from Europe, Asia, South America, and the United States.

History

D. caninum is known for a long time. In 1758, Linnaeus recognized the parasite and named it *Taenia canina*. In 1863, Leuckart created the genus *Dipylidium* and described by Diamare in 1893. Early work on the life cycle was reported by Neveu-Lemaire in 1936.

Taxonomy

The genus *Dipylidium* belongs to Family, Dipylidiidae; Order, Cyclophyllidea; Class, Cestoda; and Phylum, Platyhelminthes. *D. caninum* is the most common species that cause infection in humans. Dog-originated *D. caninum* were phylogenetic distinct from those of cat-originated *D. caninum*. *Dipylidium buencaminoi* and *Dipylidium otocyonis* are the other two species that cause infrequent infections in humans.

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Genomics and Proteomics

The complete mitochondrial genome of *D. caninum* has been sequenced. It has a size of 14,226 bp which encodes 36 genes. Protein coding genes number 12, while 22 are transfer RNA genes, and 2 ribosomal RNA genes. The excretory-secretory product of *D. caninum* has been analysed that reveals 49 small molecules of 12 different chemical groups, including amino acids, amino sugars, and amino acid lactams.

The Parasite Morphology

The Adult Worm

D. caninum adult worm is 15–70 cm in length and 2–3 mm in width and is light reddish yellow in colour. The head or scolex of the adult worm is small and measures less than 0.5 mm in diameter. It has four muscular suckers that aid in attachment and locomotion. A dome-shaped rostellum is present at the apex of the scolex (Fig. 1). The rostellum has four to seven rows of tiny "rose thorn"-like hooks which facilitate the attachment of the worm.

The body or strobila is composed of 60–175 elliptical segments or proglottids, which may be immature or mature (gravid). Immature proglottids are trapezoidal in shape. Each mature proglottid contains two sets of male reproductive organs and two sets of female reproductive organs with each set of genital apertures opening medially on the lateral edges of the proglottid. Thus the proglottids of *D. caninum* have two genital pores for fertilization which is in contrast to other more commonly encountered human-infecting cyclophyllide. Because of these bilateral genital pores, the parasite is often referred to as the "double pored tapeworm". The ovaries are bilobed and the uterus is situated posterior to the genital opening. The testes are multiple in number and occupy most of the proglottid space. Eggs accumulate within each proglottid until the proglottid becomes filled to capacity with egg capsules or egg packets containing 5–30 eggs (Fig. 2).

The gravid proglottids are convex-shaped and creamy white in colour, measure 10–12 mm in length, and resemble cucumber seeds (hence the name cucumber tapeworm).

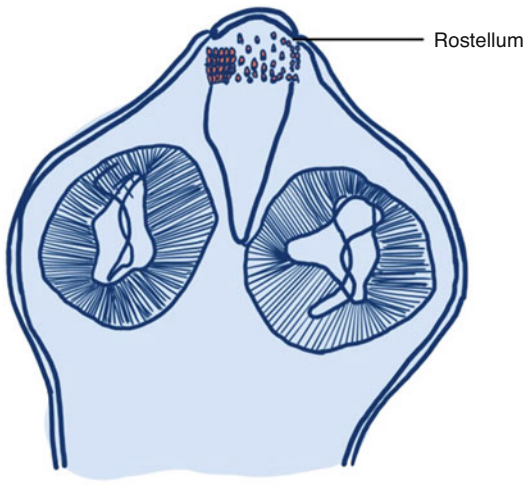

Fig. 1 A schematic diagram of *Dipylidium caninum* scolex

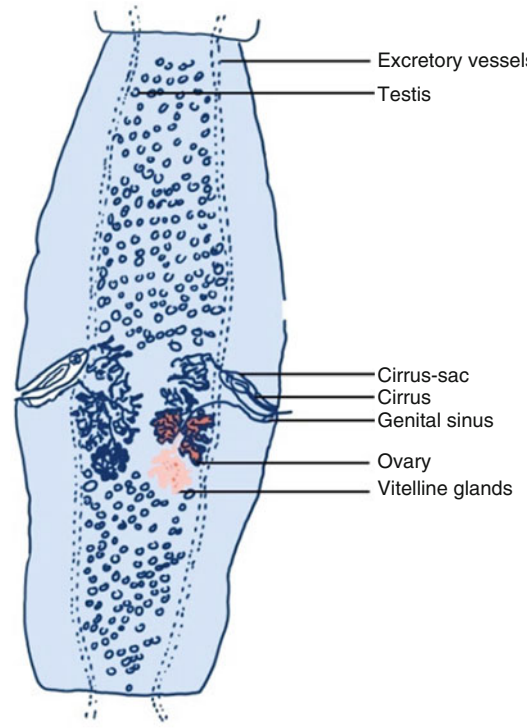

Fig. 2 A schematic diagram of *Dipylidium caninum* proglottid

Eggs

D. caninum eggs are spherical, colourless, and measure 40–50 μm in size. Each egg contains a hexacanth embryo surrounded by a thin shell. The eggs are in clusters in form of egg packets.

Cultivation of Parasites

No laboratory methods are available for in vitro cultivation of *D. caninum*.

Laboratory Animals

Dogs have been occasionally used as experimental animals in a few studies.

Life Cycle of *Dipylidium caninum*

Host

Definitive Hosts
Dogs, cats, occasionally humans

Intermediate Hosts
Dog flea, Cat flea (*Ctenocephalides canis* and *Ctenocephalides felis*)

Infective Stage

Cysticercoid in fleas

Transmission of Infection

Humans are the accidental hosts that acquire the infection by accidental ingestion of fleas that are present on the surface of the skin of pets infected with cysticercoids (Fig. 3).

The cysticercoids in the fleas, on ingestion by the humans and other vertebrate hosts, move down in the intestine and attach themselves to the intestinal wall by their scolex. Inside the gut, the cysticercoid develops and transforms into the mature adult worm consisting of proglottids. The mature gravid proglottids are detached from the strobila of the adult worm body and are shed in the faeces passed with the stool. The gravid proglottids that are shed in the faeces possess both circular and longitudinal smooth musculature. Hence, they exhibit crawling motility on the animal's perianal region, in the faeces, on the bedding, or across any surface where they are deposited. Outside the body, on the disintegration of proglottids, the egg capsules are released and the eggs are released.

The larval stages of the cat or dog flea actively feed on the freshly passed faeces of the animals containing the eggs of the parasite. The flea larvae have mandibulate mouthparts which allow it to ingest the eggs of *D. caninum*. The adult flea, however, is not able to ingest these proglottids as it can consume only liquids because of its syphon-like mouthparts. In the flea larva, the oncospheres that excyst out of the embryonated egg enter the haemocoel after piercing the digestive canal. The oncosphere continues to develop and transforms to infective cysticercoid in the adult flea after about 30 days. The flea may contain an average of 10 cysticercoids.

Pathogenesis and Pathology

Dipylidiasis in humans occurs due to accidental ingestion of the dog or cat flea infected with cysticercoids, which act as an intermediate host for *D. caninum*. Inside the small intestine of the host, cysticercoids develop into an adult within a period of 20 days. The length and diameter of adult worm measures around 10–70 cm and 2–3 mm, with a lifespan of less than a year. Studies on pathological changes due to dipylidiasis in humans are scanty.

Immunology

Studies on immune response to gastrointestinal nematodes have demonstrated the induction of Th2 dominant immune responses along with increased number of mucosal mast cells and intestinal eosinophils. Two ways of manifestation of immunity effect following GI nematode infection have been observed. First mechanism by innate

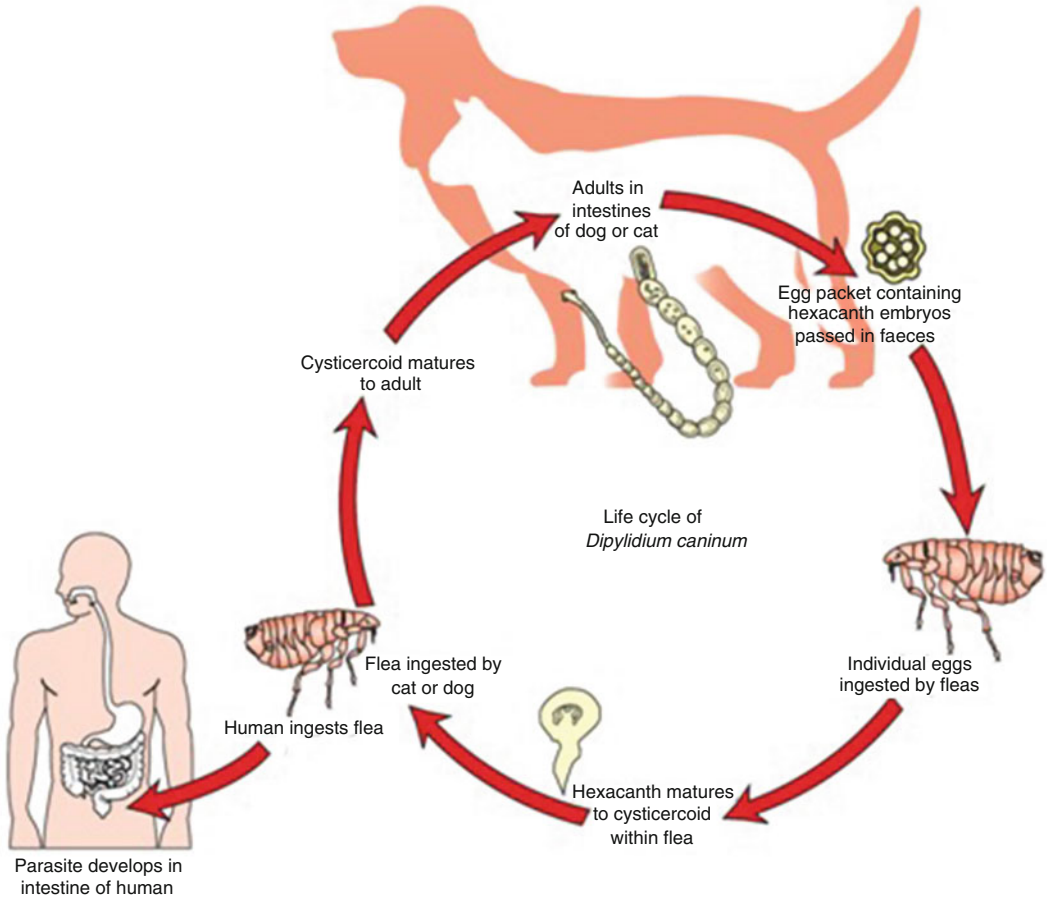

Fig. 3 Life cycle of *Dipylidium caninum*

immunity known as a self-cure phenomenon which is an ejection of nematodes from the intestines at some point in time. Second is by adaptive immune response known as sterile immunity following resolution of the infection which occurs by constant priming by regular low exposure to nematodes on pasture.

Infection in Humans

Human dipylidiasis is a very rare clinical entity, not well described due to the scarcity of records. *D. caninum* infection is mostly asymptomatic in humans. In symptomatic infection, abdominal pain, diarrhoea, restlessness poor appetite, and anal pruritis due to migrating proglottids through the anal cavity are frequently noted. The most striking feature noted by the mother of a child is the passage of proglottids in the faeces on diapers, floor coverings, and furniture. These motile proglottids are often mistaken for maggots or fly larvae. The parents and the child have anxiety because of the passage of motile segments of the worm. The worm burden is low, but sometimes more than 10 worms have been recorded.

Infection in Animals

D. caninum typically does not cause significant disease in dogs and cats; in rare instances ulceration, inflammation of mucosa, and possible bowel wall rupture take place in the definitive host. There are a few reports of young puppies having

an intestinal impact from the massive infections of *D. caninum.*

Like humans, infections in animals are asymptomatic and self limiting, except for signs of increased appetite and behavioural changes like the scraping of anal region across grass or carpet can be noticed, to relieve anal pruritis due to migrating larva. Apart from anal pruritus from proglottid migration, the natural hosts typically show no complications of infection, except in particularly heavy infections where gastrointestinal irritation can occur from the anchoring of the rostellum. At point of attachment there may be a haemorrhage leading to enteritis and diarrhoea. It is clinically manifested as retarded growth rate, reduced working capacity, and general ill health.

Epidemiology and Public Health

Dipylidiasis, caused commonly by the tapeworm of dogs and cats, is a zoonotic parasitic disease. Human cases have been reported from Europe, the Philippines, China, Japan, Argentina, Chile, and the United States (Table 1). Very few cases of human dipylidiasis have been reported from India. A total of nearly 350 cases worldwide have been reported so far, but the exact burden of disease has not been estimated because of the scarcity of clinical records. It is estimated that nearly one-third of cases occur in children below 6 months of age. Infections in young children and infants are primarily due accidental ingestion of fleas or contact with the saliva of pets which may contain cysticercoids of *D. caninum.*

Diagnosis

The diagnosis of human or animal dipylidiasis relies mainly on microscopical examination of stool, but the molecular diagnosis has also been reported (Table 2).

Microscopy

Demonstrations of *D. caninum* eggs (Fig. 4) which are round to oval containing an oncosphere with six hooklets by stool microscopy confirm the diagnosis of the condition. Expelled proglottids in stool specimen may be mistaken for fly larva or other parasites. The proglottids may appear single or in chains and identification of characteristic egg packets of proglottids which may contain 5–15 or more eggs is diagnostic.

Serodiagnosis

A study carried out in Taiwan from stray dogs for immunodiagnosis of canine dipylidiasis by ELISA using *D. caninum* mature and gravid proglottid antigens showed 100% and 50% specificity and sensitivity for mature proglottid extract and 75% and 100% for gravid proglottid extract.

Molecular Diagnosis

A conserved short fragment of 314 base pair of the mitochondrial 12S rRNA gene has been used

Table 1 Epidemiology of *Dipylidium caninum*

Parasite	Definitive host	Intermediate hosts	Geographic distribution
Dipylidium caninum	Dogs, cats, humans	Dog flea: *Ctenocephalides canis* Cat flea: *Ctenocephalides felis* Dog louse: *Thichodectes canis* Human flea: *Pulex irritans*	Cosmopolitan

Table 2 Diagnostic methods for dipylidiasis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Stool examination	Eggs	Typical egg packets
		Proglottids	Two genital pores and the egg packets
Molecular diagnosis	PCR of stool sample	314 bp of the mitochondrial 12S rRNA gene	Used in animals

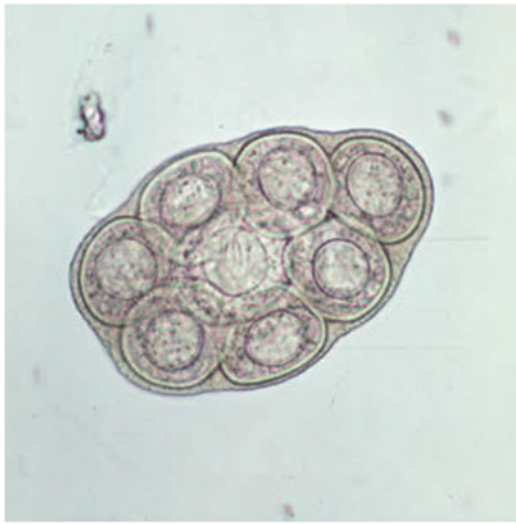

Fig. 4 *Dipylidium caninum* egg packet in a wet mount (Courtesy: CDC)

to identify *D. caninum* infection in the faecal samples in animals. A 28S rDNA PCR detection assay for identification of *D. caninum* DNA from single fleas collected from both cats and dogs had been developed which showed two genetically distinct variations of the target region during sequence analysis of the 28S rDNA fragments. These two distinct genotypes have been named based on the host origin as "*D. caninum* canine genotype" and "*D. caninum* feline genotype".

Treatment

D. caninum infection is self-limiting in humans and typically clears spontaneously in 6 weeks. The condition is treated effectively with praziquantel in a single oral dose of 5–10 mg/kg for adults. Praziquantel is not recommended for children less than 4 years old. Niclosamide is also another effective drug.

Prevention and Control

Prevention and control of *D. caninum* infection in dogs and cats include controlling fleas and lice populations in these pet animals. Deworming of the pet animals by praziquantel is effective for controlling the parasite load in the infected dogs.

Case Study

A 4-year-old male child was brought to the OPD by the mother with a complaint of passing rice grains structures in the stool for about 6 months. The child was prescribed one dose of deworming agent albendazole along with some antihistamine. Despite the treatment, the child did not respond completely and again referred to the OPD. A stool sample was collected for microscopy. Macroscopic examination of the stool sample showed small ivory-shaded structures like cucumber seeds or rice grains. Each structure was 0.5–1.0 cm long and 0.1–0.2 cm thick. The proglottid sections were prepared through paraffin implant and stained with haematoxylin-eosin. They showed inward compartmentalization of eggs in bundles.

1. What would be the final diagnosis of this patient?
2. What other relevant history need to be elicited from the patient's mother?
3. What advice should be given to parents to prevent the infection in children?

Research Questions

1. What antigens can be used to develop an immunodiagnostic test?
2. How best to control the flea population in pets to prevent human infection?
3. What is the true burden of infection in the animal population in a community?

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Hymenolepiasis

Namrata K. Bhosale

Learning Objectives

1. To understand the importance of rodents in the transmission of infection.
2. To have a knowledge about the relative mildness of the infection with no serious complications.

Introduction

Hymenolepis nana, also called the dwarf tapeworm because of its small size, is the commonest cestode infecting humans. Rodents and insects are the reservoir hosts. It does not require an intermediate host which makes its transmission easy and hence is distributed worldwide. *Hymenolepis diminuta* is a zoonotic species which is common in rodents and rarely infects humans. Though most of the infections caused by this parasite are asymptomatic, they can cause severe infection involving the central nervous system in children, people with chronic infections and immunocompromised hosts.

History

Theodor Maximilian Bilharz, a German physicist and one of the founders of tropical medicine, discovered *H. nana* in 1851. Von Seibold in the year 1851 declared it as a human parasite. He found numerous adult worms of *H. nana* in the small bowel of an Egyptian boy who had died of meningitis. *Hymenolepis microstoma* was first described by Dujardin in 1845, in the bile ducts of mice but was included in the genus *Taenia*. Grassi and Rovelli in the year 1800 ruled out the existence of an intermediate host in the life cycle of *H. nana*. The adult stage of *H. diminuta* was first recovered from a 19-month-old healthy child in the year 1852. It was Weinland who first reported a case of human infection with *H. diminuta* in the year 1858 and also coined the genus name *Hymenolepis* from two Greek words: *hymen*: membrane and *lepis*: rind.

Taxonomy

The genus *Hymenolepis* belongs to Subfamily, Hymenolepidinae; Family, Hymenolepididae; Class, Cestoda; Order, Cyclophyllidea; Subclass, Eucestoda; and Phylum, Platyhelminthes in the Kingdom, Animalia.

H. nana and *H. diminuta* are the two species that are infective for humans. These parasites are cosmopolitan. A new genus, *Rodentolepis*, has been recently proposed to replace *Hymenolepis*.

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S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
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Genomics and Proteomics

H. diminuta has a genome size of 177 Mb with 15,169 annotated protein-coding genes. Of the 13,764 bp-sized mitochondrial genomes of *H. nana* encoding 36 genes, 12 are protein-coding genes, 2 are ribosomal RNA genes and 22 are transfer RNA genes. The genome size of related *Hymenolepis microstoma* is about 140 Mb and has 12 diploid chromosomes. MicroRNAs (miRNAs), a class of small non-coding RNAs, are primary post-transcriptional regulators of gene expression and are active in many different biological processes. The new availability of parasitic helminth genomes of medical and veterinary interest, including cestodes, has provided a platform for using both computational and experimental methods to classify miRNAs. Genomic tools allow novel biomarkers to be discovered for diagnosis and/or therapeutic targets to monitor the infections they cause.

Adult *H. nana* proteomic analysis has revealed 13,738 proteins. A differential protein expression of cysticercoid and adult of *H. diminuta* found 233 cysticercoid and 182 adult proteins of which 102 were shared by both stages. A total of 131 were present only in the larval stage compared to 80 in the adults only.

The Parasite Morphology

Adult Worm

The body of an adult is divided into the head (scolex), neck and segments (strobilae).

The adult *H. nana* is 15–40 mm long and 0.5–1.0 mm wide. The head or scolex is globular and refractile and consists of four suckers. The suckers are armed with rostellum with the help of which the adult worm attaches to the ileal mucosa. Nearly, 20–30 hooklets are arranged in a linear row on the rostellum (Fig. 1). The neck is thin, unsegmented, and moderately long, leading to 200 proglottids which are immature, mature and gravid. Each mature proglottid comprises both male and female reproductive organs. It contains three dorsally situated testis and one

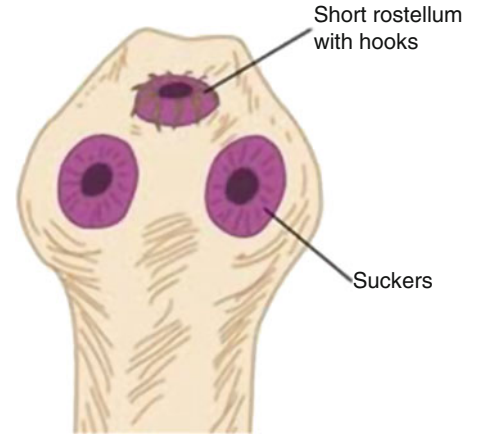

Fig. 1 Schematic diagram of *Hymenolepis* adult scolex

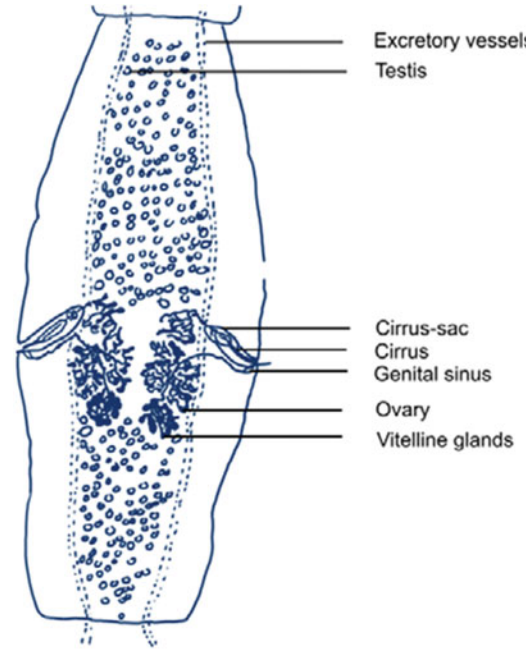

Fig. 2 Schematic diagram of *Hymenolepis* adult proglottid

centrally situated lobed ovary. The gravid proglottid is completely filled up with up to 200 fertilized eggs which are released on disintegration of the proglottid (Fig. 2).

The adult *H. diminuta* measures around 200–600 mm long and can have up to 1000 proglottids. Similar to *H. nana*, the scolex of *H. diminuta* bears four suckers and a rostellum but without any hooklets.

Eggs

Eggs are both diagnostic and infective forms of this parasite. They are round to oval and measure around 30–47 μm in size. They are non-bile stained even though they pass through the bile duct and float in saturated salt solution. The embryo has two membranes (an outer striated transparent shell and inner embryophore) with space between them filled with granules. The embryophore gives rise to two knobs at the poles, each of which gives rise to 4–8 polar filaments extending in the inter-membrane space. Four to six radially arranged hooklets can be visualized in the embryo. The eggs of *H. diminuta* differ from *H. nana* in lacking embryophore knobs, polar filaments and hooklets and are twice their size (60–80 mm).

Larva

The larval stage of *Hymenolepis* is called cysticercoid (Fig. 3). It is a well-organized cyst which is composed of triple collagen fibres and membranous cyst lining. The vesicular proximal part contains the scolex. In the indirect life cycle of *H. nana*, it acts as an infective form.

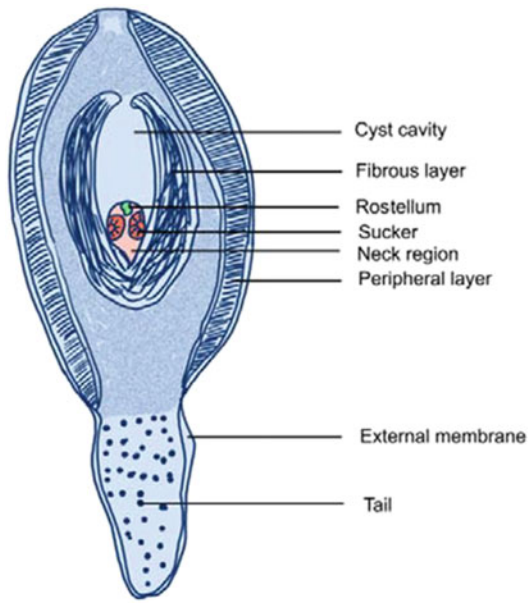

Fig. 3 Cysticercoid of *Hymenolepis* spp.

Cultivation of Parasite

The development of oncosphere to cysticercoid as well as from the stage of cysticercoids to adult worm stage in *H. nana* can be carried out successfully by a number of techniques and using different media preparations. *H. diminuta* has also been cultivated in vitro by different techniques.

Green and Wardile were the first to sustain the viability of adult *H. nana* var. *fraternal* in the year 1941 using tissue culture medium. Later in 1959 Schiller successfully demonstrated the growth of *H. diminuta* from the segments of the adult worm. Berntzen used continuous flow culture methods in the year 1961 to culture the cysticercoids of *H. diminuta* and *H. nana*. In 1975, Seidel and Voge demonstrated the growth of *H. nana* from the cysticercoid using axenic culture.

Laboratory Animals

Rats and rarely mice are the two common laboratory animals which are used for understanding the pathogenesis and investigating the action of various anthelminthic drugs used for the treatment of hymenolepiasis. These rodents are orally infected with the gravid segments of *Hymenolepis* and the faeces are examined to look for the presence of eggs. Necropsy performed on the infected rodents reveals the presence of adult worms in the intestine. Histopathological features of the intestine include mucosal ulcers, necrosis, atrophy and desquamation of the intestinal villi in case of heavy infection with these cestodes. Infiltration of the intestinal villi with inflammatory cells is also an important finding in the tissue sections.

Life Cycle of *Hymenolepis* Species

Studies have revealed two types of life cycles in *H. nana*: direct and indirect (Fig. 4).

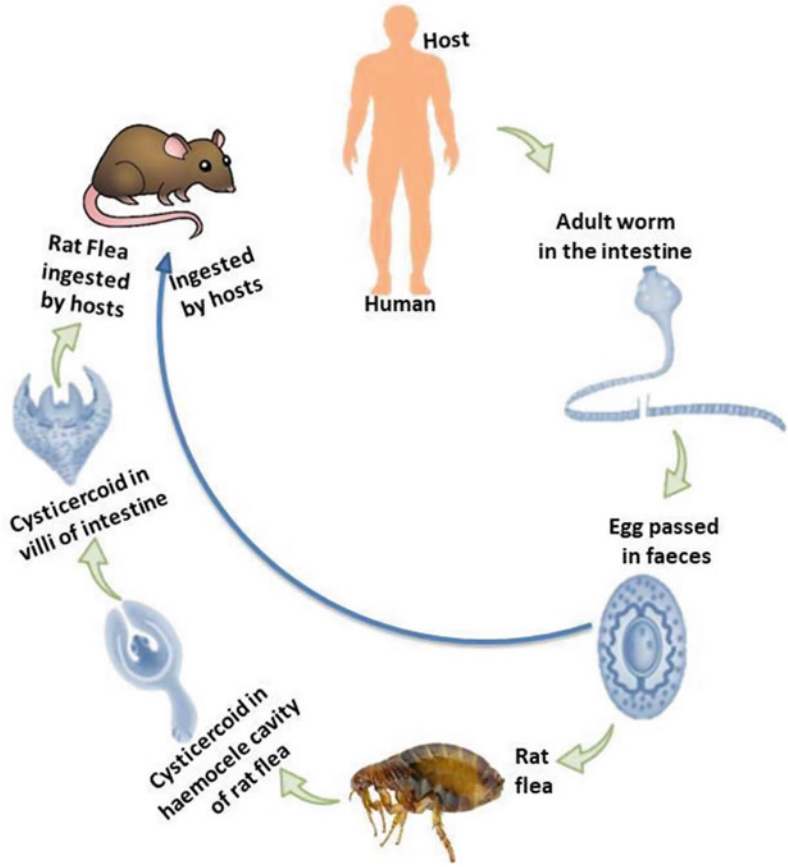

Fig. 4 Life cycle of *Hymenolepis* spp.

Hosts

Definitive Hosts

Man, rats and mice are the only definitive hosts of *H. nana*.

Intermediate Hosts

No intermediate host is required in the direct life cycle. But arthropods like rat fleas (*Pulex irritans* and *Xenopsylla cheopis*), grain beetles (*Tribolium, Tenebrio*) and moths act as the intermediate host in the indirect life cycle. In the life cycle of H. nana var. fraterna (a morphologically identical variant infecting rodents), arthropods act as intermediate host.

Infective Stage

Eggs are the infective form in direct life cycle and as mentioned previously cysticercoid larvae are the infective forms in the indirect life cycle.

Transmission of Infection

Infection is acquired through ingestion of eggs through faeco-oral route. Sometimes accidental ingestion of cysticercoid containing insects can introduce the infection.

The infection is acquired on ingestion of the infective eggs through contaminated food, water or hands. The eggs hatch to release the hexacanth motile oncospheres which enter the intestinal villi by penetration and develop into cysticercoid larvae over a period of 4 days. On rupture of the villus, the larvae are released in the gut lumen. They then get attached to the ileal mucosa with the help of the scoleces and later transform into adult worms. Eggs are released in the faeces from the gravid proglottids through the atria or by disintegration. Sometimes eggs hatch inside the host leading to autoinfection. The lifespan of the adult worm is around 4–10 weeks.

The indirect life cycle is initiated after ingestion of the eggs by the coprophagic arthropods. The ingested eggs after hatching develop into cysticercoid larvae in the body cavity of the insects. Accidental ingestion of such cysticercoid containing insects initiates the infection in humans and rodents.

The life cycle of *H. diminuta* is similar to that of the indirect cycle of *H. nana*. The definitive hosts of H. diminuta are rats (*Rattus norvegicus, Rattus rattus*), rarely mice and occasionally humans. Several arthropods like beetles, fleas, earwigs and myriapods act as obligatory intermediate hosts.

Pathogenesis and Pathology

Hymenolepis infection is normally harmless in adults and more of an annoyance than a health concern. In small children, however, severe infection with *H. nana* may be associated with pathological alterations and clinical symptoms. Within the host the cysticercoids are located mostly in the lamina propria of the small intestine but can also be found in the mesenteric lymph nodes. Hymenolepiasis symptomatology is due to host responses to worm allergens and metabolites. At sites far from the intestine, such as the brain and eye, these products are responsible for clinical manifestations. A close correlation was reported between *H. nana* and phlyctenular keratoconjunctivitis by Al-Hussaini et al. in 1979 from Egypt. Worm antigens in 73.4% of children with *H. nana* infections induce necrotic ulceration of the conjunctiva and cornea.

Immunology

The host immunity against *Hymenolepis* is found to be thymus dependent. Studies have shown that this parasite has immune-modulatory effect in irritable bowel disease (IBD) patients due to cross-reactivity of the immune system attributing to a similar structure of the host proteins to that of *H. nana*. *Hymenolepis*, like many other helminths, has thus been proposed as a candidate in the treatment of IBD.

Infection in Humans

H. nana mostly causes asymptomatic infection in humans. Heavy worm burden (15,000 eggs in faeces), chronic infection and infection in children is associated with symptomatic infections. Diffuse abdominal pain, diarrhoea, anorexia, loss of appetite, nausea, headache, dizziness and fatigability are the common symptoms caused by *H. nana*. Pruritus nasi, pruritus ani and keratoconjunctivitis, though not common, are also part of the clinical spectrum of *H. nana* infection. Infections are also associated with mild to moderate eosinophilia (5–15%). Epilepsy due to central nervous system involvement is one of the serious manifestations of *H. nana* infections. Disseminated infections are extremely rare. One case of multiorgan involvement and parasitaemia has been reported in a case of Hodgkin's lymphoma who was on immunosuppressive therapy which proves the invasive property of this parasite. Clinical effects of polyparasitemic infections are believed to be compounded by *H. nana*.

H. diminuta in humans is mostly innocuous but can cause abdominal pain, anorexia, cutaneous itch, mild diarrhoea, low-grade fever, eosinophilia and anaemia in rare cases with heavy infections.

Infection in Animals

Hymenolepiasis is a common infection of rodents like rats and mice. Similar to humans, *Hymenolepis* in rodents also mostly causes asymptomatic infection. Heavy infections can lead to weight loss, focal enteritis, mesenteric lymphadenitis, chronic abscesses and intestinal obstruction.

Epidemiology and Public Health

H. nana is considered the commonest cestode infecting humans worldwide with its prevalence ranging from 0% to 4%. It most commonly infects children in whom the prevalence is as high as 16%. Higher prevalence has been reported in males than in females. Overcrowding, poor sanitation, unhygienic practices, malnourishment and immunocompromised state are important factors which contribute towards the high prevalence of *H. nana* infections. These infections are more common in developing countries and in countries with a warm climate. Africa, Asia, southern and eastern parts of Europe, and Central and South America are endemic for *H. nana* infections. It has been evident from previous studies that human-to-human is the most common mode of transmission. The absence of intermediate host and release of matured infective embryo in the environment attribute to the high rate of transmission in *H. nana*.

H. diminuta is a zoonotic parasite which predominantly infects rodents and in humans its prevalence is rare (0.002–8.222%). To date less than 500 human cases of *H. diminuta* infection have been reported. Most of these cases have been reported in children less than 3 years old. Higher prevalence has been studied in Pakistan, Ethiopia and Bangladesh. *H. nana* is widespread in colder climates, while *H. diminuta* infects humans only rarely (Table 1).

Diagnosis

Microscopy

Laboratory diagnosis is based on the microscopic demonstration of eggs of *H. nana* in the direct wet mount preparation of stool samples. Permanent smears and Poly-vinyl Alcohol preserved samples are not preferred due to the distortion of morphology it causes. Thin-walled, round to oval non-bile stained eggs with polar filaments are seen in the wet mount preparation (Fig. 5). Stool concentration techniques and repeated examinations increase the sensitivity of microscopy in light infections. Adult worms are rarely seen.

In Vitro Culture

In vitro culture is not used for routine diagnosis.

Serodiagnosis

Few studies have evaluated the use of serological tests like ELISA to diagnose hymenolepiasis using homogenate of the adult worm as crude antigenic extract. Due to cross-reactivity with taeniasis and cysticercosis and low sensitivity, serology is used only for epidemiological and not for diagnostic purposes.

Table 1 Distribution of *Hymenolepis* species of importance in humans

Species	Distribution	Intermediate host	Definitive host
Hymenolepis nana	Africa, Asia, southern and eastern parts of Europe, Central and South America	Direct cycle: Nil Indirect cycle: Rat fleas (*Pulex irritans* and *Xenopsylla cheopis*), grain beetles (*Tribolium, Tenebrio*) and moths	Man, rats and mice
Hymenolepis diminuta	Pakistan, Ethiopia and Bangladesh	Beetles, fleas, earwigs, myriapods	*Rats (Rattus norvegicus, Rattus rattus*), mice and humans

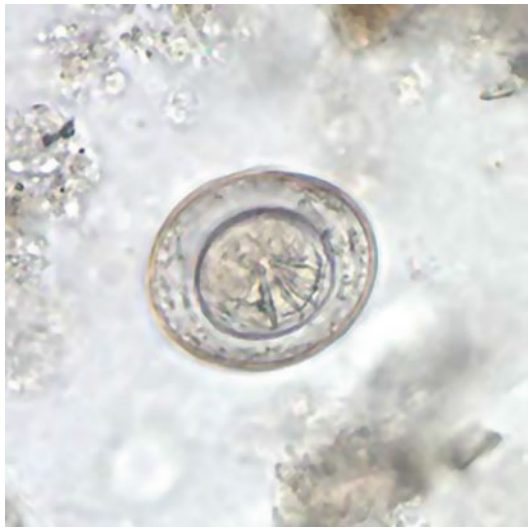

Fig. 5 Egg of *Hymenolepis nana* (Courtesy: CDC)

Molecular Diagnosis

Microscopic methods are based on morphologic identification, but due to similarities among the *Hymenolepis* species, molecular methods are more suitable for precise identification and to differentiate between the human and rodent *Hymenolepis* species. ITS1 and ITS2 of ribosomal DNA and cytochrome c oxidase subunit 1 (cox1) have been used in several studies to understand the genetic diversity between species of *Hymenolepis*. The use of molecular methods in routine diagnosis is limited due to its cost, requirement of skilled personnel and equipment (Table 2).

Treatment

The schistosomicidal drug praziquantel is the drug of choice for treating hymenolepiasis. This isochinolinpyrazin compound increases the cell membrane permeability to calcium ions, thereby causing paralysis of the parasite. It is effective against all the stages of the parasite and has a broad spectrum of action on all cestodes. A single oral dose of 25 mg/kg is effective in both adults and children and a second dose administered after 10–15 days decreases the possibility of relapses.

Alternatively, antihelminthic drugs such as niclosamide, albendazole and antiprotozoal drug and nitazoxanide are also used for treating hymenolepiasis. Compared to praziquantel, their usage is associated with adverse effects and require more frequent dosing. Once-daily niclosamide dosing of 2 gm in adults and 1–1.5 gm in children for 7 days is recommended for effective treatment. Nitazoxanide is given in a twice-daily dose (adults, 500 mg; children, 100–200 mg) for a period of 3 days.

Prevention and Control

Human-to-human transmission is the main mode of transmission of hymenolepiasis. Hence, good personal hygienic practices like washing hands with soap and water after using the toilet, before preparing food, before and after having food and proper disposal of faeces can bring down the

Table 2 Diagnostic methods for hymenolepiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Wet mount preparation of stool	Non-bile stained eggs	**Gold standard test** *Limitation*: Less sensitive in light infections
Immunodiagnostics	Antibody (ELISA)	Homogenate of adult worm	*Limitations*: (a) Significant cross-reactivity with taeniasis and cysticercosis (b) Low sensitivity Serology is used only for epidemiological and not diagnostic purposes in individual cases
Molecular assays	PCR, qPCR, LAMP	ITS-1, ITS-2 and cytochrome c oxidase subunit 1 (cox1)	High sensitivity and specificity *Limitations:* Require skilled personnel

incidence of hymenolepiasis. Controlling the rodent population will help decrease the rodent-human interaction and hence the transmission of hymenolepiasis. Foodgrains need to be properly stored to avoid insect infestation.

Case Studies

A 7-year-old boy who had convulsions was brought to the emergency department of a tertiary care hospital in Delhi, India.

The boy had a fever for the past 2 days. He had decreased appetite and nausea for over a month which resulted in fatigue and decreased concentration in school. His mother noticed oedema in his feet when he complained that he had difficulty in wearing his school shoes. He had two episodes of vomiting a day before along with abdominal cramps. He had occasional episodes of mild diarrhoea 2 weeks ago.

The boy had pallor, bilateral pedal oedema and stomatitis. After the epileptic episode, he was conscious and oriented. His temperature was 99 F; pulse, 102 beats per minute; and blood pressure, 122/80 mmHg. Cardiac auscultation revealed normal heart sounds. No organomegaly was appreciated on palpation.

His haemoglobin was 8 mg/dl and peripheral blood smear examination revealed eosinophilia. The urine samples had a specific gravity of 1030, was acidic in nature and had traces of albumin and no glucose. Microscopic examination of urine did not reveal any parasites. Microscopic examination of the wet mount revealed numerous ova of *H. nana*. The boy was treated with iron supplements for anaemia and 25 mg/kg of praziquantel for the parasitic infection. Two weeks later the faeces examination was negative for parasites and the boy's appetite was back to normal.

1. What are the differentiating features of *H. nana* and *H. diminuta*?
2. What are the preventive measures for rodent borne infections?

Research Questions

1. What are the reasons that despite multiple revisions in the nomenclature of this genus, there are still lacunae as to the host specificity and speciation?
2. Why there has been no in-depth taxonomic, epidemiological and molecular studies on *Hymenolepis* spp. in different hosts?

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Bertiellosis

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Learning Objectives

1. To have preliminary knowledge about this exotic parasite.
2. To know the importance of non-human primates and mites, and the accidental ingestion of mites through fruits, vegetables or soil responsible for disease transmission.

Introduction

Bertiellosis is a zoonotic helminthic parasitic disease caused by the members of the genus *Bertiella. Bertiella* species are non-taeniid and non-hymenolepid cestodes (tapeworms) that cause intestinal infections in non-human primates (definitive hosts). In general, identification of cestodes even at the genus level is not routinely performed in most of the clinical settings; therefore, the majority of them are arbitrarily and falsely assigned to well-known *Taenia* spp. On the basis of morphological variations among the human strains, *Bertiella studeri* is thought to be a species complex. *Bertiella satyri*, earlier thought to belong to *B. studeri* species complex, has recently been described as a separate species. Since bertiellosis is reported as singular cases, its epidemiology and clinical features are not well described.

History

Blanchard first described the infection in great apes and named it as genus '*Bertia*'. Ancey (1888) described *Bertia cambojiensis* as type strain. Subsequently, Stiles and Hassall (1992) revised the genus name to *Bertiella* because a group of terrestrial snails were already named as '*Bertia*'. The first case of human infection was reported by Blanchard (1913). He described the infection in an 8-year-old girl from Mauritius and named it as *B. satyri*, which was subsequently renamed *B. studeri* (now an Old World species). Meyner (1895) identified tapeworm from two black howler monkeys (*Alouatta caraya*) in Paraguay; now they are named as *Bertiella mucronata* (a New World species). A total of 95 cases (83 and 12 cases by *B. studeri* and *B. mucronata*, respectively) of human bertiellosis have been reported in the literature till date.

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Taxonomy

Genus *Bertiella* belongs to the Family, Anoplocephalidae; Order, Cyclophyllidea; Class, Cestoda; and Phylum, Platyhelminthes in the Kingdom Animalia.

Bertiella is the only genus in the family Anoplocephalidae known to cause infection in humans. So far, 29 species of *Bertiella* have been described; only 2 of them, *B. studeri* (Old World species) and *B. mucronata* (New World species) are identified to cause human infections.

Genomics and Proteomics

Genome analysis in *Bertiella* species is still primitive and proteomics analysis is lacking. Relatively, a few studies are available and these studies are focussed on *Bertiella* species obtained from human and primate sources in Africa, Asia and South America. Phylogenetic analysis based on NAD1 (nicotinamide adenine dinucleotide hydrogenase subunit 1), COX1 (cytochrome c oxidase subunit 1), 28S rRNA and ITS2 (internal transcribed spacer region-2) revealed a monophyletic group of *Bertiella* species within the family Anoplocephalidae. Analysis of NAD1 showed several clades and COX1 showed two clades within *Bertiella* group. COX1 analysis showed strains from Equatorial Guinea and Argentina belonged to separate clades from Sri Lankan strains. The ITS2 sequences also showed two clades. All Asian strains belonged to clade-1. Strains from Kenya, Equatorial Guinea and Brazil belonged to clade-2. *Bertiella* members from humans and non-human primates were classified into two clades based on 28S rRNA gene analysis.

The Parasite Morphology

Adult

Adult worms possess head or scolex, neck and segments (proglottids) like other cestodes. Adult worms of *B. studeri* are usually 10–30 cm long, 1.0–1.5 cm wide and 2.5 mm thick. *B. mucronata* are usually longer than *B. studeri* and can measure up to 40 cm. Scolex of *Bertiella* is sub-globose with a rudimentary, unarmed rostellum. The base of the scolex is well differentiated from the neck. The neck is approximately 2.65–5.0 mm long. Scolex measures 475 and 800 μm in diameter and has four oval suckers, two each on the ventral and dorsal sides. Suckers measure between 220 and 345 μm in diameter.

Usually, the numbers of proglottids are up to 600 in *B. studeri* and 700 in *B. mucronata*. The proglottids are usually much wider than the length. Proglottids contain both male and female reproductive organs. They are craspedote, which mean they extend transversely; they are much wider than their length. The width and length of gravid proglottids vary (7.8–11.3 mm wide [mean 9.52 mm] and 1.43–2.55 mm long). Mature proglottids lie at terminal end of the body. They have a single genital pore that opens irregularly and alternate left to right across the length. Ovary and single wide transverse uterus are centrally placed; ovary is fan-shaped and is on the poral side of the midline. Testes form a transverse band on the antero-dorsal side of the proglottid. The gravid (mature) uterus is full of eggs.

Morphometric variations of the same species from different geographical areas and in different hosts had been reported. Adult worms are usually differentiated into species based on length and width of gravid segments, size and number of testes, size of vagina and the opening of genital pore.

Egg

The eggs of *Bertiella* spp. have six hooked (hexacanth) embryo that are typical of cestode worms (Fig. 1). They are ovoid and measure 33–46 μm in width by 36–65 μm in length. They have an outer eggshell and inner chitinous membrane (inner envelope) with an albuminous layer in between. The inner envelope contains a distinct pyriform apparatus; the hexacanth embryo is located within the pyriform apparatus. The envelope extends filamentous projections around the embryo. Eggs of *B. mucronata* are smaller (36–47 μm in size) with a lot of

Fig. 1 Schematic diagram of *Bertiella* eggs with hexacanth oncosphere (embryo) in pyriform apparatus (sac)

overlapping in size with *B. studeri* and the projections are less marked.

Cysticercoid

The larvae of *Bertiella* spp. are called cysticercoids. They are found in the body of the arthropod intermediate hosts (oribatid mites). After ingestion of eggs by oribatid mites, the oncospheres (hexacanth embryos) are released and develop into cysticercoids inside the body of mites within 9 days of ingestion. Cysticercoids are pyriform in shape with everted unarmed scolex by which they attach to the intestinal wall of the definitive host and develop into adults. Cysticercoids measure 130–160 × 100–120 μm in size.

Cultivation of Parasites

Till date, there is no report of cultivation of *Bertiella* species. However, experimentally, it was observed that the cysticercoids could be demonstrated in mites as long as 76 days after exposure. Hence mites can be an experimental model for studying the biology of the larval stage of the parasite.

Laboratory Animals

Non-human primates are the natural definitive host of *Bertiella* spp. *B. studeri* usually infects monkeys of the genera *Anthropopithecus, Cercopithecus, Cynomolgus*, *Macaca* and various other species, occasionally chimpanzees *(Pan troglodytes*) and gibbons (*Hyalobates hoolock*). *B. mucronata* can infect the monkeys belonging to the genera *Callicebus* and *Alouatta*. Chimpanzees in zoo or kept in animal house can also be infected. Other animals like rodents, dermopterans and Australian marsupials can also be infected by *Bertiella* spp.

Life Cycle of *Bertiella* Species

The life cycle of *Bertiella* spp. of human importance is completed in two hosts (Fig. 2).

Host

Definitive Hosts

Monkeys of the genera *Anthropopithecus, Cercopithecus, Cynomolgus* and *Macaca* and grey langur *(Presbytis entellus*) are the definitive hosts of *B. Studeri*. Monkeys belonging to the genera *Callicebus* and *Alouatta* are the definitive hosts of *B. mucronata*. Chimpanzees (*Pan* spp.) are known to be the definitive hosts for both species. Other animals like rodents, dermopterans and Australian marsupials are also reported as definitive hosts for other *Bertiella* spp.

Intermediate Hosts

Several species of oribatid mites (arthropods) are the intermediate hosts for *Bertiella* spp.

Infective Stage

Cysticercoid, the larval stage of the parasite, is the infective stage.

Transmission of Infection

The infection is transmitted by the following ways: (a) ingestion of mites by the natural non-human primates and accidental ingestion of mites by humans, (b) consumption of mite

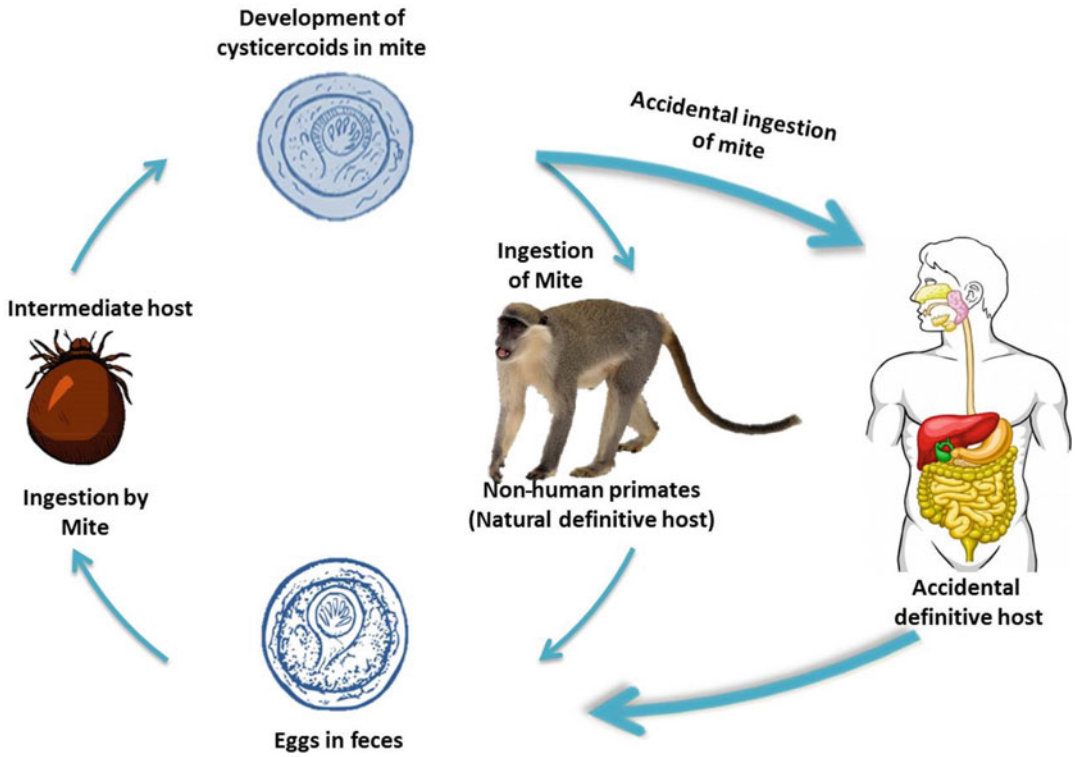

Fig. 2 Life cycle of *Bertiella* spp.

contaminated fruits and vegetations, and (c) ingestion of mite contaminated soil, especially by children.

Humans get infection accidentally by ingesting the mites and intaking contaminated fruits and vegetations. Children acquire infection by ingestion of contaminated soil. The cysticercoids released from mites attach to the small intestine with the help of unarmed scolices and subsequently develop into mature adults. The adult parasites are usually found in the lower two-thirds of the small gut. They lay eggs in the intestine, which are passed in the faeces. Around two dozen eggs are passed at a time. White, flat and actively motile segments/proglottids can also be expelled either singly or in a chain of 8–16 in stools. Characteristic proglottids in stool can be seen with naked eyes.

The intermediate hosts for the parasite (mites) acquire infection by ingestion of eggs. Oncospheres liberated from the eggs develop into infective cysticercoids within 9 days after ingestion in mites. Monkeys are infected by ingesting infected mites.

Pathogenesis and Pathology

Pathogenesis and pathology of *Bertiella* infections are not well described but presumed to be similar to other intestinal cestodes. The adult parasite attaches with its suckers to the lower part of the small intestine and then extends its rostellum into the intestinal mucosa, thereby causing damage to the epithelium. This initiates an inflammatory response involving mast and goblet cells at the initial stage followed by infiltration of neutrophils, eosinophils and lymphocytes. In young children, excessive worm burden can lead to small intestine obstruction.

Immunology

Immune response mounted by the host in *Bertiella* infection is least studied and available literatures in this area are scarce. But it is presumed to be similar to infections caused by other tapeworms. Generally, Th2 immune response is thought to be protective in tapeworm infection.

Infection in Humans

The clinical spectra of bertiellosis are not well-defined because of the limited number of clinical cases.

Most cases are asymptomatic in adults. Symptomatic cases are more common in children. The major symptoms are epigastric pain usually following meal, nausea, anorexia, diarrhoea and abdominal distension. Perianal itching in some cases had also been reported. Expulsion of segments/proglottids in stool may sometimes lead to stress and psychological problems. Heavy parasite burden in young children can cause physical obstruction of the small intestine.

Infection in Animals

Animals such as rodents, dermopterans and Australian marsupials are reported to be infected by *Bertiella* spp. However, the clinical spectra of bertiellosis in animals remain undefined. Accidental infection in dogs has also been reported.

Epidemiology and Public Health

So far, 29 *Bertiella* species have been described that cause infection among the primates, rodents, dermopterans and Australian marsupials. So far, *B. studeri* and *B. mucronata* are the two species currently known to be zoonotic and identified as human pathogens (Table 1). The first human infection by *B. studeri* (initial report as *B. satyri*) was reported from Mauritius in a child in 1913 by Blanchard. Till date, 83 cases of human infections due to *B. studeri* have been reported. It is suggested that *B. studeri* may be a species complex. *B. satyri*, which was included in this species complex, is now thought to be a separate species; however, its separation warrants further investigation.

Twelve cases of human infections caused by *B. mucronata* have been reported in literature. *B. mucronata* infections have specific geographical locations like South America (Brazil, Argentina and Paraguay) and Cuba. *B. studeri* infections have been reported from South East Asia (India, China, Bangladesh, Indonesia, Sri Lanka, Vietnam, Japan and Equatorial Guinea), the Middle East (Saudi Arabia and Yemen), Africa (South Africa and Kenya) and Mauritius. The majority of these infections are reported in children probably due to their habit of playing in the ground and eating soil contaminated with mites. In Mauritius, contaminated guava-transmitted infections in children have been reported. In adults, infection usually occurs in persons having close contact with monkeys such as zookeepers. Because of deforestation in many countries, the monkeys have moved to human settlements in suburban and urban areas, increasing the risk of human infection. Mites, the intermediate hosts, prefer to live in cool environment, especially on soil, vegetations and fruits.

Consumption of unwashed vegetations and fruits and soil eating habit of children increases the risk of infection in humans. *B. mucronata* infection is transmitted by *Dometorina* species and *Scheloribates atahualpensis*, while *B. studeri* infection by *Scheloribates laevigatus, Galumna* species, *Scutoverix minutus* and *Achipeteria coleoptrata*. Bertiellosis is considered to be one of the most neglected tropical diseases due to a lack of awareness among clinicians and other health care workers. It often remains undiagnosed because of mild symptoms. However, this disease is treatable and preventable if a timely diagnosis is made.

Table 1 Epidemiology of important *Bertiella* spp.

Species	Distribution	Intermediate host	Definitive host
Bertiella studeri (Old World species)	South America (Brazil, Argentina and Paraguay) and Cuba	Oribatid mite species: *Scheloribates laevigatus, Galumna* species, *Scutoverixminutus* and *Achipeteria coleoptrata*	*Primates:* monkeys (*Anthropopithecus, Cercopithecus, Macaca cynomolgus* and other *Macaca* spp.; baboons (*Papio ursinus* and *Papiodoguera*). Occasionally chimpanzees *(Pan troglodytes*), gibbons (*Hyalobates hoolock*) and humans
Bertiella mucronata (New World species)	South East Asia (India, China, Bangladesh, Indonesia, Sri Lanka, Vietnam, Japan and Equatorial Guinea), the Middle East (Saudi Arabia and Yemen), Africa (South Africa and Kenya) and Mauritius	Oribatid mite species: *Dometorina* and *Scheloribates atahualpensis*	Monkeys in genera *Callicebus*, *Alouatta* and *Mycetes* spp. Occasionally chimpanzees *(Pan troglodytes)* and humans

Table 2 Diagnostic methods for human bertiellosis

Diagnostic approaches[a]	Specimen	Targets	Remarks
Naked eye examination	Stool	Chain of white, flat, motile proglottids	Proglottids much more wider than length, lateral genital pore *Limitations:* Low sensitivity and require trained personnel
Direct microscopy	Stool	Eggs, proglottids, scolex	Eggs: Size 33–65 μm; oncosphere: located inside a well-developed pyriform apparatus; scolex: cup-shaped suckers (two each on ventral and dorsal sides). Gold standard for genus level diagnosis *Limitations:* Low sensitivity and require trained personnel
Molecular assays	Stool/ proglottids	18S rRNA, NAD1, COX1, ITS1 and ITS2	High sensitivity and specificity; gold standard for species level diagnosis *Limitations:* Require skilled personnel

rRNA Ribosomal ribonucleic acid, *NAD* Nicotinamide adenine dinucleotide hydrogenase, *COX* cytochrome c oxidase subunit, *ITS* internal transcribed spacer region
[a]No serological tests available till date

Diagnosis

The laboratory diagnosis of human infection is difficult (Table 2), because of a lack of knowledge and awareness about the disease. Travel history to endemic areas and contact with non-human primates should also be an indicator. *B. mucronata* and *B. studeri* have different geographic distributions. Bertiellosis should be a differential diagnosis in humans with gastrointestinal symptoms in endemic areas and in persons with risks of exposure.

Microscopy

Good microscopy with knowledge about the parasite is the key to diagnosis. Stool specimens from suspected patients require careful examination for the presence of proglottids and eggs. Repeated stool examination over several days is required since the passage of proglottids is usually intermittent. Definitive diagnosis is made by demonstration of distinctive eggs with pyriform sacs and proglottids with the appropriate size and typical morphology. Following treatment and

purgatives, sometimes the whole parasite is expelled in stool. The parasite in the stool can be fixed on a microscopic slide with AFA (2% acetic acid, 2% formaldehyde and 70% ethyl alcohol) solution and stained by carmine. Preservation of the parasite/proglottids at 4 °C for 18–24 h allows relaxation of the proglottids and better delineation of internal structures and vagina with its opening. *B. mucronata* can be differentiated from *B. studeri* by less number of testes, small size cirrus sac and elongated vagina. Notable differences are as follows: *B. studeri* versus *B. mucronata*, number of testes 280–900 versus 265–270; cirrus sac diameter 280–900 μm versus 310–322 μm; vaginal length 330–540 μm versus 1600 μm.

Serodiagnosis

Since bertiellosis is a rare neglected parasitic disease, no serodiagnostic test is available to date. Diagnosis as of today is based mostly on parasite morphology and confirmation by molecular tests.

Molecular Diagnosis

Identification of *Bertiella* up to species is made using molecular methods. Limited sequence-based NCBI GenBank data are available for strains obtained from endemic areas. Sequence-based NCBI GenBank data can be helpful in phylogenetic analysis and diagnosis at species and subspecies levels. Polymerase Chain Reaction (PCR) targeting various genes like 18SrRNA, NAD1, COX1, ITS1 and ITS2 have been used for molecular diagnosis purposes. However, they are available only in a few laboratories and consensus on a single molecular test is lacking.

Treatment

Early diagnosis and treatment can prevent disease chronicity and progression to severe infections, especially in children. Single dose praziquantel 40 mg/kg body weight followed by second dose after 3 weeks is the treatment of choice. Some cases were successfully treated by single dose niclosamide (500 mg–2 g). However, treatment failure can occur with niclosamide. The most commonly used anthelminthic such as albendazole is not effective in clinical and microbiological cure. Mild purgatives are also given following anthelminthic therapy to expel the worm, which helps in diagnosis. Young children may need supportive nutritional therapy in cases of chronic infections.

Prevention and Control

Public awareness and knowledge about the parasite among the health care personnel can help in prevention of *Bertiella* infections. Zookeepers and laboratory personnel handling non-human primates need to be educated about these infections. They should maintain strict personal hygiene. Proper hand hygiene and cleanliness are important especially in small children to prevent ingestion of soil. Proper cleaning and washing of fruits before consumption should be routinely practised.

Case Study

A 3-year-old male child (body weight 12 kg) presented with periodic episodes of epigastric pain that increased with food intake and intermittent diarrhoea with vomiting for the last 15 days. On examination his vitals and routine investigations were within normal limits. The parents gave the history of expulsion of white and flat segments in a chain, which were motile. The child used to play on the ground and also had a habit of putting soil contaminated fingers into the mouth. Moreover, the movement of monkeys in the area in search of food was a common phenomenon. Stool examination of the child showed the presence of ovoid eggs (size 36–42 μm) with hexacanth embryo in pyriform sacs. Stool sample submitted for microscopy did not show any segments (proglottids). Based on

the morphology of the eggs, the diagnosis of bertiellosis was made; species identification was not possible in the absence of proglottids. However, as per epidemiological evidences, it was likely *B. studeri* since from Asia till date only *B. studeri* had been reported. The child was treated with 40 mg/kg single dose praziqunatel and the treatment was repeated after 3 weeks. On follow up at 6 weeks after completion of treatment, the child was asymptomatic and stool examination did not reveal any egg or proglottid of *Bertiella* or any other parasite.

1. Name the parasites which may be transmitted to humans by ingestion of infected mites, beetles and ants.
2. How can you differentiate between *B. studeri* and *B. mucronata*?
3. Mention the importance of monkeys in zoonotic parasitic infections.

Research Questions

1. How to improve the limited genomic data available in database?
2. What is the exact disease burden and species involved in bertiellosis since many more species may be involved?
3. What antigens of *Bertiella* spp. can be used to develop serological tests for both diagnostic and epidemiological purposes?
4. Which genes should be targeted in PCR so as to develop a suitable, cost-effective, sensitive and specific molecular test for diagnosis?

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Raillietina Infection

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Learning Objectives

1. To have a basic idea about an uncommon parasite.
2. To have a knowledge about the peculiar mode of transmission due to accidental ingestion of arthropods.

Introduction

The genus *Raillietina* belongs to the order Cyclophyllidea of the cestode parasites.. The genus *Raillietina* consists of about 300 species confined to avian and mammalian hosts and is important pathogen for the poultry population. The zoonotic species are found in rodents and are infrequent causes of infection in humans. The parasite resides in the small intestine and commonly causes asymptomatic infections or else non-specific abdominal symptoms. Reports of *Raillietina* infections are available from various parts of the world. This chapter outlines the important features regarding the biology of the parasite, epidemiology and other relevant information.

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History

The genus *Raillietina* was named after the French helminthologist Ralliet in 1920 and the description of *Raillietina celebensis* was provided by Janicki in 1902. The parasite was first recorded from humans in 1891 from Siam by Leuckart. Daniels in 1895 first described a case of *Raillietina demerariensis* infection in an Amerindian from British Guiana and named it *Taenia demerariensis*. There had been much confusion about the aetiological agent and most of the historical reports mentioned it as *Raillietina madagascariensis*. In 1929, Joyeux and Baer concluded that many of these parasites had been incorrectly identified as *R. madagascariensis* and belong to other genera.

Taxonomy

The genus *Raillietina* (Fuhrmann, 1920) belongs to the Subfamily, Davaineinae; Family, Davaineidae; Order, Cyclophyllidea; Class, Cestoidea and Phylum, Platyhelminthes.

This genus is subdivided into four subgenera: *Raillietina, Paroniella, Skrjabinea* and *Fuhrmann*. *R. celebensis, R. demerariensis* and *Raillietina siriraji* are zoonotic species of importance to humans. A number of reports and taxonomic classification mention that *R. madagascariensis* is not a valid species and it

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most likely represents some misidentified *Raillietina* species including *R. celebensis*.

Genomics and Proteomics

The complete mitochondrial DNA sequence of the avian parasite *Raillietina tetragona* has been sequenced. The complete genome sequence is 14,444 bp in length and contains the following: 12 protein-coding genes, 2 ribosomal RNA genes, 22 t-RNA genes and 2 non-coding regions. A + T content is 71.4%.

Not much work has been done regarding the proteome analysis of *Raillietina*. The tegument proteins are considered important in parasitic survival and have implications in the development of vaccines and immunodiagnosis. The RT10 tegument protein which is a major protoscolex homologue has been studied. It is a protein of 560 amino acids and isoelectric point of 6.33. The secondary structure of the protein has revealed its antigenic property.

The Parasite Morphology

Adult Worm

The adult worm infecting humans is 10–60 cm long and 2–3 mm wide. The rostellar scolex has hammer-shaped double row of hooks (Fig. 1). The proglottides are rectangular to square in shape. They assume a rounded shape at the posterior end giving a headed appearance. The mature proglottides contain egg capsules which are polygonal in shape and contain one to four eggs. The genital atrium is unilateral which opens in the anterior part of the lateral margin of the proglottides. The zoonotic species *R. celebensis, R. demerariensis* and *R. siriraji* can be distinguished by the number and length of rostellar hooks, number of testes, length of cirrus pouch and number of eggs present in egg capsules.

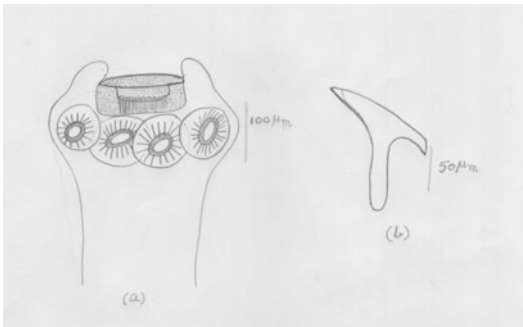

Fig. 1 *Raillietina celebensis*. (**a**) Scolex showing rostellum and suckers. (**b**) Hammer-shaped rostellar hook

Eggs

The eggs contain six-hooked oncosphere.

Cultivation of Parasites

The parasite has not been cultivated in vivo or in vitro as per the available information.

Laboratory Animals

Raillietina experimental infection in laboratory animals is not yet established.

Life Cycle of *Raillietina* spp.

Hosts

Definitive Hosts

Rodents, primarily rats (*Rattus norvegicus, Rattus rattus, Rattus exulans, Rattus demerariensis*), bandicoots (*Bandicota* species), Asian house shrew (*Suncus murinus*) are the definitive hosts. Monkeys may also act as definitive hosts.

Intermediate Hosts

Ants, beetles (ground beetles, scarab beetles, darkling beetles) and possibly cockroaches.

Infective Stage

Cysticercoids.

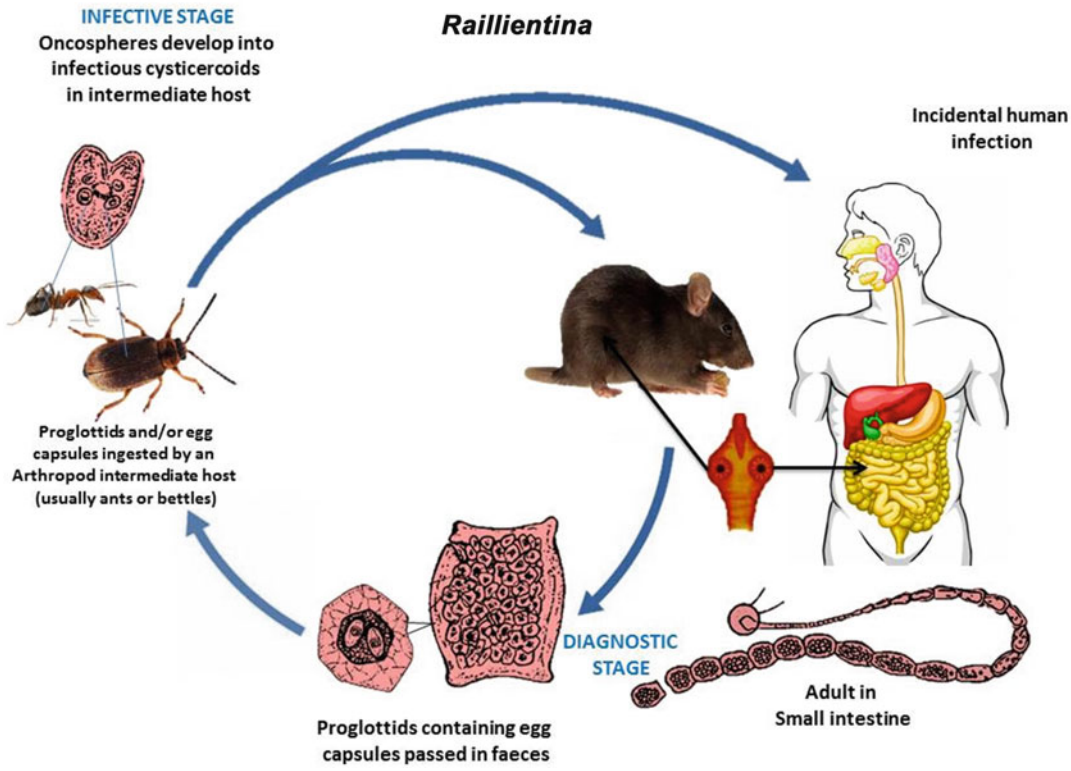

Fig. 2 Life cycle of *Raliietina celebensis*

Transmission of Infection

Humans and other definitive hosts acquire infection by accidental ingestion of the arthropod hosts infected with cysticercoids (Fig. 2).

The course of infection is not completely understood, but possibly the scolex of the ingested cysticercoid, in the intestine of the host, averts and gets attached to the wall of the small intestine. The cysticercoids develop and mature into adult worms consisting of proglottides containing egg capsules that are subsequently shed in the faeces.

The proglottides shed in the stool are motile. They migrate out of the stool and are subsequently picked up by the ants, beetles and possibly cockroaches. Oncospheres hatch out from the eggs and penetrate their intestinal wall and come to lie freely in the body cavity. They develop further into cysticercoids and become infectious in about 2–3 weeks. These are taken up by the definitive hosts when they ingest the arthropods.

Pathogenesis and Pathology

Although members of the genus *Raillietina* are important pathogens of poultry and cause intestinal pathology in the birds with the formation of nodules, information regarding its pathogenicity in the definitive hosts, particularly humans, remains largely unknown. The parasite is known to localize in the small intestine and cause mostly asymptomatic infection.

Immunology

Literature on the immunological aspects of *Raillietina* infection in rodents or humans is largely non-existent.

Infection in Humans

Raillietina infections recorded in humans are mostly asymptomatic. In a few cases, vague abdominal pain, discomfort, nausea and vomiting and diarrhoea have been recorded. Gastrointestinal distention may be present. The patient may also notice small moving white worms in the faeces.

Infection in Animals

Like humans, *Raillietina* infection in the birds does not produce any overt clinical manifestations. Weight gains and egg-laying capacity in the poultry birds may be affected. Long-term severe infections may result in diarrhoea, anaemia and haemorrhage.

Epidemiology and Public Health

Of the three species of zoonotic importance (Table 1), *R. celebensis* is the most frequently reported and is considered the Old World species. Cases have been reported from the East and South East Asian countries and Pacific Islands. *R. celebensis* infection is common in rodents. Various studies have found 54% of *Rattus norvegicus* and 9% of *Rattus rattus* in Taiwan to be infected, whereas 5% of *R. rattus* and 7% of *Bandicota bengalensis* in Bombay (Mumbai), India, have been found to be infected.

R. demerariensis is the New World species described from South America (mainly, Ecuador), Central America (Honduras) and the Caribbean. The largest endemic focus of the parasite has been found in certain areas of Ecuador, where the infection rate in school-age children varied from 4% to 12.5% during the period 1933 to 1961.

R. siriraji is the third species that has been described from Thailand. This disease seems to be restricted primarily in the paediatric population in children less than 3 years of age. Accidental ingestion of the intermediate hosts like ants or beetles containing the cysticercoid is the primary mode of infection and may be related to the practice of open-air defecations and playing with soil among the children.

Diagnosis

The laboratory diagnosis of *Raillietina* infection is based primarily on microscopy of stool for *Raillietina* proglottides or the egg capsule, similar to *Inermicapsifer* (Table 2).

Microscopy

Microscopy depends on the detection and identification of proglottides, egg capsules or scolices of *Raillietina* in the stool specimen (Fig. 3). The diagnostic dilemma occurs because of the close resemblance of these structures to those of *Inermicapsifer madagascariensis*. Nevertheless, the position of genital pore and the number of eggs in the egg capsules frequently help in the specific identification. The scolex of *Raillietina*

Table 1 Epidemiological features of *Raillietina* spp.

Species	Definitive host	Intermediate host	Geographic distribution
Raillietina celebensis	Rodents like rats, bandicoots, shrews; humans	Ants, beetle, cockroaches	Africa, Australia, Iran, Japan, Mauritius, the Philippines, Taiwan, Thailand
Raillietina demerariensis	Rodents like rats, bandicoots, shrews; humans	Ants, beetle, cockroaches	Cuba, Ecuador, Guyana and Honduras
Raillietina siriraji	Rodents like rats, bandicoots, shrews; humans	Ants, beetle, cockroaches	Thailand

Table 2 Laboratory diagnosis of *Raillietina* infection

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Carmine stain	Proglottides	Position of genital pores (anterior)
	Wet mount preparation	Egg capsules	1–4 eggs
Molecular diagnosis	PCR and sequencing	400 bp of 18 S rRNA	Species identification

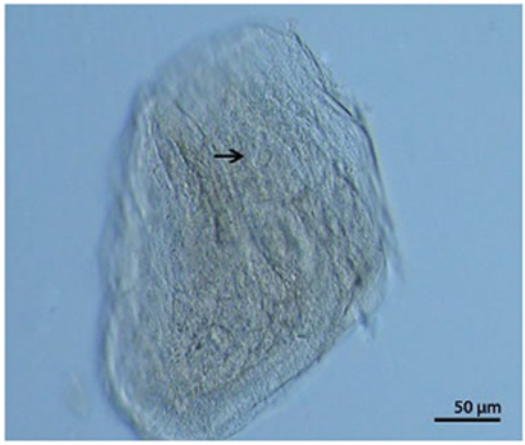

Fig. 3 *Raillietina* sp. egg capsule. One egg with six-hooked oncosphere can be seen (*arrow*). [Photos courtesy of DPDx, Centers for Disease Control and Prevention]

has armed rostellum with hammer-shaped hooks (Fig. 1) which are absent in *Inermicapsifer*. Genital pore is in the anterior portion of lateral margin in *Raillietina* while it is situated on median lateral margin in *Inermicapsifer*. Egg capsules are numerous in each gravid proglottide with 1–4 eggs in each capsule, compared to 4–15 in *Inermicapsifer*.

Serodiagnosis

Currently, no serological tests are available.

Molecular Diagnosis

As the identification of the parasite and the species is difficult morphologically, PCR has been tried for genus and species identification. DNA amplification to amplify the 400 bp partial nuclear coded small subunit (SSU) ribosomal RNA (18 S rRNA) has been employed followed by a sequencing of the amplicons.

Treatment

As the cases are rare, no studies have been done to find the optimum treatment. Praziquantel and niclosamide have been found to be effective.

Prevention and Control

Due to the rarity of the disease, *Raillietina* spp. represent a parasitological curiosity and not a public health problem. Open-air defecation and the practice of infants playing with soil should be discouraged in areas where cases have been recently reported.

Case Study

A 3-year-old child was brought to the local health centre in the village with the complaints of diarrhoea for 3–4 days. Abdominal tenderness was present with a slight fever. The parents had observed some moving white spots on the shed stool. The stool was sent to the laboratory and microscopy of the stool showed many proglottides of a tapeworm which was later identified as *R. celebensis* at the referral centre. A single dose of praziquantel caused a rapid resolution of symptoms.

1. How can you find out the presence of the parasite in the insect host?
2. What are the other parasites of which you can find the worm or its segments macroscopically in stool?

3. What is the mechanism of action of niclosamide and praziquantel?

Research Questions

(a) What antigens can be used to develop immunodiagnostic kits for *Raillietina* infection?
(b) What primers can be used for the common zoonotic species for molecular diagnosis?

Further Readings

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Inermicapsifer Infections

Abhijit Chaudhury

Learning Objectives

1. To have an overview of a rare parasite closely related to *Raillietina*.
2. To know the differentiating features between *Inermicapsifer* and *Raillietina*.

Introduction

Inermicapsifer madagascariensis is the sole species in the genus belonging to the cestode group and is an uncommon and poorly described cause of zoonotic infection. It is morphologically similar to *Raillietina* species with which it may be confused. It is a parasite of the small intestine and commonly causes asymptomatic infections. Reports are mainly confined to Cuba, but it has also been found in sub-Saharan Africa and some other African countries. This chapter is an attempt to include the available information on the parasite although recent occurrences of the infection are rare.

History

I. madagascariensis was first described by Janicki in 1910 and the first human case was reported by Kouri from Cuba in 1938. The original name assigned was *Raillietina cubensis*. Later in 1949, Bayliss reported the first human case from Kenya, Africa. Because of the presence of unarmed scolices, it was transferred from the genus *Raillietina* to *Inermicapsifer* and Baer in 1956 suggested the new name *I. madagascariensis*.

Taxonomy

The genus *Inermicapsifer* belongs to the Family, Anoplocephalidae; Order, Cyclophyllidea; Class, Cestoidea; and Phylum, Platyhelminthes.

I. madagascariensis is the species that is associated with human infection. This species has a chequered taxonomic history in relation to *Raillietina*. *Taenia madagascariensis* and *R. madagascariensis* names were previously given to this species.

Genomics and Proteomics

No study is available on the genome or proteome of this parasite.

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https://doi.org/10.1007/978-981-16-7204-0_39

The Parasite Morphology

Adult *I. madagascariensis* is 7–42 cm in length and contains 300–360 segments. The proglottids are trapezoid in appearance. The genital pore is unilateral and opens into the middle of the lateral margin of the segment. The *scolex* or head is unarmed measuring 0.4–0.5 mm with four cup-shaped simple suckers. The gravid proglottids shed in stool are white and the segments are barrel-shaped or round and motile. These segments are filled with egg capsules giving a reticulated or mosaic appearance under the microscope. The egg capsules are polygonal in shape containing 4–15 eggs.

Cultivation of Parasites

The parasite has not been cultivated in vivo or in vitro as per the available information.

Laboratory Animals

The parasite has not been maintained in any experimental infection of animals.

Life Cycle of *Inermicapsifer madagascariensis*

Hosts

Definitive Hosts

Multimammate rats (*Mastomys natalensis*), Gambian pouched rat (*Cricetomys gambianus*) and Rock hyrax (*Procavia capensis*).

Intermediate Hosts

Not definitely known. Ants, beetles and mites have been proposed but not established as intermediate hosts.

Transmission of Infection

The infective stage of the parasite is not definitely known since the identity of the intermediate host remains unknown. That the infection is acquired by the ingestion of arthropods has been proposed but not substantiated as yet. The life cycle is not completely known and it is possible that it is similar to *Raillietina* species involving arthropods as intermediate hosts (Fig. 1).

The cysticercoid in arthropods may act as an infective stage for the definitive hosts like rats and hyraxes. Humans may get the accidental infection through ingestion of ants or beetles containing the infective cysticercoids. In Africa, the transmission cycle suggested is rodent-arthropod-rodent and, rarely, rodent-arthropod-man. Outside the African continent, transmission is suggested to occur from humans-to-arthropods-to-humans.

Pathogenesis and Pathology

Data are scanty regarding the pathogenesis and the pathological changes induced by *I. madagascariensis* in the human host.

Infection in Humans

Almost all the reported cases of *I. madagascariensis* are in children, particularly in those younger than 3 years and sometimes in children of 4–5 years old. Most cases are asymptomatic, although the observation of motile proglottids in the stool has contributed to the diagnosis of the condition. Abdominal pain, anorexia, malaise and irritability and diarrhoea are the presenting signs and symptoms in symptomatic cases.

Epidemiology and Public Health

All the reports of *I. Madagascariensis* infection till date have originated either from Cuba or from countries in sub-Saharan Africa like Kenya, South Africa, Zimbabwe and Zambia and also from the island nations of Mauritius and Madagascar. The parasite is endemic among native African rodents and hyraxes, but has never been found in any animal host in Cuba or

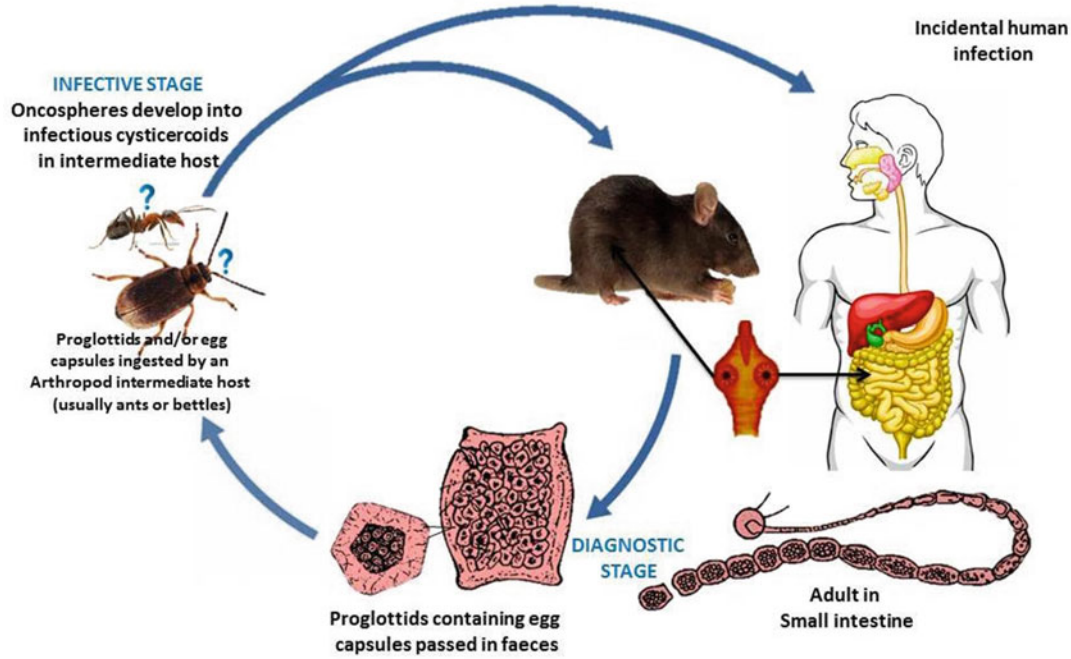

Fig. 1 Life cycle of *Inermicapsifer* spp.

the island nation of Mauritius or Madagascar. This brings up the interesting hypothesis about the adaptation of the parasite in humans as a reservoir in the absence of suitable rodent hosts in the island nations including Cuba. It is postulated that the parasite originated in Africa and was brought to Cuba and West Indies through the workers from African countries and to the African islands by the Creole workers. Hence in these areas, *Inermicapsifer* infection is an anthroponose in contrast to mainland Africa where it is a zoonotic disease (Table 1). Since no genetic studies have been performed, no conclusion can be drawn between the genetic similarities between the human adopted and rodent adapted parasite strains.

Nearly, 100 cases of *I. madagascariensis* were reported from Cuba by Kouri in 1944 and thereafter there have been no reports from this region till 1996. Since 1996 till date about 45 more cases have been reported from Cuba, Havana and Santa Clara areas. Till the 1970s, reports were available from various African countries, but in the last few decades, no cases have been reported from sub-Saharan Africa, possibly, because of a lack of awareness and research interest in this uncommon parasite.

Laboratory Diagnosis

Microscopy

Microscopy remains the sole method of diagnosis (Table 2). Diagnosis is made by observing the intact proglottids, the egg capsules and, if available, the scolex. The rice grain-like proglottids may be stained with carmine to visualize the position of the genital pore. On microscopic examination, it is possible to differentiate whether they are true grains of rice or gravid proglottids, since when compressed between the coverslip and the slide, they burst and release a large number of characteristic egg capsules. On the other hand, if it is an artefact like starch fragments, grains of starch would come off that are stained

Table 1 Epidemiological features of *Inermicapsifer madagascariensis*

Species	Definitive hosts	Intermediate hosts	Geographic distributions
Inermicapsifer madagascariensis	African rodents, hyraxes, humans	Not definitely known. Possibly ants, beetles and mites	Democratic Republic of the Congo, Kenya, South Africa, Zimbabwe, Zambia
Inermicapsifer madagascariensis (cubensis)	Humans	Not definitely known. Possibly ants, beetles and mites	Cuba, Venezuela, Madagascar, Mauritius

Table 2 Laboratory diagnosis of *Inermicapsifer* infection

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Carmine stain	Proglottids	Position of genital pores
	Wet mount preparation	Egg capsules	Hexagonal shape with 4–15 eggs

purple with Lugol's iodine. The polygonal shape of the egg capsule (Fig. 2) and the number of eggs in the capsule (4–15) are useful for the identification of the parasite. The scolex, if available, provides unequivocal identification of the parasite as it is unarmed and arostellar.

No serological or molecular diagnostic tools are available till date for diagnosis of the condition.

Treatment

Cestocidal drugs like praziquantel or niclosamide are the drugs of choice for the treatment of *Inermicapsifer* infection. Benzimidazoles are ineffective as therapeutic agents.

Prevention and Control

I. madagascariensis infection is not considered a public health problem because of the scarcity of data regarding the condition. Since the mode of infection and infective form including intermediate host are still unknown, the only preventive measures that are recommended include rodent control and personal and environmental hygiene.

Case Report

A 1-year-old child was referred to a teaching hospital with complaints of abdominal tenderness, irritability and mild fever for about 3 weeks duration. Laboratory parameters were normal. The specimen of stool was submitted for microbiological and parasitological investigations. While no bacterial pathogen was found on routine culture, a few whitish "rice grain" like structures could be seen with naked eyes. These were picked up and examined microscopically before and after crushing between slides. The crushed sample revealed a number of egg capsules with eggs. Carmine injection into one of the proglottids revealed the characteristic genital structures and a diagnosis of *Inermicapsifer* infection was made. Treatment with praziquantel at 10 mg/kg, single dose, was administered. The child expelled more such proglottids after medication, and thereafter the stool became normal and the signs and symptoms subsided.

1. How will you take up an investigative study to find the intermediate host for this parasite?
2. How will you find out the prevalence of the parasite in the animal population?

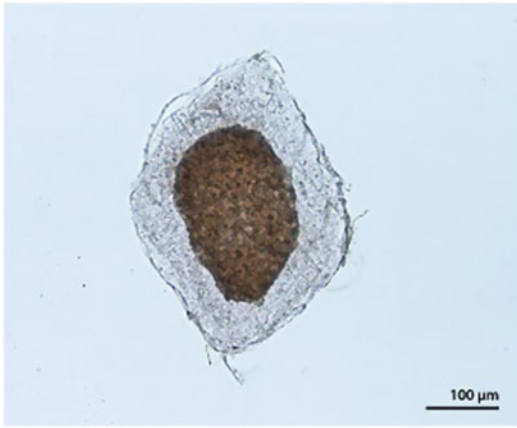

Fig. 2 Single egg capsule containing many eggs. [Courtesy DPDx, Centres for Disease Control and Prevention]

Research Questions

1. There is no genetic study which has been conducted for the parasite and no sequences are available in the GenBank. So, any case in future can be studied to get a molecular perspective of the parasite.
2. What is the exact intermediate host, and how is the infection transmitted?
3. Are the cases in Cuba and some other areas really human adapted?
4. Is there any similar definitive host in other parts of the world, particularly Asia or South America? Examination of the rodent population in these areas can address this issue.

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Part V

Zoonotic Helminth Infection: Nematode

Trichinellosis

Abhijit Chaudhury

Learning Objectives

1. To make the reader aware of the various other *Trichinella* species which have zoonotic potential, apart from the well-characterized *T. spiralis*.
2. To understand epidemiological significance with the domestic and sylvatic cycles going on in parallel in nature.
3. To emphasize importance of serological diagnosis because of non-specific and protean clinical manifestations.

Introduction

Trichinellosis is a zoonotic disease, commonly of pig origin and other animals caused by the nematode *Trichinella spiralis*. The practice of eating undercooked pork, pork products or meat of wild or game animals predisposes to trichinellosis. Although serology is useful in the diagnosis of the condition, nevertheless, the definitive diagnosis of the condition is made by demonstration of the larva of the parasite in the muscle by biopsy.

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History

The encysted larval stages of *Trichinella*, causing trichinellosis or trichinosis, were first discovered in the muscles of infected man by Tidemann in 1821 (in Germany and by James Paget and Richard Owen in 1835 in London. Joseph Leidy in 1846 observed similar encysted larvae in the pork in Philadelphia during the year 1846. Leuckart in 1855 and Virchow in 1859 showed the development of infective larva to the adult worm in the intestine in experimental animal. They observed that the young larvae, produced by the female adult worm migrated through blood vessels to reach the muscle in which they became encysted. Zenker in 1860 implicated the parasite to be the causative agent of trichinellosis in humans. The larval stage of the parasite was first demonstrated in the human blood by Herrick and Janeway in 1909.

Taxonomy

T. spiralis Owen, 1835, Railliet, 1895 belongs to the genus *Trichinella*, family Trichinellidae, superfamily Trichuroidea Railliet, 1916; order, Enoplida Chitwood, 1933; subclass Adenophorea, and class Nematoda under the phylum Nemathelminths.

Until recently, *T. spiralis* was considered to be a single species of the genus *Trichinella*. However, various workers have recognized strain

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differences within the species. Thus, the larvae of Arctic strains remain infective even by freezing the meat, while those of temperate zone strains are killed by freezing. East African strains were reported to be less infective to rats compared to strains from other areas. Because of these differences, it was proposed to subdivide *T. spiralis* into three species: *T. spiralis* (man, domestic animals and pigs), *Trichinella nativa* and *Trichinella nelsoni*. A new species, *Trichinella pseudospiralis* which has a very weak cyst and unique characteristic to develop in birds, has been included as a fourth one in the list of *Trichinella* species. In recent years, molecular methods have been used to better delineate the members of the genus to the species level and genotypes.

Currently, the genus *Trichinella* consists of nine species and three genotypes. Two clades have been defined based on the presence or absence of collagen capsule around the muscle larva:

(a) **Encapsulated**: These are found only in mammals. It includes *T. spiralis, T. nativa, Trichinella britovi, T. nelsoni, Trichinella murrelli*, *Trichinella patagoniensis* and the genotypes *Trichinella* T6, T8, T9.
(b) **Non-encapsulated**: They have a much wider distribution among mammals, birds and reptiles. It includes *T. pseudospiralis, Trichinella papuae* and *Trichinella zimbabwensis*.

The International Trichinella Reference Centre contains detailed information of the various species and genotypes (www.iss.it/site/Trichinella/index.asp).

Genomics and Proteomics

In earlier studies, a genomic approach was used using Expressed Sequence Tags (ESTs) generated from three life stages of *T. spiralis,* namely adult worm, immature larva and mature larva in muscles. A total of 3262 unique genes were found out of 19,552 genes on analysis of more than 10,000 ESTs. The GC content of protein-coding exons was found to be 39%. The *Trichinella* genome has been compared with that of *Caenorhabditis elegans* and it has been shown that there is 56% homology between them as far as EST clusters are concerned. Species and phylum based analysis has revealed great phylogenetic distance of *T. spiralis* from other nematodes. As of now, the 64-Mb nuclear genome has been sequenced using whole-genome shotgun approach and hierarchal map–assisted sequencing and is estimated to contain 15,808 protein-coding genes. The GC content of the overall genome is 34%. The 15,808 protein-coding sequences occupy 26.6% of the genome. For a detailed report of the draft genome, the readers can refer to the article by Mitreva et al. (2011).

The characterization of excretory-secretory products (ESP) in parasites is an important step in understanding their roles in host-parasite interaction and future developments in diagnostic methods and vaccine development. By using a combination of protein sequence similarity and signal peptide prediction, 345 *T. spiralis* clusters have been identified as having homology with predicted secreted or membrane proteins. Identities have been given to 43 ESP peptide spots which represent 13 different proteins, signifying the presence of substantial protein isoforms in the ESP. Some of the important proteins identified so far include serine protease, cysteine protease, zinc dependent metalloprotease, 45 KD antigen, gp43 and two unidentified open reading frames.

The Parasite Morphology

The Adult Worm

The adult *T. spiralis* is the smallest nematode which infects man. The worm is minute, whitish, thread-like and just visible to the naked eye. The oesophagus fills up from one-third to one-half of the body and is covered by a single layer of large cells. The oesophagus joins the intestine, which extends posteriorly to end in the terminal anus (Fig. 1).

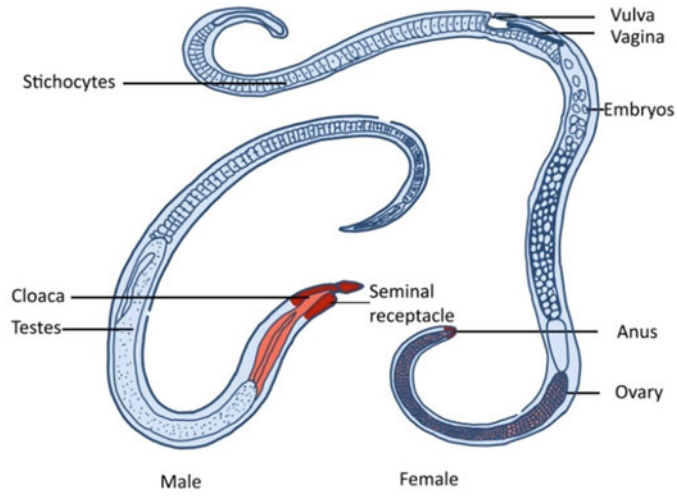

Fig. 1 Schematic diagrams of adult male and female *Trichinella spiralis*

Male worm measures 1.4–1.6 mm in length and 0.04 mm in diameter. The anterior end is delicate, filariform and possesses cephalic papillae. The posterior end is filled up with the testes and bears two conspicuous conical papillae, on either side of the cloacal orifice. The male dies usually after fertilizing the female or is excreted out along with the faeces.

The female is approximately twice as long as the male and measures 3–4 mm in length and 0.06 mm in diameter. The female genitalia consist of a single ovary, coiled uterine tube and vulva situated ventrally at the anterior fifth of the body near the middle of the oesophageal area. After fertilization by the male, the female starts producing the eggs which immediately develop into the larvae in the uterus. The females are viviparous, and by the sixth day of infection, they begin to lay motile larvae instead of eggs. Each female is capable of producing nearly 1000–10,000 larvae during its lifetime of 16 weeks. The larvae, but not the eggs, are excreted in the faeces.

Larva

The larvae measure 100 μm in length and 6 μm in breadth. They are deposited by the viviparous female in the intestine, from where they are carried by the systematic circulation and are deposited in various organs and tissues of the body. The larva becomes encysted only in the striated voluntary muscles, where it continues to develop, sexually differentiate, and attains a length of 1 mm, ten times its original size, inside the cyst. The anterior end of the fully grown mature larva is thin, while the posterior end is thick and rounded. The larva inside the encysted cyst is infective to other hosts and is viable for many years before it is calcified. The encysted cyst is lemon-shaped and lies parallel to the muscle fibres.

Cultivation of Parasites

T. spiralis is cultivated from the larval to the adult stage in artificial culture media containing 50% chick embryo extract in serum of rabbit, ox or chicken. A continuous-flow culture system with a gas phase of 85%N-5%CO_2–10%O_2 is necessary for cultivation. In a recent study, the newborn larvae were successfully cultured in 5% CO_2 at 37 °C for 18 h in the RPMI-1640 medium containing 10% fetal bovine serum.

Laboratory Animals

Laboratory animals are widely used to study the pathological and immunological reactions of the host against *T. spiralis* infection. Rat and mouse

are commonly employed in various studies. *Trichinella* is easily maintained in these animals and each developmental stage of its life cycle is recovered from these animals for in vitro studies. The pathological and immunological changes observed in experimental infections of these animals by *Trichinella* closely resemble those seen in humans.

Life Cycle of *Trichinella spiralis*

Hosts

Definitive Hosts

Pigs, rats, horses, humans

Intermediate Hosts

No intermediate hosts

Infective Stage

Larval form of *Trichinella*

Transmission of Infection

The life cycle of all species of *Trichinella* comprises of two generations, larval and adult, in the same host (Fig. 2). Although *Trichinella* has a wide host range of mammals, birds and reptiles, humans, pigs and horses are the ones most important from a public health point of view. The adult *T. spiralis* inhabits the small intestine of the pig, rat and human. The adult and larvae are the distinct stages of the parasite observed in the life cycle of the parasite.

Man acquires infection by ingestion of raw or inadequately cooked pork, infected with the larvae of *Trichinella*. On ingestion, the larvae are liberated in the stomach from the cyst by acid-peptic digestion. The larvae migrate down to the duodenum and jejunum, attach to the mucosa and grow to the adult worms by the third day of infection. The adult male and female worms mature sexually within 5–7 days and the female is then fertilized by the male, after which the male dies. The fertilized female lies deeply burrowed in the mucosa and discharges 1500–2000 larvae for a period of 5–7 weeks or till it is alive. The adult worm remains viable in the intestine for a few weeks, but may survive much longer in immunocompromised host. The larvae are carried by the portal blood circulation or lymphatics to reach the systemic circulation. These are then carried in the systemic circulation for deposition in striated muscles especially the diaphragm. Apart from the diaphragm, the intercostal muscles, muscles of the neck and other large voluntary muscles are commonly affected. The larvae inside the muscles burrow their way into individual muscle fibre causing myositis. The larva lies along the long axis of the muscle and grows rapidly over a period of 3 weeks to attain the size of 1 mm, about ten times its original length. Finally, a cyst wall develops and the larva remains locked up inside the cyst. The mature cyst measures 0.5 mm in length and 0.25 mm in breadth. In human muscle cells, the lifespan of the larva may extend for decades (up to 40 years). In other areas like the myocardium, the larvae do not encyst and die in a short time. After a variable period of time (6–-18 months) under the immune response, calcification occurs. The mature cyst when calcified can be found as fine granules in the muscle.

Trichinella infection in man is the dead end. The propagation of species however is maintained by infection in animals. Pig acquires infection by eating carcasses of other pigs (pig-to-pig) or rats (rat-to-pig) infected by *Trichinella* larvae. Rat gets an infection from an infected rat (rat-to-rat) and less commonly from a pig (pig-to-rat). Ingestion of raw flesh infected with the viable encysted larvae is responsible for the transmission of the disease to a new host.

Pathogenesis and Pathology

Pathogenesis of *Trichinella* infection is largely dependent upon the number of invading organisms and frequency of previous exposure. The larvae invade mucosal epithelium of the duodenum and jejunum and mature to the adult worms in the mucosa of the intestinal tract. The adults are responsible for the development of

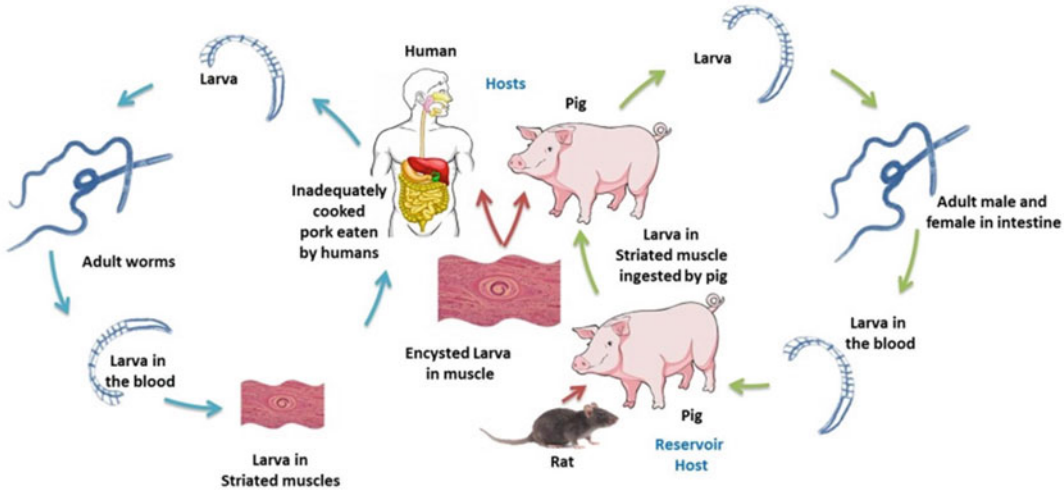

Fig. 2 Life cycle of *Trichinella spiralis*

gastrointestinal manifestations such as nausea, diarrhoea or abdominal cramps in man.

Migration of the larvae and reactions of the host to the encysted larvae within the striated muscles are responsible for the characteristic extra-intestinal manifestations of the disease such as myalgia, myositis, peri-orbital oedema, fever and prostration. The migrating larvae show predilection for their encystment in the striated muscles, particularly at the sites of attachment of these muscles to the tendons and bones. The diaphragm, tongue, larynx, intercostal, deltoid, gluteal and pectoral muscles are most commonly affected. The migrating larvae in their course evoke inflammatory reactions, which subside only after their encapsulation in the striated muscles. Encapsulation does not take place in the myocardium.

Various proteases found in parasites participate in host tissue and cell invasion in the intestinal tract and may also help in the moulting process. The transformation of the host muscle cell into the nurse cell is also under the influence of the hitherto unidentified secreted protein of the parasite. After penetration of the enterocytes, the larvae take residence in the striated muscle cells. Here, it invokes the transformation of muscle cell into "nurse cell," with the disappearance of sarcomere myofibril. Then, following encapsulation, a capillary network develops around the whole structure. The sarcoplasm becomes basophilic, the cell nucleus assumes a central position, and the nucleoli increase in number and size. Increased cell permeability leads to a release of muscle enzymes.

The intestine and the muscles are the common sites invaded by *T. spiralis*. An acute inflammatory response, predominantly neutrophilic, develops in the mucosa of the intestine around the adult worm and is usually associated with a mild and partial villous atrophy.

Migration of the larvae in various muscles provokes marked inflammatory reaction in the tissue. The muscle fibres are destroyed and an acute inflammatory reaction consisting primarily of lymphocytes and eosinophils appears in the muscles. The adjacent muscle fibres show hyaline degeneration (Fig. 3). The encapsulation of the larvae resulting in the formation of cysts of 1 mm or less in diameter takes place in the striated muscles. These cysts eventually calcify within a period of 6 months to 2 years along with the larvae. The growth or encapsulation of the larva does not take place in the cardiac muscles. In the myocardium the larva produces only inflammation, necrosis and fibrosis of the myocardial fibres. Eosinophilia is the hallmark of trichinosis and occurs after 2–4 weeks of infection by the infective larvae.

Diffuse leptomeningeal round cell infiltration and, less frequently, minute foci of gliosis around the capillaries are the major pathological changes

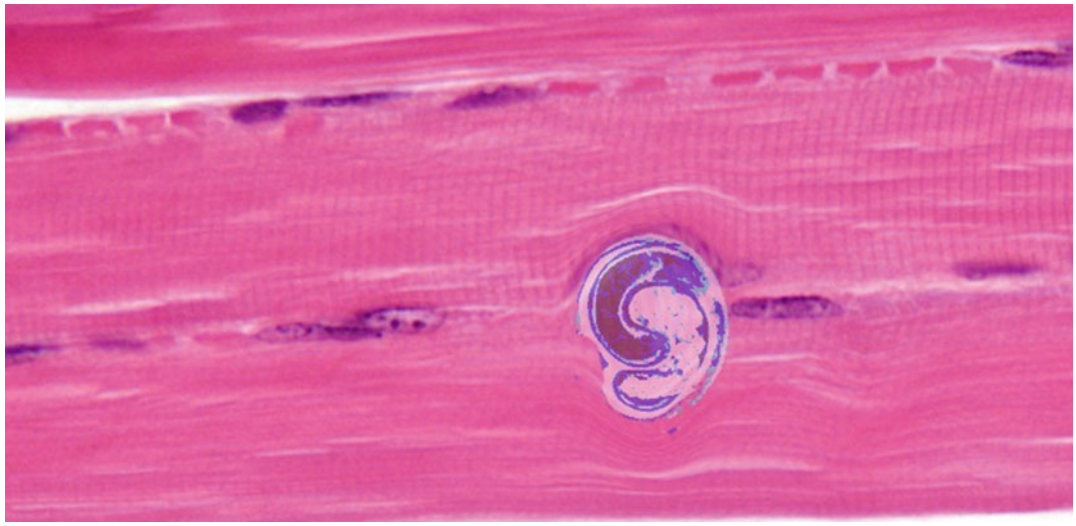

Fig. 3 *Trichinella spiralis* in skeletal muscle. (From: Rawla P, Sharma S. *Trichinella spiralis*. [Updated 2020 May 30]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020. Available from: https://www.ncbi.nlm.nih.gov/books/NBK538511/. Image courtesy S Bhimji MD)

caused by the infective larvae in the central nervous system.

Immunology

T. spiralis infection is always associated with the participation of both the humoral and cell-mediated immunities. The role of humoral and cellular responses in the development of resistance against *Trichinella* infection has been elucidated partially. The former is characterized by the elevated levels of serum IgG, IgM and IgA antibodies in experimentally infected mice after 4 weeks of infection. The parasite-specific IgG1, IgG2, and IgE antibodies are produced against the cuticula, while the IgA and IgM antibodies are produced against the membrane of the helminths. In man, *T. spiralis* infection is characterized by an increased level of circulating IgM, IgG and IgA antibodies after 10–25 days of the infection. The bulk of parasite-specific IgG1 (80%) recognize a highly immunogenic sugar, tyvelose, of parasite origin. This antibody response is associated with a strong TH2 response in the regional lymph nodes. It is to be noted that an increase in the level of IgE is not a constant feature in trichinellosis and co-relates with the degree of eosinophilia in the patients.

The cell-mediated immunity (CMI) showing delayed hypersensitivity reaction has also been demonstrated in the experimental infections of the animals. The T lymphocytes act as the regulator of the inflammatory response during muscle infection. In experimental animals, the concentration of IL-4 and the number of IL-4 producing cells increase, and it has been found that lymph nodes in infected animals produce IL-5, 10, 13 and IFN-γ. Similar findings have been noted in infected humans who demonstrated high levels of IL-5, 10 and IFN-γ. The CMI plays an important role in the development of acquired resistance against the parasite. The transfer of peritoneal exudate cells or lymph node cells from the immunized donor mice to the normal mice led to the transfer of protection against the parasite in the recipient animal. T cells are the effector cells in the development of resistance against the parasite. The gut immunity against *Trichinella* is also believed to be T cell dependent. The humoral antibodies probably play a less important role in the development of acquired resistance in

trichinellosis. No consistent correlation between levels of circulating antibodies and the number of larvae in the muscle or expulsion of adult worms from the gut has been demonstrated. The reaginic IgE antibodies are suggested to play an important role in the complex interactions between mast cells, eosinophils and helminths in the development of acquired resistance against the disease.

A discussion about the immune response in trichinellosis will be incomplete without the mention about the role of eosinophils. Eosinophilia is a prominent feature of *Trichinella* infection and the count may go up to >10,000/μL and the higher level co-relates with the degree of myalgia and central nervous system (CNS) complications. In the initial phases of infection, fall in the eosinophil count carries a grave prognosis and even death of the patient has been reported with count <1%. Eosinophils may act as double-edged swords by having a protective role together with tissue damage. On one hand they can protect the individual from severe infection by killing the newborn larvae by antibody-dependent cellular cytotoxicity phenomenon by releasing major basic proteins, peroxidase and eosinophilic cationic protein. At the same time, the release of histamine, serotonin, bradykinin, prostaglandins E2, D2, J2 and other products cause increased vascular permeability in the capillaries with leakage of fluid, electrolytes, and proteins in the surrounding tissue with resultant vascular damage. Chronic eosinophilia has been linked to severe tissue damage in muscle, myocardium and CNS.

Infection in Humans

Trichinella infection is asymptomatic in the majority of cases in humans. The clinical manifestations depend mainly on the number of larvae invading the intestine and frequency of previous exposures to *Trichinella* and may be divided broadly into (a) intestinal, (b) muscle invasion and (c) convalescence stages.

The clinical signs and symptoms of the intestinal phase are due to irritation of the gastrointestinal mucosa by the adult worm and are observed during the first week of infection. Nausea, vomiting, diarrhoea or constipation and abdominal cramps are the presenting features. In heavy infection, the patients may occasionally develop fulminant enteritis.

The symptoms in trichinellosis, due to muscle invasion by the larvae, are by far more common and are seen during the second week of infection. The condition is characterized frequently by manifestations of the peri-orbital oedema with or without subconjunctival haemorrhages and chemosis and the myositis developing in the intraocular muscles, masseters, neck muscles, limb flexors and lumbar muscles. Occasionally, a macular and petechial rash is observed. Marked peripheral eosinophilia even up to 70% is commonly seen. The patient may die because of myocarditis, encephalitis and other neurological complications. Myocarditis is seen in 5–20% of cases and presents as pericardial pain, tachycardia and electrocardiogram abnormalities. Neurological complications are rare in trichinellosis and may show multiple small cortical infarcts in magnetic resonance imaging.

The convalescence stage is marked by the beginning of the encapsulation of the larvae during the third week of infection. The systematic manifestations are usually absent in this stage; however malaise and weakness may be present for a few months. Myocarditis and less frequently bronchopneumonia, vascular thrombosis and encephalitis may be present as sequelae of the infection in this stage.

Mortality due to the condition is relatively low. Myocarditis is the commonest cause of the death. Bronchopneumonia, vascular thrombosis and encephalitis are the less frequent causes of death in this condition.

Different species of *Trichinella* may cause some variations in their clinical manifestations. It has been observed that *T. spiralis* may cause more severe infection compared to *T. britovi* possibly because females of the latter species are less prolific. *T. murrelli* may not cause peri-orbital or facial oedema but may provoke skin reactions. The non-encapsulated *T. pseudospiralis* causes more prolonged symptomatic disease.

Infection in Animals

Over 120 species of mammals including wild carnivores, felines, fur-bearing animals, rodents and insectivores are infected with *Trichinella* under natural conditions. The infection in domestic animals is rarely associated with any overt clinical manifestations. The predatory animals under natural conditions may die of trichinosis.

In rat, the infective larva shows more predilections for the diaphragm, while in pig, the masseters are most commonly affected. The skeletal muscle cysts are always round in the carnivores, round and elongated in rats and oval in pigs. The cyst undergoes calcification, the timing of which varies from host to host amongst animals. In rabbits and pigs, the onset of calcification is visible within 3–5 months and is completed by 7–9 months of infection. In mice, the process of calcification is much slower and takes a longer time of more than 1 year.

Most of the domestic and wild carnivorous animals are susceptible to infection but show no or minimal sign of infection, even though the parasite burden may be quite high. Interestingly, the household pigs and rats are resistant to the sylvatic species of *Trichinella*.

Epidemiology and Public Health

Human trichinellosis is distributed worldwide. It is most prevalent in the Northern hemisphere including the Arctic and parts of Africa and Asia. In South and Central America, autochthonous infections have been reported from Brazil, Uruguay and Chile. Recent outbreaks of trichinosis have been reported from Italy, Laos, Tanzania and France. As per the cumulative report from 55 countries, the total number of cases has been estimated to be 10,000 per year with 0.2% mortality.

Trichinella infection in humans is closely associated with the consumption of raw or undercooked meat; hence cultural and social factors play a role in the epidemiology of trichinellosis. Trichinellosis is rare in communities that consume fully cooked meat. Pork or pork products are the main source of infection especially when pigs are raised in the backyards or in the village community. Local meat consumption, particularly horse meat, has been associated with *Trichinella* infections in France and Italy. Dog meat has been incriminated as the source in China and the Slovak Republic. Human infections due to *T. nativa* has been documented in the Arctic region linked to the consumption of walrus or bear meat. Recently, *T. papuae* has been implicated in outbreaks of human trichinellosis in Thailand after eating wild boar. The first authentic case of a *Trichinella* infection in an animal was reported by Maplestone and Bhaduri in the diaphragm of a cat in the year 1942. Subsequently, it has been demonstrated in various animals like domestic pigs, rodents and wild animals like civet cats. The first human infection in India was documented in 1996 and till date at least nine case reports and one outbreak of trichinellosis have been reported. The outbreak was reported in 2014 in Tehri Garhwal district of Uttarakhand State in North India with 54 cases and one death and was associated with the consumption of raw or undercooked pork.

Nearly, 120 species of mammals and also reptiles and birds are found to be infected by *Trichinella* (Table 1). The natural cycle of *Trichinella* infection involves primarily the carnivores and is usually maintained by these carnivorous animals. The prevalence and spread of *Trichinella* infection is dependent upon the food habits of potential host species and environmental and climatic factors. In nature, *Trichinella* spp. exhibit two cycles: the domestic cycle and the sylvatic cycle.

Domestic Cycle: This is the classical well-known pig-to-pig transmission where humans get the infection by eating pork. In many parts of the world, cats, mice, dogs and various wild animals can also enter into the cycle. The domestic cycle is maintained by the pigs by consumption of meat scraps, rats or mice, dead pigs and other mammals, or ingestion of pig faeces. This type of cycle can be observed in small, local meat-producing communities or farms.

Table 1 Species and genotypes of *Trichinella* spp. and their distribution

Species (genotype)	Normal host	Sources of infection	Geographic distribution	Human cases reported	Countries from where reported
Trichinella spiralis (T1)	Pigs, rats, carnivores	Pork	Worldwide	Yes	Many countries
Trichinella nativa (T2)	Marine and terrestrial carnivores	Bear meat, walrus, dog	Arctic or Subarctic regions	Yes	Alaska in the USA, Russia, China
Trichinella britovi (T3)	Carnivores, pigs	Wild boar meat, dogs, jackals	Temperate regions, North and West Africa	Yes	Algeria, Turkey, Poland, France
Trichinella pseudospiralis (T4)	Mammals, birds	Wild boar	Worldwide	Yes	France, Thailand
Trichinella murrelli (T5)	Carnivores	Horse, bear	Temperate regions	Yes	USA, France
Trichinella papuae (T10)	Mammals, reptiles	Wild boar, soft shelled turtles	South East Asia	Yes	Thailand, Korea, Taiwan
Trichinella zimbabwensis (T11)	Mammals, reptiles	Not known	East Africa	No	–
Trichinella patagoniensis (T12)	Carnivores	Not known	Argentina	No	–
Others: *Trichinella nelsoni* (T7), T6, T8, T9	Carnivores	Not known	USA, Canada, Ethiopia, Japan, South Africa	No	–

Sylvatic Cycle: In this cycle, the carnivorous wild animals are primarily involved. Cannibalism, predation or scavenging habits of such wild animals maintain the cycle. Transmission of infection occurs by the consumption of fresh or decomposing carcasses. The species of *Trichinella* associated with the sylvatic cycle include mostly species other than *T. spiralis*. Humans and pigs normally do not enter this cycle, but they may become infected by eating infected meat of wild animals.

Diagnosis

Clinical diagnosis of trichinellosis is difficult due to protean manifestations of the disease. The condition needs to be differentiated from other similar conditions. The gastrointestinal symptoms may cause confusion with various other conditions of gastroenteritis, while systematic manifestations may mimic those of influenza, typhoid fever, sinusitis, glomerulonephritis or angioneurotic oedema. The presence of cardinal features of peri-orbital oedema, myositis, fever and high grade eosinophilia are highly suggestive of trichinosis. The history of ingestion of the inadequately or poorly cooked pork supports further the clinical diagnosis of the disease. The diagnosis of trichinellosis depends on (a) clinical findings (b) laboratory findings (Table 2) and (c) epidemiological investigation.

Microscopy

The definitive diagnosis is made by demonstration of free or encapsulated *Trichinella* larvae in the skeletal muscles (deltoid, biceps, gastrocnemius or pectoralis) at either autopsy or biopsy. In light or early infection, the larvae are difficult to show by these methods. Moreover it involves surgical invasive procedure and the amount of muscle taken for biopsy may influence the sensitivity. Sample should be collected from the muscle, free from fat or skin, weighing about 0.2–0.5 g. Examination of a muscle biopsy can be carried out by artificial digestion using 1%

Table 2 Laboratory diagnosis of Trichinellosis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Histopathology of muscle biopsy sample	Larval forms; basophilic transformation of muscle cells	Not very sensitive
In vitro cultivation	Continuous-flow culture system	Transformation of larval form to adult	Complicated procedure; live larva needed
Serology	ELISA, indirect fluorescent antibody test (IFAT), bentonite flocculation test	TSL-1 antigen for ELISA; larva or infected muscle in IFA	IFAT shows cross-reactions with *Onchocerca* and *Schistosoma*
	Immunoblotting	Excretory-secretory antigens	Confirmatory test
Molecular diagnosis	PCR-RFLP	ITS-1 and ITS-2 of rRNA	Species and genotype identification
Other laboratory tests	Blood biochemistry and haematological investigations	Elevated serum levels of CPK, LDH and aldolase. Leucocytosis and eosinophilia	Detected from second to fifth week of infection

pepsin and 1% hydrochloric acid, or histological analysis by haematoxylin-eosin staining. Even in the absence of visualization of the larva, the basophilic transformation of muscle is an excellent indicator of trichinellosis. The larvae may be looked for, though difficult to find, in the faeces during the intestinal stage or in the blood, spinal fluid or milk during the migratory phase of the disease.

Serodiagnosis

A variety of immunological tests are currently available for diagnosis of the condition in individual cases and for epidemiological studies of trichinellosis.

The intradermal skin test using *Trichinella* antigen, used earlier, shows positive immediate hypersensitivity reaction after 11–16 days of infection and remains positive for many years.

During the acute stage of infection, IgE levels rise early in a majority of the cases, but the absence of raised IgE cannot rule out trichinellosis. Hence IgE detection is of no diagnostic importance. Circulating IgG antibodies appear 12–60 days after infection and the window period depends on the number of ingested larvae, the particular species involved and the individual's immune response. ELISA and the indirect fluorescent antibody test (IFAT) are the most commonly used approach at present, replacing the bentonite flocculation test, latex agglutination (LA) and indirect haemagglutination (IHA) employed earlier in the serodiagnosis of trichinellosis. The antigens used for IFAT include infected rodent muscle or free muscle larva. Cross-reactions have been seen with *Onchocerca* spp. and *Schistosoma mansoni* in IFAT. Immunoblotting using excretory/secretory antigens of muscle larvae of *T. spiralis* is used as a confirmatory test after initial screening by ELISA or IFAT.

Molecular Diagnosis

Molecular methods have been employed to identify the *Trichinella* species or genotype of the isolated larva. PCR-RFLP can be used for species differentiation and genotype determination. Database derived from ITS 1 and ITS 2 as well as from expansion segment V region of the rRNA repeat of various *Trichinella* species and genotypes are available for this purpose.

Other Laboratory Findings

Leucocytosis along with high-grade eosinophilia (even up to 70%), elevated serum creatinine phosphokinase (CPK) and lactic dehydrogenase (LDH) is the non-specific finding of *Trichinella* infection in humans, especially during the stage

of muscle invasion. Eosinophilia appears early during the second to the fifth week of infection prior to the onset of clinical signs and symptoms. During the same period, there is a rise of serum CPK, LDH and aldolase levels in 75–90% of infected persons and may persist for up to 4 months.

Epidemiological Diagnosis

For epidemiological purposes, the patient should be asked for the place from where he had bought the meat or meat products, the time and the mode of consumption (raw or undercooked). The triad of high fever, peri-orbital oedema and myalgia occurring in a cluster in a community or household points towards an outbreak of trichinellosis and needs appropriate investigation.

Diagnosis in Animals

Diagnosis of trichinellosis in pigs is routinely carried out by the use of "Trichinoscope" to detect *Trichinella* larvae in the muscles. Trichinoscopy is a reliable procedure to diagnose moderate to heavy infections but occasionally fails to detect light infection. The pooled sample digestion method and the immunoassays such as IFAT and ELISA to demonstrate *Trichinella* antibodies in the serum are the alternate methods recently followed to establish the diagnosis of trichinellosis in the swine. Molecular methods are also employed.

Treatment

The benzimidazole group of anthelmintics like albendazole remain the mainstay of treatment. However, although useful against adult worms and early larval stages, they are ineffective against the encapsulated larva in muscle cells. Hence, treatment should be directed against the adult worms or the migrating larval stages and should be initiated within the first 3 days of infection.

Albendazole is administered in a dose of 400 mg twice daily for 8–14 days; and for mebendazole, it is 200–400 mg three times a day for 3 days, followed by 400–500 mg thrice daily for 10 days. Pyrantel pamoate is safe in pregnancy and in children and given as a single dose of 10–20 mg/kg of body weight and repeated for 2–3 days. However, it acts only against the adult worms and not against the larval stages.

Corticosteroids are helpful in relief of symptoms which result from inflammatory and allergic reactions to the larvae. Caution should be exercised in prolonging the steroidal treatment because of the danger of an increased number of larvae in muscles and more extensive muscle invasion. The standard treatment for severe symptoms is with prednisone at a dose of 30–60 mg/day for 10–15 days.

It needs to be emphasized that delay in initiation of treatment increases the probability of establishment of viable larva in the muscle which is no longer amenable to medical treatment. In such a scenario, the larva will persist in the muscle causing persistent myalgia. Although prolonged therapy can be started in the late stages of infection, it has been observed that it is useless against long-term sequelae and chronic trichinellosis.

Prevention and Control

Thorough cooking, deep freezing at −20 °C or refrigeration at 4 °C for more than 20 days are the effective methods of killing *Trichinella* larvae in the pork. Smoking, curing and drying of meat are unreliable and are not effective procedures to kill the larvae.

Control of infection in the swine and destruction of *Trichinella* larvae in the pork will prevent transmission of infection to man. Avoidance of the habit of feeding raw, infected garbage to

swine and the boiling of garbage prior to feeding of pigs will help to significantly reduce trichinellosis in swine.

Case Study

A 30 years old male attended outpatients department with symptoms of fever, headache and pain in the left calf muscle with restricted knee movement. On examination, tenderness in the left gastrocnemius muscle with mild rise in local temperature was noted. He gave a history of regular intake of pork and pork products. The patient was admitted for further evaluation. Laboratory findings revealed: TLC: 26,000/dL; eosinophils: 10%; CPK: Elevated. With a clinical suspicion of trichinellosis, biopsy from gastrocnemius muscle was performed. Histopathological examination showed typical coiled larva surrounded by inflammatory cell and nurse cell-larva complex. A definitive diagnosis of trichinellosis was made and the patient was prescribed Albendazole 400 mg twice daily for 14 days and discharged. On follow-up after 3 weeks, there was complete subsidence of signs and symptoms.

1. What other parasites can cause musculoskeletal involvement?
2. How can you diagnose this case serologically?
3. Apart from pork, what other animal meat can transmit this infection?

Research Questions

1. What are the drugs that can kill the muscle larva? How the study of bioinformatics can help in drug design?
2. Which genus and species specific antigen can be utilized for immunoassays?
3. What are the protective antigens which can help in vaccine development?

Further Readings

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Dracunculiasis

Abhijit Chaudhury

Learning Objectives

1. To understand that dogs and other domestic animals may act as reservoirs and may cause the resurgence of infection in areas where the disease might have been controlled or eradicated.
2. To study the key measures which can be used to eradicate the disease from a region.

Introduction

Dracunculiasis or Guinea worm disease is a disease, restricted to a few countries of the world, caused by the nematode helminth *Dracunculus medinenesis*. The disease is characterised primarily by the formation of a blister in the affected part which later develops into a skin ulcer. Its incidence has declined in the last few decades because of concerted public health and global eradication measures. At present, the infection is confined to only a few countries in the world. Treatment of the condition mainly rests on manual or sometimes surgical removal of the worm. From about 3.5 million cases in 1986, the number of the cases has drastically been reduced to 148 cases in 2013. Nevertheless, the disease has re-emerged in Chad, Africa, in 2016 after an absence of 10 years.

History

Dracunculiasis was known in Africa and Middle East since time immemorial. The Greeks in second century BC gave a clear description of the disease among the people living near the Red Sea. It was also mentioned by Avicenna, the Arab physician. In 1674, a cure for the disease by winding of the worm out with a stick was first mentioned by Velschius. The British Army doctors working in India from early nineteenth century were familiar with the disease. In 1870, the Russian scientist Alexei Fedchenko first gave the full description of the worm and its life cycle. Finally, Robert Leiper, a British physician, described the complete life cycle and recommended measures to prevent the disease in a series of work carried out in Africa in 1905–1910.

Taxonomy

The genus *Dracunculus* belongs to Phylum, Nematoda; Class, Secementea; Order, Camallandia; Superfamily: Dracunculoidea; and

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Family, Dracunculoidae. The members of family Dracunculidae are widely distributed in mammals, birds and reptiles. A total of 12 species have been described, primarily from reptiles.

D. medinensis is the most important species that causes infection in animals. *D. insignis* is another species that is common among carnivores and may occasionally infect humans.

Genomics and Proteomics

The draft genome assembly of *Dracunculus* was produced by the Wellcome Trust Sanger Institute. The parasite has a genome size of 103.8 Mb, with nearly 11,000 coding genes. Recent discovery of the prevalence in non-human hosts, particularly dogs, in some African countries, coupled with the re-emergence of human cases, has prompted studies into genetic variation among human and non-human isolates of *Dracunculus* species in these endemic areas. For investigating phylogenetic relationship between species, the 18srRNA sequence analysis has been adopted in most studies, with the addition of the CO1 (Cytochrome Oxidase subunit 1) gene in recent years.

A total of 10,920 proteins have been studied in *D. medinensis* and many of them remain uncharacterized. The interested reader can refer to https://www.uniprot.org/uniprot/?query=proteome:UP000038040 for more information.

Parasite Morphology

Adult Worm

The male and female adult parasites obtained from humans have been described, although the scarcity of the male parasite has hampered its detailed description.

The female adult measures 60–100 cm in length, with a diameter of about 1.5 mm. It is milky white in colour and has a slender, long and smooth body (Fig. 1). The posterior end is tapering, bent in the form of a hook. The mouth, present at the anterior end, is small and triangular and is surrounded by a plate. The oesophagus has a large protruding glandular portion. In young adults, the vulva is situated midway in the body, but is atrophied in adults. The gravid uterus is filled with a large number of embryos, causing the intestine to become compressed and non-functional. The female is ovoviviparous and discharges embryos in successive batches. It has a life span of about 1 year.

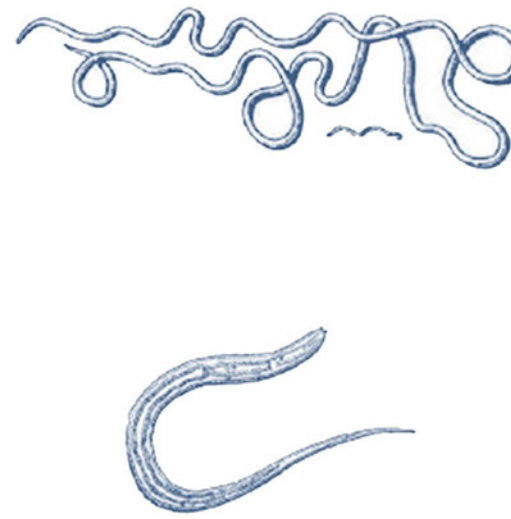

Fig. 1 *Dracunculus medinensis* Adult worm and larva

The male adult is much smaller than the female and measures 12–30 mm by 0.4 mm in size. Spicules are unequal in size and there is considerable variation in the genital papillae. A definitive description of the male worm is not available. The life span is about 6 months.

Infective Larvae

Larvae have rounded head and long slender tapering tails and coiled bodies. They measure about 700 × 20 μm in size. These embryos are set free at the time of parturition in contact with water. Further development only takes place if ingested by freshwater cyclops. If not taken up by the crustacean, they die in 4–7 days' time.

Cultivation of the Parasite

There is no report of in vitro cultivation of the parasite.

Laboratory Animals

The laboratory study of dracunculiasis has suffered due to the lack of a suitable, readily available animal model.

The most suitable definitive host for laboratory transmission for *D. medinensis* was found to be the rhesus monkey (*Macaca mulatta*) and the parasite has been maintained in monkeys for four laboratory cycles. Successful infection of *D. insignis* has been reported in ferrets (*Mustela putorius furo*), which remains the most preferred experimental animal. Several other species, including raccoons, rhesus macaques (*Macaca mulatta*) and mink have been successfully inoculated with *D. insignis.*

Life Cycle of *Dracunculus medinensis*

Hosts

Definitive Hosts
Humans, dogs.

Intermediate Hosts
Cyclops (*Mesocyclops* species, *Thermocyclops*, *Eucyclops*, *Tropocyclops*).

Infective Stage

Larva inside the cyclops.

Transmission of Infection

Humans acquire infection by drinking water contaminated with the cyclops harbouring the infective larva of *D. medinensis* (Fig. 2). In the stomach, the cyclops are digested and larvae are released. The larvae penetrate the duodenal wall, cross the abdominal mesenteries, pierce the abdominal muscles and enter the subcutaneous connective tissue, from where they migrate to the inguinal region and occasionally to the axillary region. A third moulting occurs 20 days after infection and the final moult, nearly after 40 days. Males fertilize the females 3 months after exposure and the males die in 3–6 months' time and degenerate. The gravid females then migrate to the skin in about 6–12 months and select those areas which are likely to come in contact with water. These areas commonly include the back of water carriers, arms and legs of washer men and legs of those who fill water in step wells and small water bodies. In the skin, a blister is produced, which then bursts, forming an opening for the exit for young worm. Muscular contractions, stimulated by cold water, cause the worm to protrude through the skin wound and forces a large number of coiled embryos to be discharged into the water. Eventually, the uterus gets empty, the worm is exhausted, and the wound heals by itself. The larvae have round head, long slender tapering tails and coiled bodies. They measures about 700 × 20 μm in size.

Further development of the larvae takes place only if ingested by freshwater cyclops. If not take up by the crustacean, they die in 4–7 days' time. A cyclop can ingest 15–20 embryos from water and the embryos enter the intestine of the crustacean intermediate host. They penetrate the gut wall within 1–6 h of ingestion and enter the body cavity (haemocoele) of the cyclop, whereby they undergo moulting and increase in size to about 1 mm. This development is completed in 12–14 days' time.

Pathogenesis and Pathology

The principal pathology is seen if the adult *Dracunculus* worm is unable to emerge out of the body. The parasite products elicit an intense inflammatory response in the subcutaneous tissue. The site of exit can serve as the entry point of bacteria, causing secondary bacterial infections, including the formation of abscess.

Immunology

The few studies carried out to understand the host immune response to Guinea worm infection have

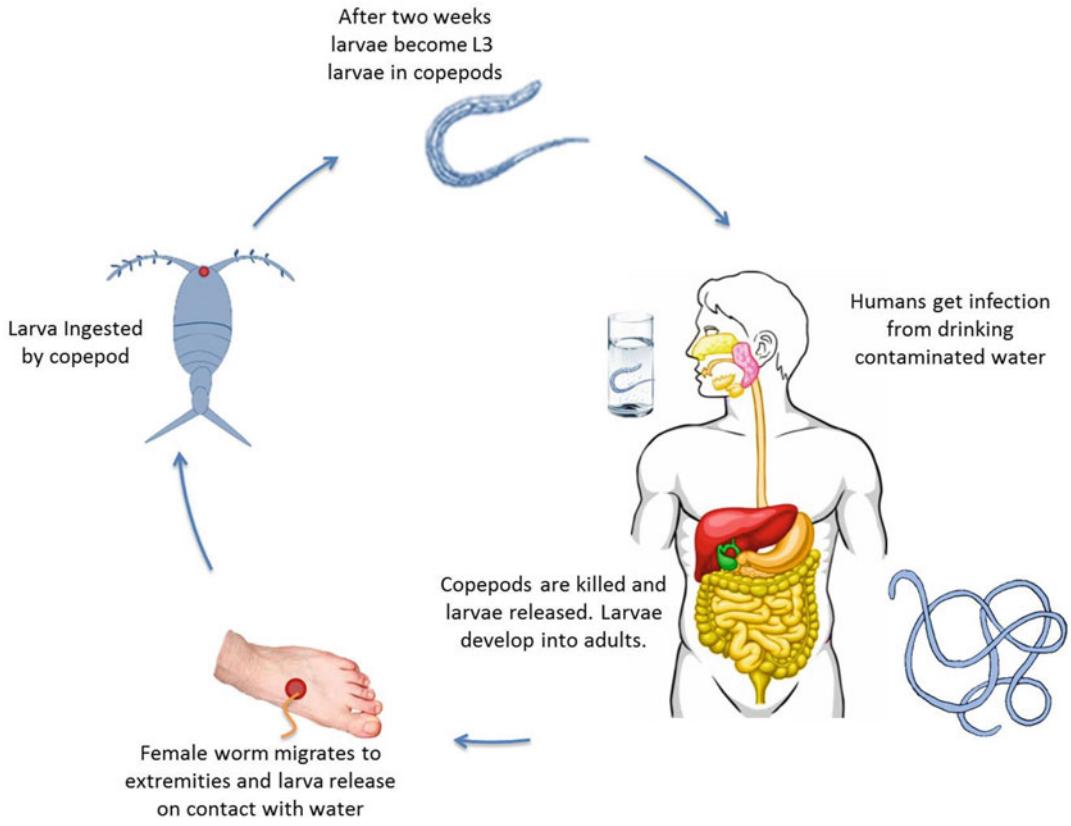

Fig. 2 Life cycle of *Dracunculus medinensis*

indicated some differences in patent and pre-patent infection states. A depression of IFN-γ production has been seen in patent infection state, but TH2-specific IL10 levels were similar in both conditions. The mean antibody titres have been found to be similar in both patent and post-patent infection categories. Among the antibody sub-classes, parasite-specific IgG4 as well as IgG1 reactivity corresponds with *D. medinensis* infection state and a clear distinction between patent and post-patent patients could be found. In general, patent infection causes a depression of cytokine release in the patients. The IgG1 and IgG4 production during patency may play a role in protecting the ingested larva from destruction by the immune system. Infection by the parasite does not confer any immunity to the host and repeated infections in the same host are common.

Infection in Humans

The third-stage (L3) larva is not pathogenic. The adult female is only pathogenic and is responsible for various clinical manifestations. After initial infection, the host remains asymptomatic for about 1 year till the adult gravid female reaches the skin. The clinical features may be due to the emerging adult female, secondary bacterial infection and worms which are not able to emerge.

(a) **Emerging Adult Worm**: This is the commonest scenario and occurs whenever the worm makes an attempt to come out of the body to discharge the embryos. The lower extremities are the commonest sites, but cases have been reported in the arm, trunk, buttocks, head and neck. At first, there is blister formation on the skin at the

point of exit. An allergic reaction is elicited due to the release of metabolic wastes of the worm which appear to act as parasitic toxins. The patient may experience nausea, diarrhoea and localized oedema. There is intense local itching often associated with intense burning pain (hence the name *Fiery Serpent* in the Bible). The blister is filled with a yellow white fluid containing numerous larvae and white blood cells and is bacteriologically sterile. The blister ultimately bursts and symptoms subside. The site may develop an ulceration, which heals rapidly. A tiny hole remains through which the head of the worm can be seen protruding whenever the part comes in contact with water. The larvae come out when in contact with water and pain is relieved once all the embryos are released. The worm slowly dies and gets absorbed in the tissue, followed by healing of the ulcer.

(b) **Secondary Bacterial Infection**: In almost half of the cases, the local ulcer is secondarily infected with bacteria, which may lead to abscess formation. This causes severe pain. Bacterial infection becomes more serious when the retreating worm draws the bacteria inside, which leads to cellulitis. This may also facilitate the entry of tetanus spores. Other complications include arthritis, chronic ulcer, synovitis and bubo formation.

(c) **Non-emergent Worm**: In conditions where the adult worm is unable to emerge from the skin, it begins to degenerate and in the process releases its degenerated products. This may lead to aseptic abscesses and arthritis. Ultimately, the worm dies and is calcified.

Infection in Animals

The manifestations of Guinea worm disease in dogs are similar to those in humans. Purulent fistulated skin nodules in dogs caused by *D. insignis* have been reported.

Epidemiology and Public Health

Dracunculiasis is primarily a disease prevalent in Asia and Africa. Currently, the disease is restricted to only a few countries (Table 1; Fig. 3). From an estimated 3.5 million cases in 1986, the total cases reported in 2018 were only 28. The countries reporting one or more infections include Chad, Mali, Burkina Faso, Angola, South Sudan and Ethiopia in Africa and Yemen in Asia. As per the latest report in 2019, there has been a resurgence of cases in Chad and 54 cases were reported from Chad and the rest from South Sudan, Angola and Cameroon. An outbreak of seven suspected cases with the emerging worm was reported from Ethiopia in April 2020. In India the disease was limited to Rajasthan, Punjab, Gujarat and some other states before these states were declared disease-free by the WHO in 2000.

Water contaminated with infected cyclops remains the primary source of infection. Humans acquire the infection by drinking water from shallow ponds, wells or cisterns contaminated with cyclops harbouring the infective larvae. Poor sanitation and personal hygiene facilitate the transmission of infection in the community.

Dracunculus is a parasite with an aquatic life cycle but it also thrives in a desert environment or in conditions of draught. In such areas all the animals and humans depend on isolated water bodies for existence. Dried up rivers reduced to deep isolated pools, step walls and man-made ponds are areas where planktonic organisms thrive. The warm stagnant water causes the cyclopean population to multiply tremendously. Humans come into contact with such water for all daily activities like washing, bathing and even for drinking. An infected person during his contact with water sheds the embryos for ingestion by copepods. Drinking of such water predisposes to human infections.

Table 1 Epidemiological features of *Dracunculus medinensis*

Species	Definitive host	Intermediate host	Geographic distribution
Dracunculus medinensis	Man, dogs. Possibly baboons and cats.	Cyclops	Chad, Ethiopia, South Sudan, Angola, and Cameroon

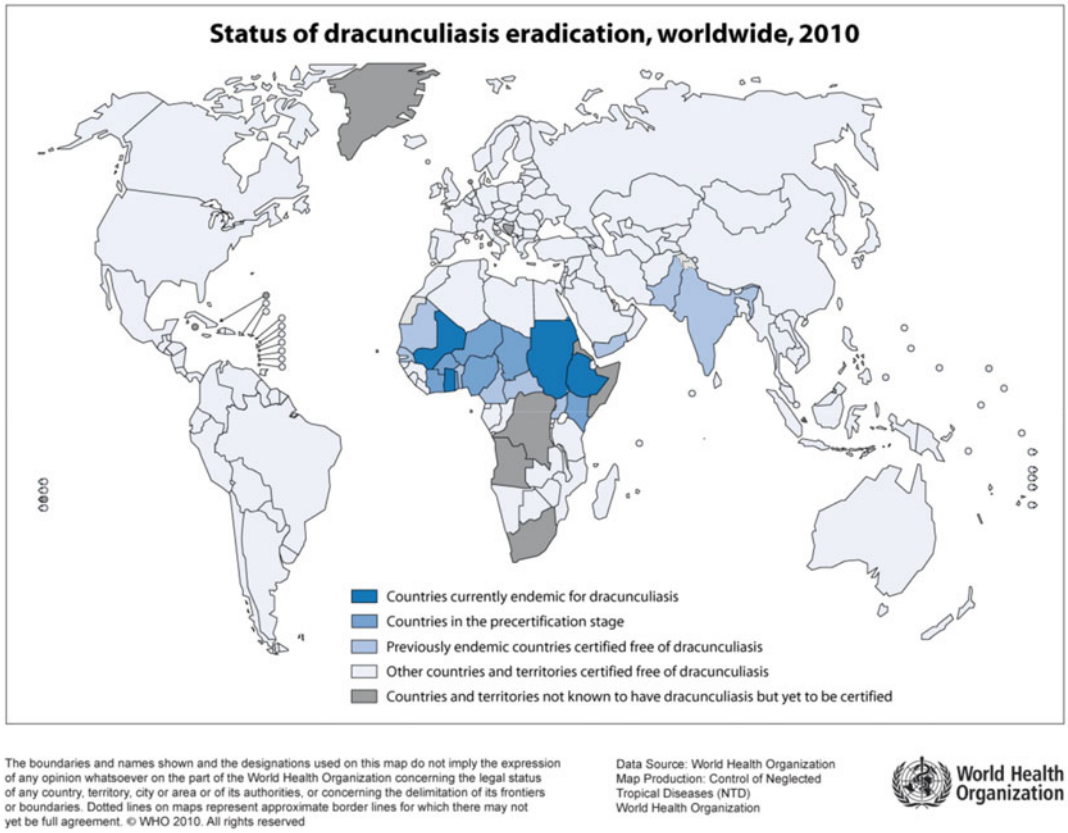

Fig. 3 Prevalence of *dracunculiasis* worldwide (reproduced with permission from the WHO)

Is Dracunculiasis a Zoonotic Disease?

Humans have been considered to be the only reservoir of dracunculiasis till now but recent studies have revealed some new facets. Although traditionally considered a water-borne anthroponosis, recent studies from Chad, where dracunculiasis has re-emerged, have thrown up some unusual findings. In certain African countries, two important facts have emerged. First is the existence of some animal reservoirs like dogs, cats and baboons. Second, an alternate mode of transmission by the ingestion of a paratenic host like frog or a transport host like fish. Small fish or "fingerlings" may act as important vehicles for the transmission of the cyclops. These fingerlings ingest copepods as a major food source. In Chad, Ethiopia and possibly other countries, children or adults may eat poorly cooked or raw fingerlings or they may be fed to dogs or cats. In Chad, a large number of dogs have been found to be infected and they get the infection in the dry seasons by consumption of cyclops-contaminated water. Guinea worm eradication programmes have traditionally aimed at the water-borne route, and hence, these new routes,

Table 2 Laboratory diagnosis of *dracunculiasis*

Diagnostic approach	Method	Target	Remarks
Naked eye examination	Visualization of worm	Whole worm	Specific method.
Microscopy	Affected part douched with water.	Embryos	700 × 20 μm in size, coiled structures.
Serodiagnosis	ELISA, IFAT for IgG, IgG4	Whole worm antigen Larval antigen	Can diagnose patent and pre-patent infections.

particularly food-borne transmission and to a lesser extent, spill-over from dog populations, may be the potential mode of re-emergence of this disease. In 2015, 459 dogs in 150 villages of Chad were found to be infected and it was believed that *Dracunculus* species in animals were different from those of humans. But subsequent genome sequencing disproved the hypothesis and showed that these dogs were infected with the human species of *D. medinensis*.

Diagnosis

Various approaches are being followed in the laboratory diagnosis of dracunculiasis (Table 2).

Microscopy

Detection of adult worms or embryos: Adult worms are detected when the worms protrude out of the skin and appear on the skin surface. For demonstration of the embryos, the affected part may be douched with water to encourage discharge of embryos. The milky fluid is pipetted off and examined under the microscope, which shows numerous embryos.

Serodiagnosis

Circulating antibodies in the serum are detected by ELISA or indirect immunofluorescent test, particularly in patent infections. Whole-worm parasitic-antigen-specific IgG4 demonstration detects even pre-patent infections upto 6 months before the emergence of the worm. Use of first-stage larvae antigen in ELISA to detect IgG4 antibodies was found to be 83% sensitive and 97% specific.

Other Tests

Dead worms in deeper tissues may become calcified and can even be visualised in plain X-ray. Blood examination reveals a marked eosinophilia.

Treatment

Treatment of dracunculiasis includes either extraction of the worm, surgical removal of the worm or medical treatment.

1. **Extraction of the worm**: This remains the mainstay of treatment in patients where the worm is seen protruding from the skin lesion. The worm is wrapped or rolled on a stick and a few centimetres gradually extracted each day (Figs. 4 and 5). The affected part is immersed in cold water so that more of the remaining portion comes out and wrapped on the stick. The whole process may last for a few weeks because of the length of the worm. Care needs to be taken to use gentle traction to prevent breakage of the worms. Tropical antibiotics are applied to prevent secondary bacterial infections. The affected part is bandaged with fresh gauze after each manoeuvre to protect the site.
2. **Surgical removal of the worm**: Non-emerged worms can be removed by surgical method using local anaesthesia. Surgical intervention is also necessary if, during extraction, there is

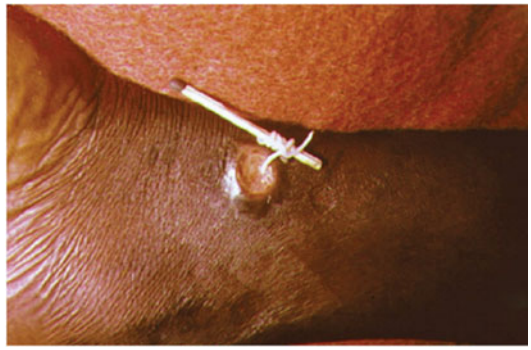

Fig. 4 Removal of adult guinea worm by winding it around a matchstick. (Source: https://wtcs.pressbooks.pub/pharmacology/chapter/3-18-anithelmintic/#return-footnote-288-1)

breakage of the worm. In such cases, a severe inflammatory response with abscess formation may occur due to worm retraction and release of larvae in the tissue. This requires incision and drainage along with the removal of the rest of the adult worm.

3. **Medical treatment**: The role of anti-helminthic drugs remains controversial. Various broad-spectrum agents like albendazole may help in elimination, but it has been reported that it may be associated with aberrant migration of the worm. Ivermectin was not found to be effective.

Prevention and Control

Prevention of dracunculiasis is possible and various measures taken in this direction have helped in large-scale eradication of the disease in most countries of Asia and Africa. The strategies include the following:

(a) **Active surveillance**: The surveillance network detects and reports human or animal infection within 24 hours of worm emergence. A cash award has also helped in improved reporting. Village volunteers have taken an active part in this.

(b) **Education**: The village communities are educated not to enter a drinking water source if a worm is emerging and also not to allow any individual or animal to enter the water bodies if found with emerging worms.

(c) **Case containment**: These centres provide treatment and support to people with Guinea worm disease and help prevent them from contaminating water.

(d) **Water filtration**: Potentially contaminated water used for drinking purposes needs to be filtered to remove the cyclops. Use of fine-mesh cloth filters in households is a highly effective method. For travel or work away from the household, individuals can be provided with pipe filters, which act like straws, to drink water from unsafe sources. Cotton cloth filters or a synthetic gauze of mesh size below 0.15 mm is effective in holding back the copepods.

(e) **Chemical treatment of water**: The contaminated water sources can be treated with Temephos, which is an insecticide capable of destroying the cyclops. This is an expensive method and requires careful calculation of water volume for adding the requisite safe dosage of the insecticide. A 50% emulsifiable concentrate added at a rate of 2 ml/m^3 of water may be used at 4–6 weeks' intervals.

(f) **Boiling of water**: It kills all the copepods present in the contaminated water.

(g) **Provision of safe water**: To prevent dependence on potentially contaminated water for drinking purpose, safe drinking water provisions need to be provided at the village level. These include walled bore wells and hand-dug wells.

In 1981, the Inter-agency Steering Committee for Co-operative action for the International Drinking Water Supply and Sanitation Decade (1981–1990) proposed the elimination of dracunculiasis as a measure of success of the decade. This led to a WHO-CDC (the United States) collaboration to formulate the strategies and technical guidelines for an eradication campaign, which the Carter centre and UNICEF also joined as strategic partners. This consortium, in partnership with the national Guinea Worm Eradication

Fig. 5 Extraction of *Dracunculus* worm being done in the field (Photo Credit: WHO)

Programme of the Ministries of Health of affected countries, has pioneered the eradication of the disease. Compared to 1986, the disease burden has been reduced by 99.99% from an estimated 93.3 million cases to 28 human cases in 2018. To date, the WHO has certified 187 member states as free of disease transmission. However, in recent years, the emergence and persistence of worm infection in animals in Chad, Ethiopia and Mali have challenged the eradication efforts. It is now understood that dogs may act as reservoir animals and they and other domestic animals such as cat can acquire *D. medinensis* infection by consuming raw fish entrails and other inadequately cooked or raw aquatic animals like frogs. The worms affecting humans and other animals have been found to be genetically similar. Thus, intervention measures are being adapted to reduce animal infection through food-borne routes. The final goal is to certify all countries as free of Guinea worm disease. The remaining work to be done has been divided into three phases: transmission interruption in remaining countries, pre-certification and the final phase of certification by the International Commission (ICCDE, WHO) for Dracunculiasis Eradication in the remaining seven countries of Chad, Ethiopia, Mali, South Sudan, Sudan, Angola and the Democratic Republic of Congo.

Case Study

A 50-year-old male from a tribal African village presented with the chief complaints of pain in his left foot. An ulcer was found, with some portion of an emerging worm. According to the patient, the ulcer was preceded by a blister, which then broke down to form the ulcer. He was a resident of a remote village and the village people used the water from a well for drinking and other purposes. The worm was surgically removed and subsequently the ulcer healed. Examination of the worm confirmed it to be a Guinea worm.

1. What is the simplest method which has been adopted in many countries to eradicate this infection?
2. How will you proceed to find out the presence of any reservoir host in the community?
3. What is the danger of manual removal of the worm?

Research Questions

1. Are the sporadic cases of Guinea worm disease from parts of India and other countries due to the close proximity of animal reservoirs like dogs, cats and monkeys?
2. Is the consumption of raw or under-cooked fish a possible mode of infection transmission?

Further Readings

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Capillariasis

Vibhor Tak

Learning Objectives

1. To study the differentiating features of *Capillaria phillipensis* and *Capillaria hepatica*.
2. To have the knowledge that *C. phillipensis* can cause hyper infection and prove fatal.
3. To be aware of spurious infection due to *C. hepatica* and differentiate it from true infection.

Introduction

Capillariasis is a zoonotic infection caused by nematodes of the genus *Capillaria*. Nearly, 300 species of *Capillaria* are known to cause infections in various fish, amphibians, reptiles and mammals. The majority of human infections are caused by three *Capillaria* species, which includes *Capillaria phillipinensis*, *Capillaria hepatica* and *Capillaria aerophila*, causing intestinal, hepatic and pulmonary capillariasis, respectively. However, a fourth species, that is, *Capillaria plica*, has also been implicated in a few human infections.

History

There are evidences of finding *Capillaria* eggs in coprolites, that is, fossilized faeces of canines from Patagonia dating back to 6500 BC. There is also historical evidence of human infections in France during the Paleolithic and Neolithic eras and from Belgium during the sixteenth century.

The first case of human capillariasis caused by *C. phillipinensis* was reported by Chitwood in 1964 in a 29-year-old male school teacher from Northern Luzon in the Philippines. The patient presented with intractable diarrhoea, recurrent ascites and emaciation for 3 weeks before admission to the hospital and died within a week. On post-mortem examination, a huge number of worms were retrieved from the patient's intestines. However, species-level identification of these worms could not be done at that time. In 1923, a case of hepatic capillariasis was first reported from a British soldier who died in India. During the post-mortem examination, large number of *C. hepatica* eggs were observed on histopathologic examination of liver samples.

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S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
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Taxonomy

All capillarids are members of superfamily Trichelloidea and are closely related to the genera *Trichuris* and *Trichinella*.

They belong to Phylum, Nemathelminthes; Class, Aphasmida; Order, Trichocephalida; Superfamily, Trichinelloidea; Family, Capillaridae; and Genus, *Capillaria*. Genus *Capillaria* includes nearly 300 different species that cause infection in a wide range of hosts. In 1982, Moravec reclassified the Capillarids into 16 different genera, after which a lot of new genera, synonyms and reclassifications have been proposed.

Currently, *C. phillipinensis* has been reclassified as *Paracapillaria phillipinensis* and *C. hepatica* is classified as *Calodium hepaticum*. However, this new taxonomic classification is used in a limited manner and still the use of the genus name *Capillaria* is more popular. Therefore, in this chapter we would be discussing these two important pathogens *C. philippinensis* and *C. hepatica* (old nomenclature), as the human infections caused by the other two species are very rare.

Genomics and Proteomics

Molecular and phylogenetic studies done on capillarids are scarce, mostly focusing on the 18 S r DNA and cox1 targets. Borba et al. in 2019 elucidated the worldwide paleodistribution of capillarids. However, more genetic studies are required to solve the conflicts in taxonomy and to aid in the proper systematic knowledge about family Capillaridae. El-Dib et al. in 2015 have submitted the Capillaria DNA sequence to GenBank with the access number KF604920.

Parasite Morphology

C. philippinensis

Adult Worm

C. philippinensis adult worms have characteristically thin and filamentous anterior end, and a thicker and shorter posterior end (Fig. 1). Sexual dimorphism is present and females are longer than the males. Females maybe both oviparous and viviparous, and their uteri may contain thick- or thin-shelled ova and larvae. *C. philippinensis* may be considered as a bridge between the genera *Trichuris* (oviparous) and *Trichinella* (viviparous).

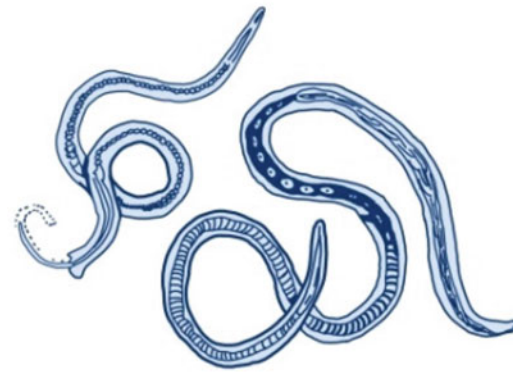

Fig. 1 Adult worms of *Capillaria philippinensis*

Adult males measure 1.5–3.9 mm in length and 3–5 μm in width at the head, 23–28 μm at the stichosome, and 18 μm at the cloaca. The male possesses a spicule, which is 230–300 μm long, and is covered by the unspined specular sheath, which may measure up to 440 μm in length. The tail has ventrolateral expansions containing two pairs of papillae. The anus is sub-terminal,

Females are much longer than the males, 2.3–5.3 mm in length, with widths of 5–8 μm at the head, 25 μm at the widest part of the stichosome, 28–36 μm at the vulva, and 29–47 μm post-vulva. The vulva is located behind the oesophagus. The uterus of the female worm may contain abundant thick-shelled eggs, thin-shelled eggs, with or without embryos, or larvae.

Eggs

C. philippinensis eggs are peanut- or barrel-shaped, with striated shells and with flattened bipolar mucoid plugs. They measure 36–45 μm in length and 20 μm in breadth.

Larvae

Larvae have rounded anterior ends and the oesophagus occupies more than three-fourth of

the entire body length. The larvae, present in the fish, are infective for humans.

C. hepatica

Adult Worm

C. hepatica adult is a slender nematode, with a narrow anterior end, and a swollen posterior part. The females measure about 53–78 mm in length and 0.11–0.20 mm in width. Males are smaller than the females and are approximately 24–37 mm in length and 0.07–0.10 mm in width. The oesophagus occupies nearly half of the female body and only one-third of the male body. The tail end of *C. hepatica* carries a copulatory spicule and sheath.

Eggs

The eggs of *C. hepatica* are similar to those of *Trichuris trichiura*, but differ in their size. *C. hepatica* eggs measure about 48–66 μm × 28–36 μm in size. They are elliptical, bi-operculated, surrounded by a double envelope. The external envelope is thinner than the internal envelope and the two separated by sagittal striations present between them. Numerous minipores are present on the outer shell. Eggs containing yellowish-white nodules are characteristic of *C. hepatica.* Eggs are the infective stage of the parasite.

Cultivation of the Parasite

Cultivation of the parasite is not a very common technique used for diagnosis of Capillarids and therefore there is not much published data regarding the culture of *Capillaria* spp. in the laboratory. But due to similarity between the life cycles of *Strongyloides stercolaris and C. phillipinensis* the cultivation of this parasite is also possible in the laboratory. *C. phillipinensis* can be cultured using Harada Mori technique and agar plate techniques.

Laboratory Animals

Various animals such as rats, Mongolian gerbils, monkeys, and so on have been used as animal models for understanding the pathogenesis and life cycle of *C. phillipinensis.* Rats with hepatic capillariasis have been used as experimental animals models of *C. hepatica* for testing drugs with antifibrotic properties such as pentoxyphylline, gadolinium chloride and vitamin A.

Life Cycle of *Capillaria phillipinensis*

Host

Definitive Hosts

Fish-eating aquatic birds are the definitive hosts. Humans are the accidental hosts.

Intermediate Hosts

Tiny fish. Besides fish, shrimps, crabs and snails may act as intermediate hosts.

Infective Stage

Larvae present in fish are the infective stage for humans.

Transmission of Infection

The natural cycle of *C. phillipinensis* consists of a bird-fish-bird cycle (Fig. 2). Fish-eating aquatic birds, and occasionally humans, acquire infection on consumption of improperly cooked fish harbouring the larval stages of the parasite. The larvae, released in the intestine, develop into sexually mature adult males and females followed by conjugation. Adult female worms are oviparous; however, certain females can be viviparous and produce the larvae directly. These larvae are responsible for autoinfection and hyper-infection in the definitive hosts. In about 4–6 weeks' time post-infection, the females start releasing the characteristic elliptical eggs in faeces. These

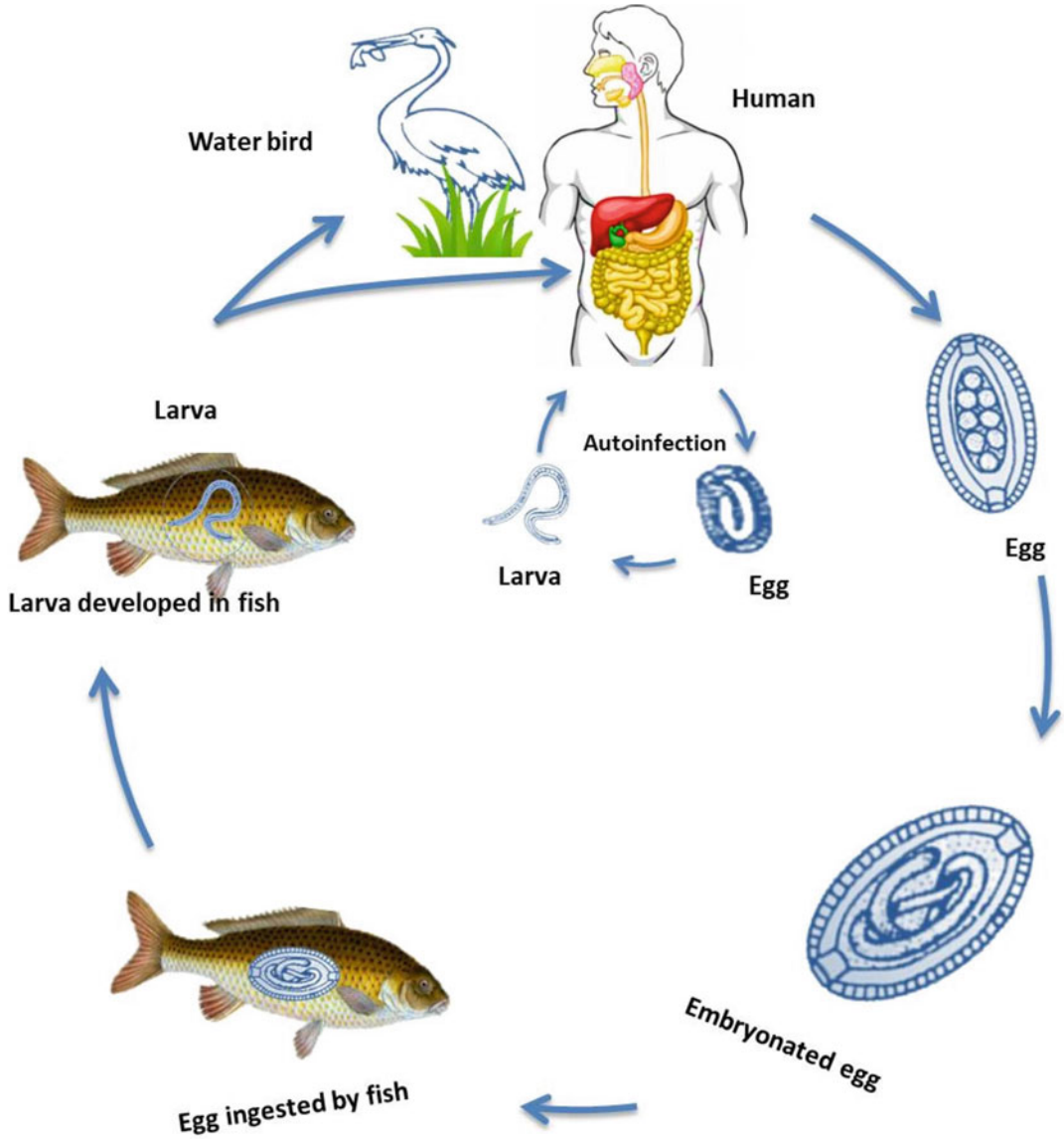

Fig. 2 Life cycle of *Capillaria phillipinensis*

eggs reach water bodies and are further consumed by fish, where they again develop into larval forms in about 3–4 weeks.

Life Cycle of *Capillaria hepatica*

Hosts

Rodents and other small mammals are natural hosts of this infection. Humans are the accidental hosts.

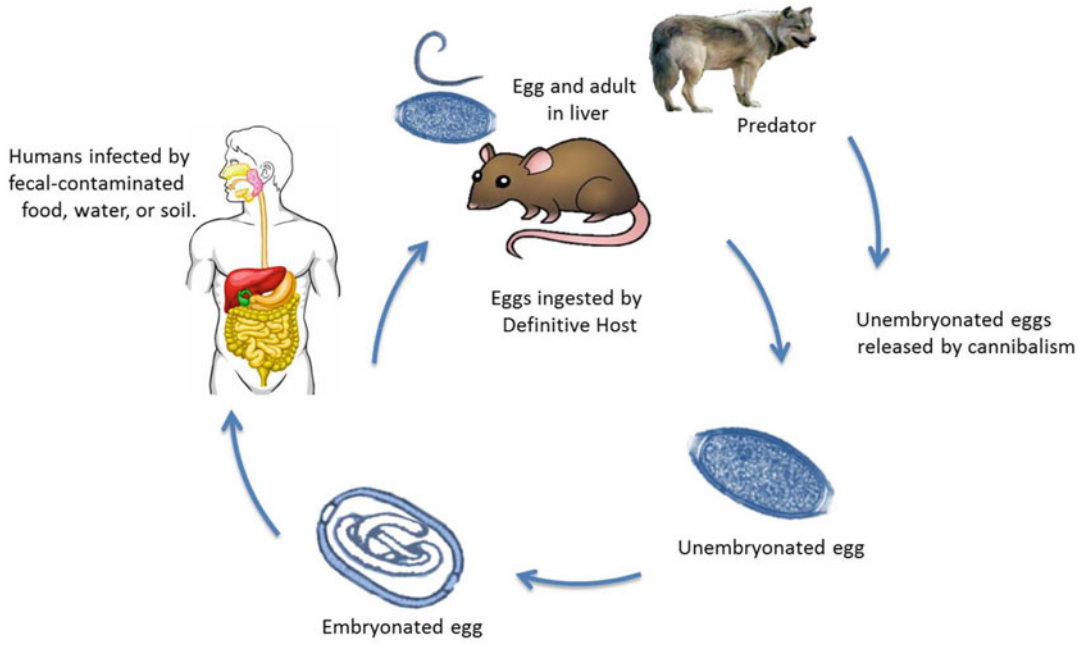

Fig. 3 Life cycle of *Capillaria hepatica*

Infective Stage

Embryonated eggs of *C. hepatica* are the infective stage of the parasite.

Transmission of Infection

C. hepatica infection is transmitted to susceptible hosts either by ingesting the food or through soil contaminated with embryonated eggs or non-embryonated eggs, which results in true hepatic and spurious infection, respectively.

C. hepatica has a monoxenic life cycle (Fig. 3). Hosts, including humans, acquire infection on ingestion of embryonated eggs from contaminated food or water. The eggs hatch in the caecum, giving rise to the larvae. These larvae penetrate the intestinal mucosa and gain entry into the portal system and thereafter migrate into the liver parenchyma. In approximately about 3–4 weeks post-infection, the larvae mature into adult worms. Adult females deposit hundreds of fertilized unembryonated eggs in the surrounding liver parenchyma. These eggs remain in the liver, and unembryonated forms are passed to the environment either on death/decomposition of the animal or by faeces of its predator or scavenger or cannibal animals. These non-infective forms embryonate in the environment and become infective in about 5–7 weeks' time.

Pathogenesis and Pathology

Adult *C. phillipinensis* reside in the small intestine, mainly in the jejunum, in large numbers. Endoscopic examination reveals non-specific inflammation of small intestinal segments associated with erythema, superficial erosions and exudates. Radiological examination of the upper gastrointestinal tract using barium meal series may show mucosal thickening and segmentation in the small intestines suggestive of malabsorption syndrome. The worms cause mechanical compression of intestinal epithelial cells, leading to compressive degeneration and production of micro-ulcers in the intestinal epithelium. The degenerative and ulcerative lesions caused by

the heavy worm burden leads to malabsorption, with loss of fluids, proteins and electrolytes.

C. hepatica larvae migrate along the wall of the large intestine. The larva has high affinity for liver and, in about 2 days, it reaches the liver via the portal vein, where it invades and resides in the sinus hepaticus. In about 18-20 days, the larvae develop into adult worms, which produce thousands of eggs in the liver parenchyma. The adult worms and numerous eggs cause foci of chronic focal inflammation with areas of necrosis and granuloma formation, containing infiltrates of eosinophils, macrophages and multinucleate giant cells. This process of persistent inflammation may lead to calcification or encapsulation and eventually septal fibrosis. It is proposed that the release of disintegrated products from encapsulated parasitic lesions in a slow and continuous manner activates the Kupffer cells. This subsequently leads to synthesis of fibrous connective tissue disproportionately, causing liver fibrosis, which may progress to cirrhosis in a relatively short time.

Immunology

Published literature on immune responses to human capillariasis is scarce. Rosenberg et al. in 1970 reported acquisition of humoral antibodies, including circulating IgE, in patients infected with *C. phillipinensis*.

Infection in Humans

C. phillipinensis in humans cause intestinal capillariasis. Patients suffering from intestinal capillariasis present with abdominal pain, borborgymi and passage of 8–10 voluminous stools per day. This may lead to dehydration, weight loss, malaise and features of malabsorption. Severe muscle wasting, abdominal distension and oedema occur due to malabsorption together with fluid and electrolyte imbalances. If the infection remains undiagnosed and untreated, it may lead to fatal outcomes due to severe hyperinfection. Death may occur due to complications like pneumonia, hypokalemia, heart failure and cerebral oedema within a few weeks to months after infection.

C. hepatica infection can be classified as true hepatic infection or spurious infection based on ingestion of embryonated or unembryonated eggs, respectively.

True infection of hepatic capillariasis presents as acute or sub-acute hepatitis along with ascites and eosinophilia. Infection is characterized by a triad of persistent fever, hepatomegaly and eosinophilia. The condition is also associated with abdominal pain, splenomegaly, kidney enlargement, anaemia and weight loss. Severe liver damage and hepatic failure can result in fatal outcomes.

Spurious *C. hepatica* infections occur on ingestion of unembryonated eggs directly from the undercooked liver of rodents. These people hardly exhibit any clinical symptoms, except passage of unembryonated eggs of *C. hepatica* in their faeces.

Infection in Animals

C. philippines infection in fish-eating birds, the natural hosts, usually does not show any particular signs. Hepatic capillariasis rarely shows any disease manifestations in infected rodents, lagomorphs and a few mammals.

Epidemiology and Public Health

Intestinal capillarisis caused by *C. phillipinensis* was first reported from the Phillipines in 1964 and since then its geographical distribution has been expanding. It has spread to many other countries, including Thailand, Japan, Indonesia, Taiwan, South Korea, India, the UAE, Iran, Egypt and so on. The infection is prevalent in areas where there is a practice of eating raw or undercooked fish and where defecation in and around water bodies is common. This helps in the sustenance of the life cycle of *C. phillipinensis*. Shrimps, crabs and snails act as intermediate hosts that facilitate transmission of infection.

Table 1 Epidemiology of *Capillaria* spp. infecting humans

Species	Distribution	Intermediate hosts	Definitive hosts
Capillaria phillipinensis	The Phillipines, Thailand, Indonesia, Taiwan, South Korea, Japan, India, Iran, the UAE, Egypt, Spain, the UK	Various small fish, crabs, snails, shrimps	Piscivorous birds, humans
Capillaria hepatica	Japan, China, India, Indonesia, Iran, Egypt, South Africa, Former Czechoslovakia, Brazil, Mexico, the USA	Life cycle can be completed in a single host. No intermediate hosts are required	Rats, rodents, small mammals, human beings are accidental hosts
Capillaria aerophila	Worldwide	Life cycle can be completed in a single host. Sometimes earthworms may act as intermediate hosts	Dogs, cats, foxes, wolves and other mammals, occasionally humans
Capillaria plica	North America, Europe, Asia and Africa	Earthworms	Dogs, cats, foxes, wolves and other mammals, occasionally humans

Till date, total of 72 cases of human hepatic capillariasis have been reported worldwide, predominantly in Japan, China, India, Indonesia, Iran, Egypt, and also in the European countries.

Rodents belonging to family Muroidea are the natural hosts of *C. hepatica*. Ninety Muroidean rodent species and other small mammals in over 60 countries of the world are found to be the natural hosts of the parasite. Globally, *Rattus norvegicus* (Norway rat) is the principal host for hepatic capillariasis. The infection is maintained in the nature by acts of cannibalism, predation and scavenging activities prevalent in the natural hosts. Human beings are the accidental hosts (Table 1).

Diagnosis

Intestinal Capillariasis

Based on clinical history and geographical location of the patient intestinal capillariasis is suspected. The diagnosis is established by a battery of laboratory tests (Table 2).

Microscopy

Demonstration of characteristic eggs of *C. phillipinensis* (Fig. 4) in stool sample either by direct wet mount examination or by using stool concentration techniques is diagnostic. Occasionally, larvae as well as adult worms may also be detected during stool examination. At times, the parasite may also be picked up from duodenal aspirates.

Serodiagnosis

An ELISA to detect *C. phillipinensis* coproantigen in faeces is currently available. An immuno-chromatographic test based on *Trichinella spiralis* larval antigen has been developed in Thailand for detecting antibodies in serum against *C. phillipinensis* with 100% sensitivity and 96.6% specificity.

Molecular Diagnosis

Molecular methods like nested PCR have been developed for diagnosis of *C. phillipinensis* targeting SSU r DNA. A specific nested PCR has been successfully for detection of *C. phillipinensis* in faecal samples.

Other Tests

Other laboratory investigations may show hypokalemia, hypoalbuminemia and microcytic

Table 2 Laboratory diagnosis of human intestinal capillariasis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Stool; duodenal aspirate	Characteristic eggs, larvae and adults	Most commonly used test *Limitation*: Eggs may be confused with *Trichuris trichiura* eggs
Serology	Sandwich ELISA	Coproantigen detection in stool	Good sensitivity but not very specific as cross-reactions are seen with other parasites like *Fasciola gigantica, Clonorchis sinnensis, Schistosoma mansoni, Toxocara canis*, and hydatid antigen. Limited geographical availability
	ICT	Antibody detection by using *Trichinella spiralis* antigen	Rapid results with 100% sensitivity *Limitations*: Cross-reactivity with trichuriasis, gnathostomiasis, angiostrongyloidiasis; not readily available
Molecular methods	Nested PCR	SSU r DNA	Highly sensitive and specific test. *Limitations*: Technically demanding; limited availability

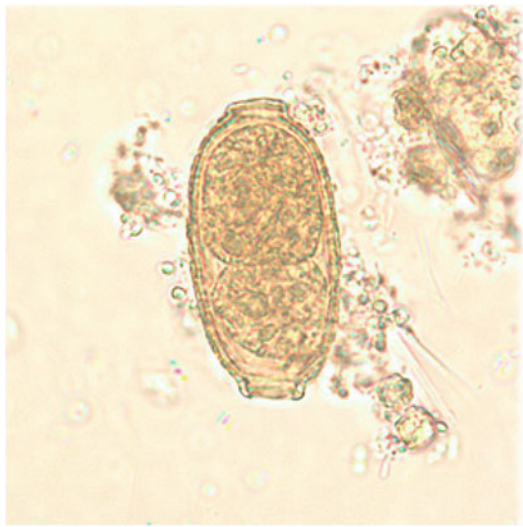

Fig. 4 Ovum of *Capillaria phillipinensis* (Courtsey: CDC)

hypochromic anaemia. There may be mild eosinophilia.

Hepatic Capillariasis

Microscopy

In suspected cases, a liver biopsy is carried out to demonstrate the characteristic eggs of *C. hepatica* (Table 3).

Larvae or adults may also be seen on histopathological examination of liver tissue (Fig. 5). In cases of spurious infections, unembryonated eggs of *C. hepatica* may be passed in faeces and may be detected during stool examination.

Serodiagnosis

Indirect immunofluorescence assay and ELISA have been developed to detect antibodies in the serum, but are restricted to a few laboratories.

Other laboratory investigations may reveal hypergammaglobulinemia and elevated levels of ALT (alanine aminotransferase), AST (Aspartate aminotransferase) and LDH (Lactate dehydrogenase).

Molecular Diagnosis

Due to advancements in the molecular techniques, diagnosis of *C. hepatica* can be done using PCR techniques. The SSU rRNA gene of *C. hepatica* has been used to amplify the nucleic acid for detection of *C. hepatica* infection in liver tissue of wild rats.

Treatment

Mebendazole given orally in a dose of 200 mg twice a day for 20–30 days; supplemented with fluid and electrolytes, is effective for treatment of intestinal capillariasis, in both children and adults.

Table 3 Laboratory diagnostic approaches of human hepatic capillariasis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Hepatic biopsy; stool	Characteristic eggs. Sometimes larvae and adult worms may also be seen	Most commonly used test *Limitation*: Eggs may be confused with *Trichuris trichiura* eggs
Serology	Indirect immunofluorescencetest (IIFT); ELISA	Antibody detection in serum	Good sensitivity and specificity *Limitations*: Not readily available
Molecular methods	PCR and sequencing	SSU r DNA	Highly sensitive and specific test *Limitations*: Technically demanding; limited availability

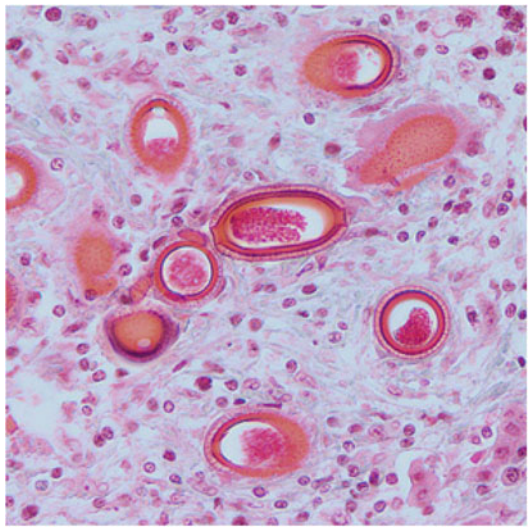

Fig. 5 Histological section of liver tissue showing characteristic eggs of *Capillaria hepatica* (Courtsey: CDC)

Alternatively, albendazole in a single dose of 400 mg or in two divided doses for 10 days is also useful. Mebendazole 200 mg given twice daily for an additional period of 30 days; or albendazole 400 mg per day for additional 20 days, is useful for treatment of relapse or recurrence. Repeat stool examination may be performed post treatment to check the efficacy of the anti-helminthic treatment.

No specific chemotherapy is available for treatment of hepatic capillariasis. Various antihelminthic agents such as albendazole, thiabendazole, mebendazole and pyrantel tartarate, in combination with corticosteroids, have been used for treatment of the condition with variable efficacies.

Prevention and Control

Health education such as avoidance of eating uncooked or improperly cooked fish, safe disposal of human faeces and improved personal hygiene prevents *C. philippinensis* infection.

In endemic regions, thorough washing or cooking of vegetables and boiling of water contribute in prevention of hepatic capillariasis. Appropriate disposal of animal carcasses is also a significant measure to prevent contamination of food and water, as well as transmission to other animal hosts.

Case Study

A 27-year-old male from Thailand was admitted to the hospital with a history of diffuse abdominal pain, borborgymi and passing 8–10 loose stools for past 3 weeks. He also gave a history of weight loss. On examination the patient looked dehydrated and severely cachectic. Blood tests revealed a 6% eosinophilia, anaemia and hypoalbuminemia. Microscopic examination of stool revealed the presence of numerous peanut- or barrel-shaped ova with striated shells and with flattened bipolar mucoid plugs, measuring 36–45 μm in length by 20 μm in breadth.

1. What are the reasons for the emergence of intestinal capillariasis in recent years?
2. Why hepatic capillariasis is difficult to diagnose?
3. What are the important differences in the morphology of the adult worms of *C. philippinensis* and *C. hepatica*?

Research Questions

1. What is the recent information on taxonomy of Capillrids?
2. What are the new developments in understanding the life cycle of *C. philippinensis*?
3. What are the newer developments in the diagnosis of hepatic capillariasis?

Further Readings

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Strongyloidiasis

Kashi Nath Prasad and Chinmoy Sahu

Learning Objectives

1. To understand the significance of strongyloidiasis as an emerging disease due to immune suppression as a result of transplants and therapy.
2. To understand the importance of autoinfection resulting in hyper-infection, which may have severe consequences.
3. To gain knowledge of pulmonary disease and sepsis due to Gram-negative bacteria.

Introduction

Strongyloidiasis is caused by the intestinal round worm belonging to the genus *Strongyloides.* There are more than 50 species in *Strongyloides* genus; however, the most common and pathogenic species is *Strongyloides stercoralis.* The parasite has a soil-to-human transmission cycle. The infection usually occurs following penetration of the skin by filariform larvae. *S. stercoralis* can cause reinfection in the same host (also called autoinfection) and can thus become a chronic disease. This occurs because a few rhabditiform larvae while passing through the bowel into the stool can transform into infective filariform larvae that penetrate the bowel mucosal layer and perianal skin. In healthy immunocompetent individuals, chronic infection is usually not apparent, while in immunocompromised individuals, it may cause hyper-infection syndrome, which is usually severe and often fatal. Differentiation between strongyloidiasis and hookworm is important, since the larvae of both these parasites enter the body through skin penetration, causing skin irritation and pruritus at the portal of entry. Strongyloidiasis is diagnosed by demonstrating rhabditiform larvae in stool rather than eggs, as in hookworm infection. Measures like personal and environmental hygiene are very important for control of the infection.

History

In 1876, there was an outbreak of diarrhoea among French troops who returned from the Indochina border. *S. stercoralis* larvae were first demonstrated in their stools, which were further identified as the intestinal nematode by the French physician Louis Alexis Normand. Life cycle of the parasite was first described by the German parasitologist Rudolf Leuckart. The mode of infection by the larvae and the

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autoinfection mechanism were described by the Belgian physician Paul Van Durme and the German parasitologist Friedrich Fülleborn, respectively. In 1940s, it was observed that strongyloidiasis-infected persons who were subsequently treated with immunosuppressive agents developed hyper-infection syndrome.

Taxonomy

The genus *Strongyloides* belongs to Phylum, Nemathelminths; Class, Secernentasida; Sub Class, Rhabditia; Order, Rhabditorida; Suborder, Rhabditina; and Family, Strongyloididae.

Two clades within the genus *Strongyloides* have been suggested based on molecular phylogenetic analysis of the parasite.

Genomics and Proteomics

Expressed sequence tag (EST) analyses have revealed different clusters in free-living L1 and infective L3 larvae of *S. stercoralis*. A total of 11,000 ESTs were reported for *S. stercoralis* and they were grouped into 3311 clusters.

The Wellcome Trust Sanger Institute is engaged in sequencing the whole genome of *Strongyloides ratti* as a reference strain. The whole-genome sequencing of *S. stercoralis* by using the shotgun method is also in the pipeline. The whole-genome sequencing would lead to the discovery of gene targets and enhance our knowledge on the biology of these parasites. This will further help in developing immunoassays and vaccines.

Transgenic methods for *S. stercoralis* and *Parastrongyloides trichosuri* (another related nematode) have been developed. This would be helpful for translational research on this parasite. Recombinant antigens-based immunoassays are in the process of development for diagnosis of *S. stercoralis* infection.

Parasite Morphology

S. stercoralis exists in both parasitic and free living forms. In the parasite form, the female worms reside in the small intestine of humans.

Adult Worm

Female Parasite

Female *S. stercoralis* measures 2500 μm in length and 40–50 μm in breadth and is transparent. The buccal cavity has four small lips. The oesophagus is cylindrical and muscular in nature, and it comprises the anterior third of the body, while the intestine comprises the posterior two-thirds. The anus is present mid-ventrally. The posterior end of the female parasite is extremely pointed, which helps to differentiate it from the male parasite. The female genital organs consist of a paired uterus, an oviduct and ovaries. The opening of the vulva is found at the meeting point of the middle and end part of the body. The females are ovo-viviparous. Each female worm gives 30–40 eggs per day in the mucosal layer of the human small intestine.

Male Parasite

Male *S. stercoralis* is shorter and broader than the female parasite. As such, these males do not have penetrating power and remain parasitic in the large intestine. Males possess spicules and gubernaculum and thus they can be further differentiated from females.

Eggs

In the gravid females, the eggs lie within their body antero-posteriorly in a single chain of 5–10 eggs. The size of the eggs is 55 μm in length and 30 μm in breadth. Eggs are oval, transparent and thin-shelled, with larvae inside, ready to hatch. Rhabditiform larvae start hatching as soon as the eggs are laid. Hatched larvae enter the lumen of the intestine and are excreted in stool.

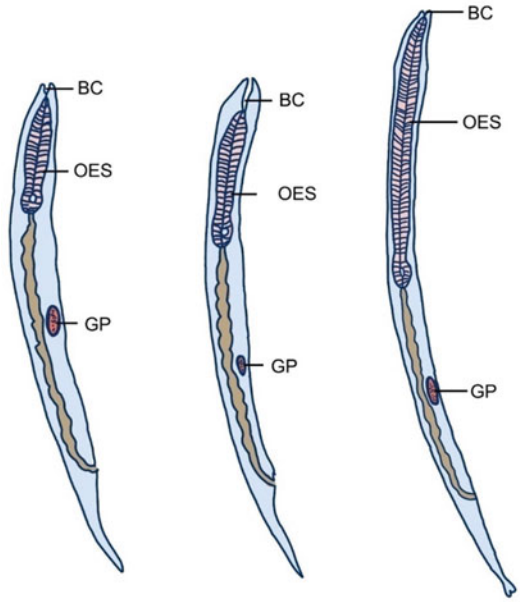

Fig. 1 (**a**) Rhabditiform larva of *Strongyloides stercoralis*, (**b**) rhabditiform larva of hookworm, (**c**) filariform larva of *S. stercoralis*. *BC* buccal cavity, *OES* oesophagus, *GP* germinal primordium

Larvae

The larvae of *S. stercoralis* are of two types:

1. **Rhabditiform larvae**
   Rhabditiform larvae (Fig. 1) are the first-stage larvae that are hatched out as soon as the eggs are laid by the gravid female and enter the intestinal lumen. They are actively motile and measure 180–380 μm in length and 14–20 μm in breadth. They possess a short buccal cavity (mouth) and a double-bulb oesophagus. They have a prominent genital primordium.
2. **Filariform larvae**
   Filariform larvae (Fig. 1) are longer and slender than rhabditiform larvae. They are 500–600 μm in length and 16 μm in breadth. They possess a short buccal cavity and a long cylindrical oesophagus with notched tail ends. Filariform larvae are the infective stage of the parasite.

Cultivation of the Parasite

Adult *Strongyloides* worms are not cultivable in vitro but they are maintained in animals such as marmosets and dogs for research purposes. Larvae are usually cultured from stool for diagnostic purpose. Culture of larvae is more sensitive than direct microscopy.

The different culture techniques are as follows:

Agar plate culture: In this method, usually 1–2 g of stool is placed in the central part of a culture plate; the plate is incubated at room temperature for 2 days and examined daily. Larvae present in the stool move away from the centre and they carry commensal stool bacteria along with them. Tracks containing bacterial colonies in sinusoidal pattern on the culture plate reveal the presence of larvae under microscopic examination. This culture technique is usually considered the most sensitive method for recovery of larvae from stool.

Harada-Mori technique: In this technique, the suspected stool specimen is smeared on a filter paper, which is then dipped in 3 ml water in a sealed 15 ml centrifuge tube. The tube is incubated in the dark for 7–10 days at room temperature. The tube is centrifuged and the deposit is collected and examined microscopically for the presence of larvae.

Modified petri dish culture: In this procedure, filter paper or watch glass is smeared with the suspected stool specimen and placed in a petri dish containing water. Larvae, if present, move out of the specimen to the fresh water in the petri dish. Larvae are demonstrated in the sediment of the centrifuged water.

Baermann technique: This is a funnel-based method where the funnel is clamped to a stand. A rubber tube is attached to the lower part of the funnel and the tube is pinch-clamped. A wire mesh with gauze padding is placed on the funnel and a reasonable amount of stool is smeared on the gauze pad and covered with water. Around 10 ml of water is collected through the pinch clamp after 2–4 h and centrifuged. The sediment is examined under microscope for the presence of larvae.

Strict precautions are required during this culture because the transformed filariform larvae can penetrate intact skin and can cause healthcare-associated infection. Sometimes, if stool specimens of hookworm-infected individuals are left for several days at room temperature, hookworm larvae may be seen in such specimens. However, they have a long buccal cavity, an inconspicuous genital primordium and pointed tails compared to filariform larvae of *S. stercoralis*, where the tails are notched (Fig. 1).

Laboratory Animals

A good animal model for *S. stercoralis* is lacking. However, subclinical infection can be established in mixed-breed laboratory dogs. Adult worms are being maintained in the intestine of such dogs in some veterinary laboratories. Dogs are infected by usually inoculating 3000 infective filariform larvae subcutaneously. Attempts to infect rats and mice with *S. stercoralis* have failed; however, infection and autoinfection have been established in gerbils, and this small animal may be a valuable model for *S. stercoralis* research. Both autoinfection and hyper-infection/disseminated infection, resembling that of humans following steroid therapy, typically occur in marmoset (*Callithrix penicillata*) when 100–500 infective filariform larvae are inoculated subcutaneously. Rat (*Rattus norvegicus*) is used as a model for *S. ratti* to study the pathophysiology of the parasite.

Life Cycle of *Strongyloides* Species

Hosts

Life cycle is completed in a single host, principally human, within 4 weeks after larval entry in the body.

Infective Stage

Filariform larva is the infective stage and rhabditiform larva is the diagnostic stage of the parasite.

Transmission of Infection

Soil contaminated with human faeces is the main source of infection. Humans acquire infection mainly through penetration of the skin by the filariform larva (infective L3), and ingestion of food and drink contaminated with larva. Humans can acquire infection less frequently through transplantation of the infected organ. Transmission of infection may also occur from mother to infant through milk (Fig. 2).

Strongyloides have two distinct life cycles, one within the human body called parasitic cycle and the other in the soil called free living or environmental cycle. The larvae enter the venous circulation following penetration of the skin. They are carried from the right side of the heart to the pulmonary capillaries through blood circulation. From, pulmonary capillaries the larvae enter the lungs, crawl up to the bronchi, trachea, larynx and epiglottis, and finally swallowed back into the intestinal tract. Here, the larvae become adult worms by moulting twice.

The females burrow deep into the small intestinal mucosal layer and lay eggs by parthenogenesis (asexual reproduction without fertilisation). The eggs are quickly hatched to produce the non-infective diagnostic stage, rhabditiform larvae. These larvae are carried to the bowel and passed with faeces. The rhabditiform larvae may then progress to the parasitic (direct) cycle or the free living (indirect) cycle.

In the parasitic autoinfective cycle, the rhabditiform larvae while passing through the intestine in stool may metamorphose into filariform larvae. These filariform larvae then penetrate the intestinal mucosa and enter venous

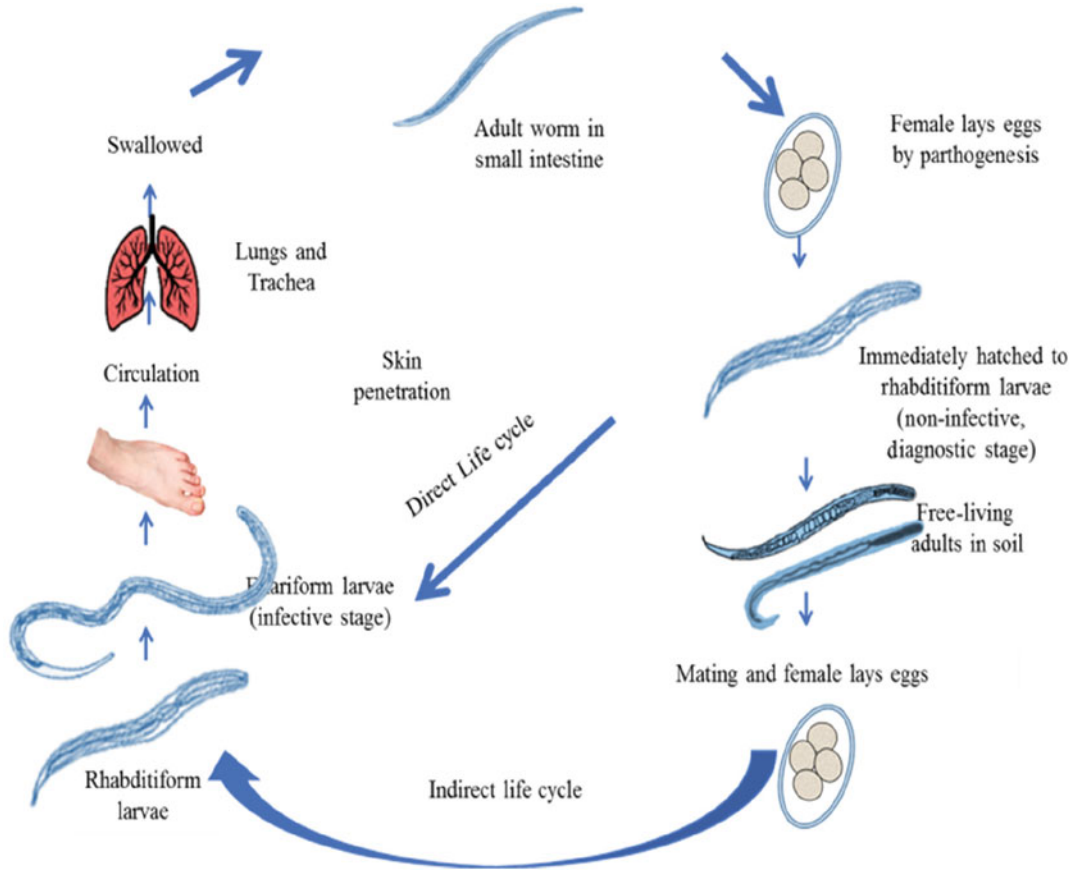

Fig. 2 Life cycle of *Strongyloides* spp.

circulation, causing internal autoinfection. The filariform larvae while being voided through stool can also penetrate the skin around the anal region, causing external autoinfection. Thus, the infection is continued by separate migratory cycles throughout the life of the infected host, if not adequately treated.

Cycle in the Environment

In the soil, the rhabditiform larvae in faeces may either develop into infective filariform larvae (direct cycle) or into free-living adults (indirect cycle). In direct cycle, the rhabditiform larva in the soil develops into the second-stage rhabditiform larva and finally into infective filariform larva by undergoing a second moulting (infective L3) within 3–4 days. The infective filariform larva then enters the human body through penetration of the skin and the cycle is repeated. In indirect cycle, the rhabditiform larvae in the soil develop to free-living adults, male and female, usually within 24–30 h. Sexual mating occurs in the soil between free-living male and female adult parasites, which results in the production of eggs. Second-batch rhabditiform larvae are hatched immediately from these eggs. In 3–4 days, these larvae are metamorphosed to

infective filariform larvae, which enter the host (human) through skin penetration.

Pathogenesis and Pathology

Adult female of *S. stercoralis* lives in the mucosal and submucosal layers of the proximal small intestine. Acute symptoms of strongyloidiasis like itching and pruritus occur at the portal of entry and during the migration of filariform larvae through the skin to the venous system. Local symptoms can also occur while the larvae migrate through the lungs and trachea to the intestine. Various symptoms during the migratory phase of the larvae occur as a result of the host immune response to the parasite. Most of the gastrointestinal symptoms are usually related to the adult worms. The female burrows deep into the intestinal mucosa and lays eggs by a process called *parthenogenesis*. Autoinfection and hyper-infection may lead to a severe form of disease.

Autoinfection

Some rhabditiform larvae are transformed to the infective filariform larvae while passing through the intestine. These transformed larvae reenter venous circulation through penetration of the bowel wall, resulting in internal autoinfection. Occasionally, filariform larvae pass into the stool, adhere to the peri-anal skin, buttock or thigh, and penetrate the skin, at the site of adherence, resulting in external autoinfection.

In immunocompromised patients, repeated autoinfection results in a heavy parasite burden and infection may persist for many decades if not treated adequately.

Hyper-infection Syndrome

Hyper-infection syndrome occurs due to dissemination of larvae in different organs like the central nervous system, skin, lungs, liver, heart and so on. These organs are not the parts of normal parasitic life cycle. Hyper-infection occurs due to reactivation of a previous asymptomatic or mildly symptomatic infection or due to acquisition of a new infection. Susceptible populations for hyper-infection syndrome are those who are on corticosteroid therapy or having impaired Th2-type immune response. However, disseminated strongyloidiasis is less common among HIV/AIDS patients even in highly endemic areas for *S. stercoralis* infection.

Immunology

The immune response to *S. stercoralis* in humans has not been well studied. Most of the facts about the immune response and protective immunity have been contributed by animal studies. Both innate and adaptive immune responses are important in conferring protection against the parasite. In cellular immunity, Th2 response plays an important role in mediating protection against disseminated infection. Strongyloidiasis elicits the production of *S. stercoralis*-specific antibodies of all isotypes. The immune response differs between HIV and human T-cell lymphotropic virus type 1 (HTLV-1) infections. In HIV infection, Th1 response is reduced, while Th2 response is usually increased or unaffected. In HTLV-1 infection, Th1 response is enhanced and Th2 response is reduced. Moreover, there is a shift in the immune response from Th2 to Th1 due to enhanced production of interferon-γ in HTLV-1. Since Th2 response is protective against helminthic infection, reduced Th2 response in HTLV-1 explains the increased cases of disseminated strongyloidiasis hyper-infection syndrome in HTLV-1.

Evolution of the immune response in humans due to strongyloidiasisis not well studied. There are some studies that suggest the rapid production of parasite-specific antibodies like IgE, IgG1, IgG2 and IgG3 followed by a rise in parasite-specific IgG4. IgG4 antibodies are thought to block IgE-mediated effector responses, resulting in a shift to Th2-mediated inflammatory response.

There is a predominance of Th2-associated immune response with increased levels of anti-inflammatory cytokines due to the expansion of Th2/T9 cells and IL-10-mediated suppression of Th1/Th17 cells. This usually occurs after

6–8 weeks of infection. Almost all cytokine levels and cellular disarray return to normal within 12 months if adequately treated.

Infection in Humans

Acute Strongyloidiasis

Clinical features of acute strongyloidiasis are related to the migration of larvae from the site of skin penetration to the small intestine. The larvae incite irritation or localised urticaria at the site of skin penetration. Sometimes, dry cough or throat irritation can occur. Anorexia, abdominal discomfort and loose motion may also occur due to infection of the small intestine.

Chronic Strongyloidiasis

Chronic infection with this parasite is usually clinically silent and the majority may only present with eosinophilia. In symptomatic cases, patients usually complain of various gastrointestinal symptoms like loose motion, abdominal discomfort and intermittent vomiting. They may also present with skin manifestations such as recurrent urticaria along the lower trunk, thighs and buttocks. These skin manifestations are due to the inflammatory response to the migrating larva called *larva currens*. Sometimes, unusual clinical manifestations involving various extra-intestinal internal organs can be seen.

Hyper-infection Syndrome and Disseminated Infection

Hyper-infection is defined as augmented autoinfection because of the immunosuppression status of the host. The diagnostic parameters differentiating autoinfection and hyper-infection are not well characterized. Hyper-infection usually denotes the signs and symptoms related to augmented migration of larvae. Exacerbations of pulmonary and gastrointestinal symptoms are common. In disseminated strongyloidiasis and a hyper-infection, organs other than those of normal parasitic pathways are also affected, and sometimes involvement of such organs may be fatal. In non-hyper-infection, though there may be enhanced larval migration, but the organs of normal larval pathways such as gastrointestinal and pulmonary are involved. The migrating larvae may carry enteric bacteria along with them and cause bacteraemia or septicaemia in both autoinfection and hyper-infection.

There is a wide range of clinical features of *S. stercoralis* hyper-infection that depend on the organs involved and the immune status of the patient. The frequent symptoms are chills and fever. Other symptoms such as fatigue, weakness and body ache are also reported. Usually, eosinophil count is increased in hyper-infection and it carries better prognosis than those cases with normal eosinophil count.

Gastrointestinal Manifestations

Abdominal pain, abdominal fullness, constipation, diarrhea, nausea and so on are non-specific gastrointestinal symptoms. In severe cases, mucositis, ulceration and oedema can be found throughout the intestine, leading to gastrointestinal bleeding. In such cases, direct stool examination usually reveals many rhabditiform larvae along with occasional filariform larvae. Larvae may be also seen in intestinal ulcer biopsies.

Cardiopulmonary Manifestations

Cardiopulmonary symptoms include cough, palpitations, atrial fibrillation, chest pain, dyspnoea, haemoptysis, hoarseness of voice and, rarely, respiratory failure. Sometimes, larvae can be demonstrated in sputum.

Sepsis

The filariform larva induces ulcers in the small intestine. The gut flora can gain access through these ulcers or can travel along with the larvae to

systemic circulation, causing bacteraemia and sepsis. Sepsis can be severe and sometimes fatal in immunocompromised patients. *Enterococcus* and *Streptococcus* spp., members belonging to the family *Enterobacteriaceae* and *Pseudomonadacae,* coagulase-negative staphylococci, *Streptococcus pneumoniae* and *Candida* spp. are the most commonly involved organisms.

Central Nervous System Manifestations

The most common CNS manifestations in hyper-infection syndrome are due to meningeal inflammation by the migrating larvae, resembling mostly as aseptic meningitis. Features of aseptic meningitis such as normal glucose, elevated protein and pleocytosis in the cerebrospinal fluid (CSF) are observed. Occasionally, features of Gram-negative meningitis can also occur with negative bacterial culture results. Larvae can be demonstrated in CSF, meningeal vessels and spaces.

Strongyloides stercoralis Infection in Human Immunodeficiency Virus (HIV)

Earlier, strongyloidiasis was considered as acquired immune deficiency syndrome (AIDS) defining disease. Subsequent studies failed to establish the direct role of CD4 cell depletion and disseminated strongyloidiasis because of unaffected Th2 response. Effective treatment by highly active antiretoviral therapy (HAART) has decreased the clinical manifestations of AIDS and associated strongyloidiasis.

Strongyloides stercoralis Infection in the Transplanted Patient

All types of transplantations, including solid organ transplants and haematopoietic stem cell transplants (HSCTs), along with their pre-conditioning and subsequent immunosuppressive therapy, pose a significant risk for the development of disseminated strongyloidiasis. HSCT has the highest incidence of dissemination, graft vs. host disease and mortality compared to other transplants.

Infection in Animals

There are more than 50 species in the genus *Strongyloides* infecting wide spectra of mammals, birds, reptiles and amphibians. So far, only two species *S. stercoralis* and, rarely, *S. fuelleborni* are known to infect humans. Important *Strongyloides* species and their final hosts are shown in Table 1.

S. stercoralis is considered zoonotic. Dogs are the potential source of zoonosis, and rarely cats can also serve as source of zoonosis. *S. stercoralis* is reported in dogs throughout the world. Infections in dogs and cats are either asymptomatic or mildly symptomatic. High parasite load with severe diarrhoea and extensive skin lesions and bronchopneumonia with fatal outcome in dogs has been reported. Hyper-infection in calves due to *S. papillosus* has been reported from Japan. Calves can develop severe bronchopneumonia and outcomes are usually fatal.

Epidemiology and Public Health

Strongyloidiasis is categorised as an emerging infectious disease and it is prevalent throughout the world. The disease is increasingly being reported in Europe, Southeast Asia, African and Caribbean countries (Table 1). It is estimated that, nearly, 30–100 million people are infected with *S. stercoralis* worldwide. In specific areas of certain endemic countries, the infection rate is as high as 10%. The prevalence of strongyloidiasis was 3.2% in a community-based survey from North India.

Lack of awareness, poor hygiene and sanitation, and migration from highly endemic areas are the factors that contribute to the increased prevalence of the disease. Further, introduction of new treatment modalities like solid organ and haematopoietic transplants, increased use of

Table 1 Epidemiological aspects of *Strongyloides* species

Parasite species	Hosts	Geographical distribution
Strongyloides stercoralis	Humans, dogs, non-human primates, wild canids (cats)	Endemic in Southeast Asian, African and South American countries. Frequently reported from Europe, Australia and the USA
Strongyloides fuelleborni	Monkeys, humans	Africa, Asia
Strongyloides felis	Cats	Worldwide
Strongyloides tumefaciens	Cats	Worldwide
Strongyloides papillosus	Cattle, sheep/lamb, goat, rabbits	Worldwide
Strongyloides westeri	Horse and other equines	Worldwide
Strongyloides ransomi	Pigs, wild boars	Worldwide
Strongyloides planiceps	Cats, wild canids, weasles	Worldwide
Strongyloides ratti	Rats (*Rattus norvegicus*)	Worldwide
Strongyloides venezuelensis	Rats (*Rattus norvegicus*)	Worldwide

chemotherapy, anticancer and immunosuppressive drugs and so on favour the occurrence of strongyloidiasis.

S. fuelleborni, a non-human nematode of primates found in African monkeys, rarely infects humans in specific geographical locations in western and eastern Africa. Another species resembling *S. fuelleborni* has been reported from Papua New Guinea. *S. fuelleborni* is an important cause of protein-losing enteropathy in humans and *swollen belly syndrome* due to abdominal distension in infants. However, abdominal pain and diarrhoea remain the major clinical manifestations. The eggs of *S. fuelleborni* morphologically resemble the eggs of hookworms. Unlike *S. stercoralis*, the eggs of *S. fuelleborni* are excreted in large numbers in faeces of infected humans. Demonstration of eggs in the stool is an important diagnostic criterion of *S. fuelleborni* infection. However, treatment of *S. stercoralis* and *S. fuelleborni* remains the same.

Diagnosis

Diagnosis of strongyloidiasis remains a challenge in asymptomatic chronically infected persons, Sometimes, eosinophilia is the only diagnostic feature, but it is a non-specific sign and may occur intermittently. Moreover, patients on immunosuppression drugs may not show eosinophilia. However, in hyper-infection and disseminated strongyloidiasis, large numbers of larvae are found in stool or body fluids such as CSF and, pleural and bronchoalveolar fluids, making the diagnosis easy.

The following methods are used for diagnosis of strongyloidiasis (Table 2).

Microscopy

Demonstrations of larvae in the stool by microscopy is the definitive diagnosis of strongyloidiasis (Fig. 3). The sensitivity of microscopy is usually low in chronically infected asymptomatic and mildly symptomatic persons due to intermittent excretion of larvae in small numbers in faeces. Hence, multiple stool samples in such patients need to be examined over a period of several days. The number of larvae excreted in stool correlates with disease severity. Large numbers of larvae are passed in stool in patients with hyper-infection syndrome/disseminated strongyloidiasis. Larvae can be demonstrated in the duodenal aspirate collected during endoscopy. It is thought to be more sensitive than stool examination. But endoscopy being an invasive procedure, it is not a preferred method. Adult female worms along with eosinophilic infiltration in lamina propria and duodenal crypts are also demonstrated on histopathology.

Table 2 Diagnostic methods of strongyloidiasis

Diagnostic approach	Method	Target	Remarks
Microscopy	Wet mount of stool, duodenal aspirate	Larva, rarely eggs/ adult	Multiple stool examinations may be needed. Duodenal aspirate more sensitive
In vitro cultivation	Coproculture by agar plate/ Harada-Mori/Baermann techniques	Recovery of larva	More sensitive than microscopy
Immunodiagnosis	Antigen detection in stool	Rabbit polyclonal antibody against excretory/secretory antigen	Mainly used in research settings
	Antibody detection (IgG) by ELISA, luciferase immunoprecipitation systems	Somatic antigens; recombinant antigens 32 kD NIE and SsIR	Cross-reactivity, lower sensitivity in immunocompromised patients, cannot differentiate active from past infections
Molecular diagnosis	PCR, LAMP	18S rRNA, IST1, cytochrome *c* oxidase subunit 1	Can identify active infection

In Vitro Culture

Demonstration of larvae in coproculture is a more sensitive method for diagnosis of strongyloidiasis. Coproculture is recommended in all suspected strongyloidiasis patients, especially those from endemic regions and patients with organ transplant and on immunosuppressive therapy. Stool specimens are transported at normal atmospheric temperature without any preservative. Different coproculture techniques like agar plate culture, Harada-Mori filter paper, modified petri dish culture and Baermann techniques, as described earlier in the chapter, are frequently used for larval recovery.

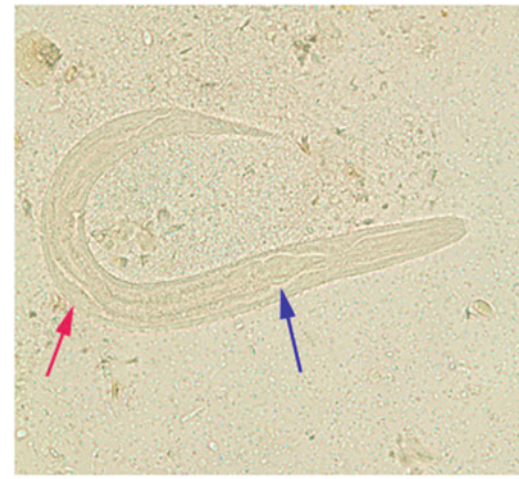

Fig. 3 Direct microscopy of stool showing rhabtidiform larva of *Strongyloides stercoralis* under 400× magnification. The oesophageal bulb (*blue arrow*) and prominent genital primordium (*red arrow*) can be seen (Courtesy: CDC)

Serodiagnosis

Antibody detection: A number of immunoassays/enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of antibody as an adjunct for other diagnostic modalities like microscopy and eosinophil count. Besides improving the sensitivity, the absence of antibody rules out strongyloidiasis. Results of antibody-based ELISAs need to be interpreted with caution, as they have several shortcomings like cross-reactivity with filarial infections, lower sensitivity in patients with haematologic malignancies or HTLV-1 infection due to low antibody titre and their inability to differentiate between current and past infection. Also the current immunoassays use crude larval antigens of *S. stercoralis* or *S. ratti*, which also limits the sensitivity and specificity of the tests. To overcome these limitations, *S. stercoralis*-specific recombinant-antigen-based immunoassays

are being developed. Use of a number of immunoassay formats such as luciferase immunoprecipitation systems and diffraction-based biosensors is likely to improve the diagnostic accuracy.

Antigen detection: ELISA is frequently used to detect coproantigen in stool specimens. Although several capture ELISAs have been developed for *S. stercoralis* coproantigen detection, they are yet to be used routinely, but mostly used for research purpose.

Molecular Diagnosis

Polymerase chain reaction (PCR) and loop-mediated isothermal amplification assays are being increasingly used in stool samples for diagnosis of strongyloidiasis. They show a high degree of specificity and sensitivity. To increase the specificity, DNA targets like 18S rRNA, IST1 and cytochrome *c* oxidase subunit 1 are being used. Better methods for DNA extraction from stool have also improved the sensitivity of these molecular tests. These methods are also used as prognostic markers to monitor treatment, as they become negative after successful therapy.

Treatment

Although *S. stercoralis* infection is asymptomatic in the majority of patients, treatment is required in proven cases. It not only clears the parasite but also prevents complications like autoinfection, hyper-infection and disseminated infection.

Oral ivermectin (200 μg per kg for 2 days) is the drug of choice for uncomplicated *S. stercoralis* infection. It is both adulticidal and larvicidal. The common anthelminthic drug albendazole is not as effective as ivermectin, since it only targets the adult worm. But it can be used as a second choice or alternative therapy. It is prescribed at 400 mg twice a day for 3–7 days. It is not recommended in first trimester of pregnancy and children below 12 months of age.

Hyper-infection syndrome should be considered as a potential medical emergency. It requires prompt therapy, since delay in treatment may have fatal outcome. Ivermectin is the treatment of choice for a minimum period of 2 weeks. Patients on immunosuppressive therapy may require balanced reduction of immunosuppression drugs based on indication. In some cases, a combination therapy with ivermectin and albendazole has produced better results but case-control studies are lacking.

Prevention and Control

Estimation of disease burden in endemic areas through regular surveillance is the key to develop preventive measures. As a policy matter, all travellers returning from endemic areas should be routinely examined for *S. stercoralis* infection. Organ donors and patients undergoing organ transplant, patients receiving chemotherapy, chemotherapeutic and immunosuppressive agents, including steroids, need to be regularly monitored for *S. stercoralis* infection. Overall economic growth and improvement in environmental health help in the prevention of the disease.

The following measures may help in reducing the disease burden in endemic areas: (a) proper disposal of human faeces, (b) reliable water supply, (c) use of toilets instead of open-field defaecation, (d) practice of hygienic habits like footwear use in endemic areas, (e) monitoring for strongyloidiasis before and after organ transplants, chemotherapy and immunosuppressive therapy and (f) regular examination of pet animals, especially dogs and adequate treatment of infected pets.

Case Study

A 30-year-old female patient (body weight 45 kg), known case of systemic lupus erythematous, was on prednisolone (0.5 mg/kg/day) for 6 months. She complained of abdominal pain and diarrhoea (loose stools, 5–6 episodes/day), anorexia, vomiting and mild fever for 5 days.

She was non-diabetic, non-hypertensive. She lived in a village and often worked in the field barefooted.

On examination, her vital parameters were normal. She was mildly dehydrated, had pallor and bilateral pedal oedema. Investigations revealed haemoglobin- 7.0 g/dL, total leucocyte count- 4200/μL (neutrophil: 67%, lymphocyte: 27%, eosinophil: 05%, monocyte: 01%), ESR: 70 mm/1st h, S. creatinine: 2 mg/dL, S. protein: 5.2 g/dL and S. albumin: 2. 9 g/dL. Urine and blood culture yielded no growth. Stool microscopy revealed the presence of many actively motile rhabditiform larvae of *S. stercoralis* measuring 200 μm in length and 20 μm in breadth with a short mouth and a double-bulb oesophagus. Based on morphology, they were identified as rhabditiform larvae of *S. stercoralis.* No other abnormality was detected. Stool culture did not reveal enteropathogenic bacteria.

The patient was treated with oral ivermectin 9 mg/day for 2 days followed by albendazole 400 mg twice for 7 days. Prednisolone was tapered to 0.3 mg/kg/day. Subsequent stool microscopy was negative for larvae after 1 and 2 weeks. The patient is currently asymptomatic and on follow-up. It is possible that she was an asymptomatic carrier before prednisolone therapy or she acquired infection during the therapy. Prednisolone therapy led to an increased larval load and precipitated symptoms.

1. Why hyper-infection syndrome is common in certain immunocompromised conditions?
2. Why serology is not very useful in the diagnosis of strongyloidiasis in HIV-infected patients?
3. Apart from improving the sanitary conditions, what additional measures may be needed to control strongyloidiasis in the community?

Research Questions

1. What is the exact pathogenicity and immune response in human hosts in strongyloidiasis?
2. Which protective antigen(s) of *Strongyloides* spp. can be used to develop effective vaccines?
3. Which small animal model is good for translational research in strongyloidiasis?
4. What point-of-care test will be ideal for rapid diagnosis?

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Ancylostomiasis

Utpala Devi

Learning Objectives

1. To learn about the importance of zoonotic hookworm infections caused by various species of *Ancylostoma* and its clinical significance.
2. To study the limitations of diagnostic modalities available for diagnosis of zoonotic ancylostomiasis.

Introduction

Hookworms that infect animals can be transmitted to humans when their eggs are shed in the environment and their larvae penetrate unprotected skin in humans. The hookworm species that are known to have zoonotic potential are *Ancylostoma brazilense*, *Ancylostoma caninum*, *Ancylostoma ceylanicum* and *Uncinaria stenocephala*. *A. brazilense*, *A. caninum* and *Uncinaria stenocephala* cause cutaneous larva migrans (CLM) in humans. *Bunostomum phlebotomum* and *Ancylostoma tubaeforme*, which are parasites of calves and cats, respectively, are also known to infect humans. The larvae of *A. caninum* cause eosinophilic enteritis in humans. People can get infected while walking barefoot or sitting on contaminated soil. Infection of humans may also occur by ingestion of the infective form. A. *ceylanicum* and *A. caninum* infections may also be acquired by oral ingestion. Cutaneous larva migrans is diagnosed clinically based on the signs and symptoms and history of exposure to zoonotic hookworms. Rarely, zoonotic hookworms may infect humans, causing diarrhoea, discomfort and abdominal pain. Measures focussing on health education, using coverings to prevent direct contact with contaminated soil, regular deworming of pets/animals, and proper and prompt disposal of animal faeces are important to prevent infection.

History

A. braziliense was first described by Gomes de Faria in 1910. *A. ceylanicum* was described by Arthur Loossin in 1941. Initially, it was thought to be synonymous with *A. braziliense*, but later studies concluded that they were distinct species. Lee for the first time described a creeping eruption on the skin of a patient in the year 1874, and about 50 years later, Kirby-Smith and colleagues discovered a nematode larva in a skin biopsy sample of such an eruption.

Utpala Devi, (deceased), Professor Devi wrote this book while at ICMR-RMRC, Dibrugarh, India

S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
https://doi.org/10.1007/978-981-16-7204-0_44

Taxonomy

The genera *Ancylostoma* and *Uncinaria* belongs to Family, Ancylostomatidae; Order, Strongylida; Class, Secernentea; and Phylum: Nematoda.

A. caninum, *A. braziliense, A. ceylanicum* and *U. stenocephala* are the species that cause zoonotic infection in humans.

Genomics and Proteomics

The knowledge of the genes that are expressed during hookworm infection contributes towards the development of new drugs or vaccines against the same. The study of the mitochondrial genomes is suggested to be an important source of population genetic markers for epidemiological studies of hookworm. The mitochondrial genome of *A. caninum* is 13,717 bp in size, and contains 12 proteins, coding 22 transfer RNA and 2 ribosomal RNA genes, representing a rich source of population genetic markers.

A. ceylanicum genome sequence is of 313 Mb, with transcriptomic data throughout infection showing the expression of 30,738 genes. Approximately, 900 genes are up-regulated during early infection in vivo and those down-regulated included ion channels and G-protein-coupled receptors.

Parasite Morphology

Adult Worm

Adult *Ancylostoma* worms have a dorsally curved anterior end and possess a buccal capsule which is lined by teeth. The body is covered with cuticle. The alimentary canal is composed of the oesophagus, intestine and rectum. The oesophagus is muscular and stout. Males have a copulatory bursa, with rays on it, which helps to distinguish between the different species of *Ancylostoma*. *Ancylostoma* females are difficult to be differentiated based on their morphological appearance. In all the species female adults are larger than the male adult worms (Fig. 1).

A. caninum: The female adult worm measures about 14–16 mm in length and 0.5 mm in width, while the male measures about 10–12 mm in length and 0.36 mm in width. The teeth of *A. caninum* are located inside the buccal capsule and arranged in three sets, two prominent ventral sets which form the lower jaw and one less prominent dorsal set forming the upper jaw. The adult females have the vulva at the junction between the final two-third and one-third of the body. The

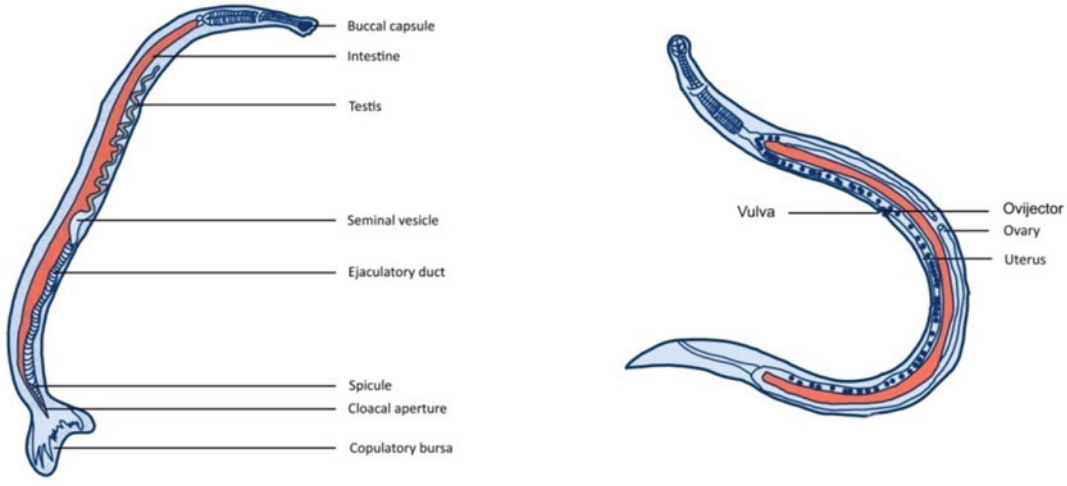

Fig. 1 Adult male and female of *Ancylostoma* spp.

copulatory bursa in males has spine like spicules located on three muscular rays.

A. brasiliense: Males have a tubercular process in the buccal capsule of the mouth. The copulatory bursa in males possesses a pair of lateral lobes and single dorsal lobe, which have rays on them.

A. ceylanicum: Adults are about 6–10 mm in length. The mouth of the adult worm has a cutting plate with a sharp dorsal end and a less distinct sharp ventral end, which is not seen among the other hookworm species.

U. stenocephala: Adults measure 10–20 mm in length and 0.4–0.5 mm in width. They are equipped with a large, dorsally curved, ventral buccal capsule which contains cutting plates. A single pair of small teeth is found within the ventral capsule. The copulatory bursa of males has a single dorsal and a pair of lateral lobes. Long and thin spicules are attached to the bursa.

Eggs

A caninum eggs are 38–43 μm in width and have a thin wall (Fig. 2). *U. stenocephala* eggs are about 71–93 μm × 35–58 μm in size. All the species have eggs of similar appearance.

Larvae

Ancylostoa has four larval stages. The first and second larval stages are free-living rhabditiform, with a tapered buccal cavity and a flask-shaped oesophagus. The third-stage larva is the infective filariform larva and measures 0.4–0.6 mm in length. It is the non-feeding form of the parasite, with a closed-mouthed, prolonged oesophagus possessing a dorsal bulb and a pointed non-forked tail (Fig. 3). The fourth-stage larvae are found in host tissue.

Cultivation of the Parasite

The larval stage of the parasite can be hatched from the egg in stool specimens by a number of techniques. These include the following:

Baermann technique: In this method, about 5–10 g of stool is placed in the centre of a double-layered cheesecloth, which is then suspended on a piece of wire gauze. Two layers of cotton gauze are spread in a plastic glass funnel that is attached with rubber tubing and the later consist of a pinch of clamp attached at the bottom. The glass funnel is filled with warm water and is left to stand for 2 h. After 2 h of incubation, the clamp is opened to collect 10 mL of the liquid and is centrifuged. A drop of the

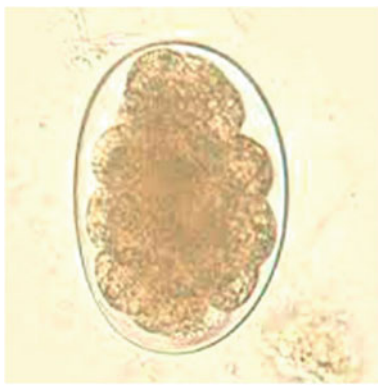

Fig. 2 Ovum of *Ancylostoma caninum*

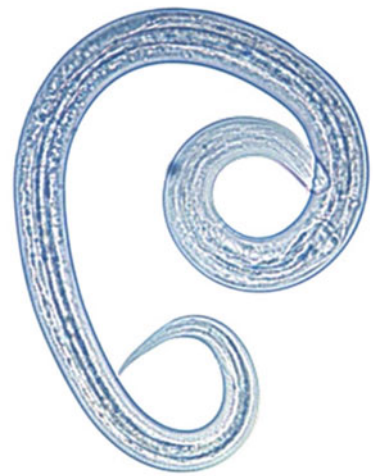

Fig. 3 Infective larval form of *Ancylostoma*

centrifuged deposit sediment is smeared on a glass slide and observed for larvae by microscopy.

Harada-Mori technique: In this technique, a strip of narrow filter paper with slightly tapered ends is taken to which the fresh faeces is added in the centre and placed in a centrifuge tube containing about 4 mL of distilled water. The water level is maintained slightly below the faecal spot. The tube is then screw-capped and kept in an upright position, incubated at 25–28 °C for up to 10 days and daily observed for the water level. After 10 days of incubation, a drop of fluid is put on a glass slide and a smear is then prepared and examined under microscope for the presence of infective third-stage motile larvae.

Agar plate method: This method has been used for culturing infectious larvae of *A. ceylanicum* which showed a better yield than the Harada-Mori or Baermann technique.

A. caninum larva has also been cultured using a solid medium, consisting of beef extract (3 g), peptone (10 g), sodium chloride (5 g), agar (20 g) and distilled water with filter paper.

Laboratory Animals

Golden Syrian hamsters *(Mesocricetus auratus)* have been used as a laboratory animal for *A. ceylanicum* for vaccine and drug studies. The beagle (*Canis lupus familiaris*) has been used to study the pathophysiology, vaccine efficacy and immunological parameters for *A. caninum*.

Life Cycle of *Ancylostoma* spp. (Fig. 4)

Hosts

Cats, dogs and foxes are the definitive hosts. Humans are the accidental hosts. Rodents may act as paratenic or transport hosts.

Infective Stage

The third-stage larvae, known as filariform larvae, are the infective stage of the parasite for humans.

Transmission of Infection

Ancylostoma infection is transmitted by the infective larvae penetrating through the skin. In addition to this, *A. ceylanicum* and *A. caninum* infections may also be acquired by oral ingestion.

The eggs are excreted in stool by the adult females residing in cats or dogs. These eggs contaminate the soil and environment. Under favourable conditions the larvae hatch out from these eggs in 1–2 days and develop to free-living larvae in the soil. The first-stage larval form is known as the rhabditiform larva, which is not infective. The rhabditiform larva matures in the soil or faeces by feeding on faecal bacteria or other organic matter and after two moults is transformed into the third stage filariform larva of the parasite. This larva is the infective form and has the capacity to survive in the environment for about 3–4 weeks under favourable conditions. The filariform larvae infect the definitive host by penetration of the skin. When they come in contact with the host skin, they penetrate and enter the blood vessels, and moving towards the heart. They subsequently enter the lungs, and after perforating the alveoli, they ascend the bronchioles and gain entry into the pharynx, before being engulfed into the small intestine. In the duodenum and jejunum, they mature into the fourth-stage larva consisting of the buccal capsule, which helps them to feed on intestinal mucosa and blood. The buccal capsule is temporary; it is shed when this larval stage moults finally to become the adult. After 6–8 weeks of penetrating the skin, the worm reaches its maturity and eggs start appearing in stool.

In case of human infections caused by *A. caninum*, *A. braziliense*, *A. ceylanicum, A. tubaeforme, B. phlebotomum* and *U. stenocephala*, filariform larvae penetrate the skin, usually up to the dermis, but do not enter the circulation as observed in their canine host. Thus, in infected humans, the pathology is limited to the skin.

A. ceylanicum and *A. caninum* cause infections in humans through the oral ingestion of eggs. Their infective forms do not migrate through the lungs and instead settle in the intestine.

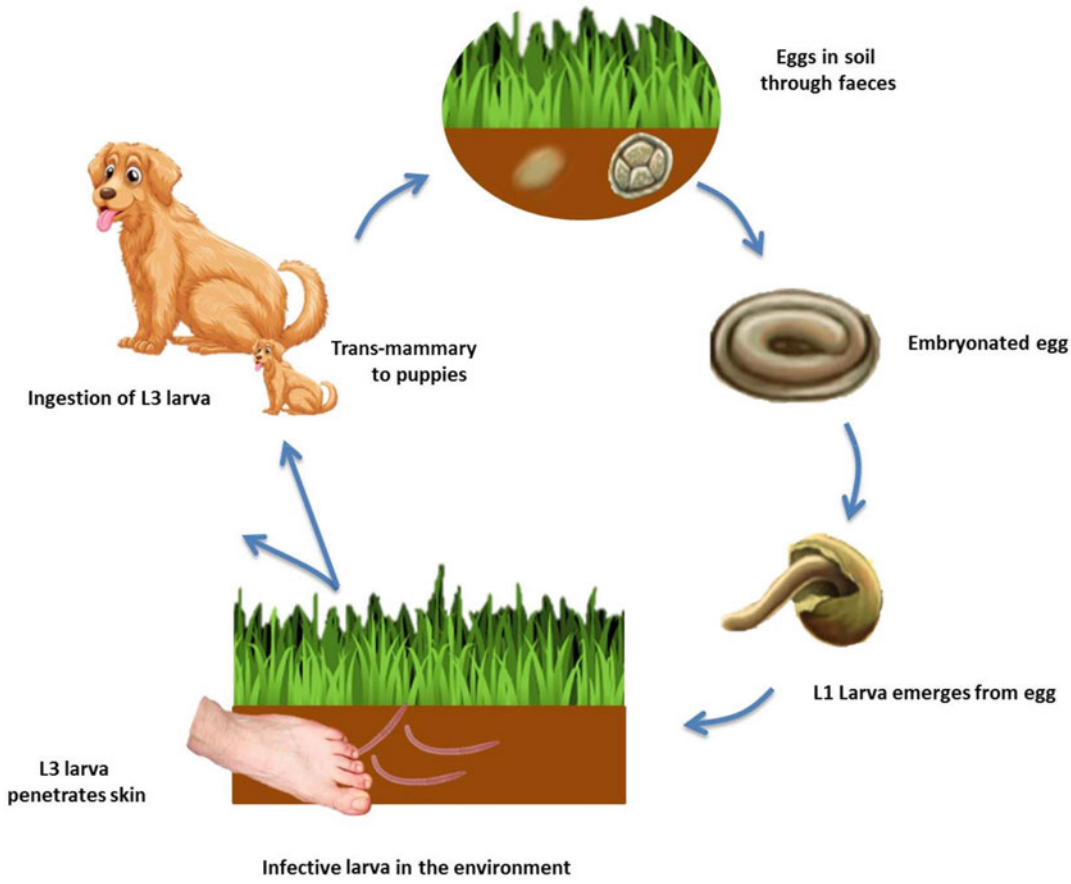

Fig. 4 Life cycle of *Ancylostoma caninum*

Pathogenesis and Pathology

Infections by zoonotic *Ancylostoma* species in humans lead to a skin condition called cutaneous larva migrans, or CLM.

The hookworm larva creeps into the skin of the host and thus its movement underneath the skin results in psoriasis. The larva penetrates into the stratum corneum layer of the epidermis. The penetration of the skin is aided by the excretion of protease and hyaluronidase.

Sometimes in rare cases, animal hookworms such as *A. ceylanicum* and *A. caninum* may reach the intestine usually as single worm and cause painful abdominal disorder called eosinophilic enteritis. *A. ceylanicum* causes patent intestinal infection in humans, while *A. caninum* is not known to cause any such patent infection.

Immunology

Antibody responses are seen against both the larval and adult hookworms when they enter the human host. The entry and migration of the worm are aided by the secretion of various enzymes and antigens. The larvae enter the host tissue by secretion of enzymes and, sometimes, they perish at this stage and release a number of immunoreactive molecules. Once the larva enters the circulation, it interacts with the immune system in the pulmonary capillaries and the gut.

In human infections, the cellular immune response against zoonotic hookworms is characterised by eosinophilic infiltration at the site of entry of the larva. It usually depends on the stage and intensity of infection as well as on various host factors. Most of the canine

hookworm L3 larvae that penetrate the human skin settle in the tissues at this site, from where individual L3 larvae mobilize to move into the gut in sporadic cases. T-helper type 2 response is predominant for both the larval and the adult stages of the parasite in the human as well as in experimental animal models. There is an increased production of IgE along with mast cell activation and elevated eosinophil levels in the tissue and blood. The features are similar to type 1 hypersensitivity reaction.

Eosinophilic enteritis in *A. caninum* infections is characterised by eosinophilic inflammation of all the layers of the gut. It has been observed that even a single worm of *A. caninum* could cause severe eosinophilic enteritis. It has been observed that pulmonary involvement is seen only with a high number of invasive L3 larvae of *A. caninum*, whereas in case of *A. brasiliense,* it is seen even with a few invading larvae.

Infection in Humans

Cutaneous larva migrans, a skin eruption, is most commonly seen in cases of human infections. The lesion is usually seen as papules, vesicles or snake-like elevated erythematous lesions on legs, buttocks or hands. These lesions are very itchy, but sometimes maybe associated with pain. These lesions progress daily from several millimetres to a few centimetres. These lesions usually subside by themselves within a few days to weeks, but sometimes may last for up to a year. The hookworms of dogs are associated with cutaneous larva migrans (CLM). *B. phlebotomum* is known to cause short-lived CLM in humans. Sometimes, the larva may migrate to the muscles, causing myositis.

In chronic Ancylostoma infections, the signs and symptoms in humans are mainly due to anaemia and hypoalbuminaemia. *Ancylostoma*, which causes intestinal infections also causes eosinophilic enteritis. The condition is manifested as increasingly severe episodes of abdominal pain associated with peripheral eosinophilia, but without any blood loss.

Infection in Animals

Depending upon the worm burden, Ancylostoma infections in animals may remain asymptomatic or turn symptomatic. During the early stages of larval migration dermatitis usually occurs on the feet, which disappears after a few days. Pneumonia may occur during their migration through lungs.

Ancylostoma infection in cats and dogs results in anaemia, haemorrhagic diarrhoea, dehydration, anorexia and weakness. In severe cases of anaemia, and in cases of infections of neonatal pups, it may prove fatal. Malabsorption and protein loss may result in weakness and poor growth. Anaemia is not seen with *U. stenocephala* and *A. braziliense*. They usually cause intestinal symptoms.

Epidemiology and Public Health

Zoonotic *Ancylostoma* spp. are usually found in tropical and subtropical countries worldwide and their transmission to humans represents a significant public health problem.

In animals, the canine hookworm is commonly seen in warmer regions as well as in colder regions. *A. caninum* and *A. tubaeforme* are distributed worldwide. *A. braziliense* infection is reported in tropical and subtropical countries, including Central and South America, the Caribbeans and parts of the USA. A. *ceylanicum* has been reported in parts of Asia, Africa, Australia, the Middle East and Brazil. *B. phlebotomum* and *U. stenocephala* are distributed in temperate and colder regions, respectively (Table 1).

Human *A. ceylanicum* infections have been found in southern India, Sri Lanka, Indonesia, Malaysia and neighbouring countries in Southeast Asia and western New Guinea. *A. caninum* is a major cause of eosinophilic enteritis in north-eastern Australia. Hookworm-related cutaneous larva migrans (CLM) is prevalent in India, Brazil and West Indies. The geographical distributions of intensity of infection vary depending upon climatic factors. Such factors include rainfall in adequate amount with ideal humidity and temperature. A survey in Brazil amongst the rural people during the rainy season showed that the prevalence

Table 1 Epidemiological aspects of some *Ancylostoma* spp. of human importance

Species	Distribution	Paratenic host	Definitive hosts
Ancylostoma brazilense	Central and South America, the Caribbeans and parts of the USA, India, Brazil, Africa, Indonesia, the Philippines	Rodents	Dogs, cats
Ancylostoma caninum	Sri Lanka, Southeast Asia, Malaysia, Australia	–	Dogs, wolves, foxes, cats
Ancylostoma ceylanicum	Asia, Africa, Australia, the Middle East, Brazil	Rodents	Dogs, cats, humans
Uncinaria stenocephala	Canada, northern regions of the USA	Dogs, cats rodents	Dogs, cats, foxes
Ancylostoma tubaeforme	Worldwide	Rodents	Cats

Table 2 Diagnostic methods for zoonotic hookworm infection

Diagnostic approaches	Methods	Targets	Remarks
Clinical examination Cutaneous larva migrans	Examination of skin	Linear serpinginous tracks on the skin that are very itchy	Creeping eruption as a clinical sign is diagnostic
Direct microscopy Cutaneous larva migrans	Skin biopsy	Presence of larva	*Limitation*: Not useful, as it rarely identifies the parasite
Intestinal disease	Stool microscopy	Eggs	*Limitation*: Difficult to differentiate between species
Immunodiagnostics	Antibody detection (ELISA using excretory-secretory antigens from adult *Ancylostoma caninum)*	IgG and IgE antibodies	*Limitation*: No data on sensitivity and specificity
	Western blot (Ac68) protein	IgG4 antibodies	More sensitive than IgG and IgE ELISA using excretory-secretory antigens
Molecular assay	PCR, PCR-RFLP, qPCR	ITS1, 5.8S, ITS2 of rDNA	*Limitation*: Require skilled manpower

rate of CLM to be 14.9% and 0.7% in less than 5-year-old children and in adults aged 20 years or older, respectively. Frequent occurrences of such cases have been reported in areas where stray dogs or cats are common.

The exact global burden of zoonotic hookworm infection in animals and humans is not known. The prevalence of *Ancylostoma* spp. in dogs in resource-poor settings was found to range between 66% and 96%. Many people are infected by walking barefooted or sitting naked on the ground where animal faeces have contaminated the soil. In Brazil, up to 4% of the general population and 15% of children are infested with hookworms. It was reported that CLM had significantly impaired the skin-disease-associated life quality in child and adult patients living in urban slums in North Brazil, which normalized rapidly after treatment with ivermectin. Hookworm-related CLM in high-income countries has been reported during the winter season, when people live in close contact with their pets. In hot climatic areas the infection is seen as sporadic cases.

Diagnosis in Humans

Diagnosis of CLM in humans is usually clinical indications of symptoms and a history of exposure to zoonotic hookworm. On examination of the skin, linear pruritic serpinginous tracks are observed on the feet or lower part of the legs.

Stool microscopy supplements the diagnosis of enteritis (Table 2).

Microscopy

Skin biopsy is frequently useful to demonstrate the larva in CLM. In enteritis, stool microscopy may reveal the typical hookworm eggs. Eggs are not shed in stool constantly; hence, repeated sampling may be necessary to detect infection.

In Vitro Cultivation

The eggs of different species of *Ancylostoma* are morphologically similar; hence, they cannot be distinguished from each other. Therefore, the copro-culture of stool specimen is frequently carried out for species-specific identification of *Ancylostoma* based on the identification of the larvae that hatch out of *Ancylostoma* eggs during the stool culture. The centrifuged deposit of the culture fluid after incubation is examined for the larvae under the microscope for their further identification. Various techniques such as agar plate culture, Harada-Mori technique and so on are frequently used for the purpose.

Serodiagnosis

Western blot and ELISA are tests of choice in the serodiagnosis of the condition. ELISA using excretory-secretory antigens from adult *A. caninum* is available for detection of IgG and IgE in the serum. Western blot to identify IgG4 antibodies to a 68 kDa (Ac68) protein is more specific and sensitive.

Molecular Diagnosis

Real-time PCR targeting the ITS2 sequence, followed by high-resolution melting analysis, has been used in research settings for detection and differentiation of *N. americanus*, *A. duodenale*, *A. ceylanicum* and *A. caninum.* PCR-RFLP has also been employed for detection of *Ancylostoma* species.

Diagnosis in Animals

Ancylostoma infection in animals may present with lethargy, weight loss, weakness, roughness of the hair coat and pale mucous membranes. Stool concentration by a floatation method and microscopy for eggs are frequently used for diagnosis of the infection, followed by in vitro cultivation for species identification.

Treatment

The signs and symptoms of CLM usually resolve without medical treatment. Ivermectin is the drug of choice for treatment of cutaneous larva migrans in humans. It is contraindicated in children below 5 years and in pregnant women. Oral albendazole is also effective. Topical thiabendazole is used for treatment of the lesion and is as effective as oral ivermectin.

Prevention and Control

Preventive measures include (a) avoidance of walking barefoot and wearing of shoes; (b) reducing environmental contamination of the soil or sand with animal faeces by regular deworming and prompt and proper disposal of their faeces, covering the sandboxes when not in use and using sodium borate to sterilize lawns, kennels or other areas; and (c) housing of animals to prevent the development of infective larvae.

To prevent *A. caninum* infections in puppies, bitches should be free of hookworms, and they need to be kept out of contaminated areas during their pregnancy.

Case Study

Umbrello and his co-workers reported a case of an Italian 2-month-old breastfeeding female

infant who was hospitalised with complaint of vomiting and weight reduction. One month prior to this, she was admitted in a different hospital for the same health issues. After the examination of urine and blood, her eosinophil count was found to be 2900/μl, while ammonium concentration, ultrasound of abdomen and Rotavirus/Adenovirus faecal antigens test showed negative. During this period, she suffered from mild diarrhoea, which was cured after treating with probiotics. Thereafter, she was released with a sound health condition. Upon investigation, it showed that the mother had a travel history of 70 days to Vietnam and Thailand (Southeast Asia) during her first stage of the gestation period, where she experienced several episodes of nausea with vomiting. Blood test report of the infant showed a white blood cell count of 19,060/μl, eosinophil (5170/μl), platelets (756,000/μl) and haemoglobin (9.1 g/dl). The blood smear showed the presence of microcytic hypochromic red blood cells. Considering the haemoglobin level, iron and folic acid were supplemented orally. The other parameters such as serum protein level, bilirubin level and albumin level were in normal range. Liver function test and abdomen test showed normal. On the other hand, C-reactive protein (CRP) was also negative. Electrolytes, kidney function test and coagulase test were reported as normal. The stool looked normal, while viral antigens and bacterial culture were reported as negative. The microscopic examination of the stool revealed the presence of eggs of hookworm, which was further confirmed by real-time PCR in the parasitology laboratory of "Ospedale Sacro Cuore Don Calabria" of Negrar as *A. duodenale*. The absence of the parasite in the parents' faeces proved the vertical transmission of the parasite during the gestation period. The patient was administered two doses of mebendazole (100 mg) orally twice a day for 3 consecutive days. The infant was released in a good health condition. In the follow-up visit scheduled after 1 month, the infant's clinical condition and medical reports appeared to be normal (Umbrello G et al. 2021).

1. What are the various methods for speciation of *Ancylostoma* spp.?
2. Which species of *Ancylostoma* can cause a patent infection in humans? What are the clinical manifestations?
3. What are the other parasites which can cause CLM?

Research Questions

1. What is the worldwide burden of zoonotic diseases in cats and dogs?
2. What is the infection prevalence and intensity of zoonotic ancylostomiasis in human populations?
3. How to map *A. ceylanicum* in areas other than Southeast Asia and the Pacific region?

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Ascariasis

Utpala Devi

Learning Objectives

1. To make the reader aware that Loeffler's syndrome can also be due to zoonotic ascariasis transmitted by pigs.
2. To study the limitation of microscopy in diagnosis, since the more common *Asacris lumbricoides* eggs are of similar morphology as those of *Ascaris suum.*

Introduction

Ascariasis caused by *Ascaris lumbricoides* and *Ascaris suum* is a common parasitic disease in both humans and pigs. *A. lumbricoides* is known to infect humans while *A. suum* infects pigs. However, in rare cases, *A. suum* is known to infect humans also. Ascariasis in humans occurs due to the ingestion of infective eggs present in the contaminating environment. *A. suum* usually infects pigs worldwide and causes huge economic losses and is an important zoonotic pathogen. In most of the infections, a person may remain asymptomatic, but, in some cases, fatal situations like visceral larva migrans (VLM) can be seen. It is often difficult to diagnose specifically whether ascariasis is due to *A. lumbricoides* or *A. suum* due to the similarity between the two. Molecular markers targeted for the nuclear ITS1 region are used to differentiate *A. suum* and *A. lumbricoides,* where the G1 genotype is usually found in humans and G3 in pigs. Measures focussing on health education and better farming practices, along with personal and food hygiene, are important to prevent this infection.

History

The disease ascariasis has been documented in the written records of Egyptian hieroglyphics. The disease was first studied by Edward Tyson during the later part of the seventeenth century. *Ascaris* eggs were found in humans from archaeological material dated 30,000 years back. *A. suum* was first described and named by Goeze in the year 1782.

Taxonomy

A. suum belongs to the genus *Ascaris* of Ascarididae family, Ascaridida order, Secernentea class, Nematoda phylum and Animalia kingdom. *A. suum* is a closely related species of *A. lumbricoides* and has high host specificity for pigs, although *A. suum* infections have also been reported in humans. There has

Utpala Devi, (deceased), Professor Devi wrote this book while at ICMR-RMRC, Dibrugarh, India

S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
https://doi.org/10.1007/978-981-16-7204-0_45

been considerable debate as to whether *A. lumbricoides* and *A. suum* are a single and the same species or two distinct separate species. Numerous studies have been carried out to differentiate between A. *lumbricoides* and *A. suum*, based on their morphology, immunology and biochemistry, but with inconclusive results. Recent studies have identified genetic markers which could possibly differentiate between the two.

Genomics and Proteomics

A. suum draft genome of 273 mega bases published by Jex et al. in 2011 reports a mean GC content of 37.9% and a few repetitive sequences (4.4%) as compared to other metazoan genomes sequenced. It was found to encode about 18,500 protein-encoding genes. The secretome of *A. suum* was found to be rich in peptidases, and was suggested to be responsible for host tissue degradation and penetration, and an assembly of molecules which contribute to evade host immune response.

The excretory-secretory (ES) products of the larval stages of A. *suum* revealed that the majority of ES proteins from L3-egg were distributed between 10 and 120 kDa, whereas those of L3-lung varied between 30 and 100 kDa, and for L4-ES the major bands lie between 37 and 150 kDa. The proteins involved in metabolic pathways varied in numbers from the L3-egg stage to the L3-lung and L4-stage larvae. The motor activity proteins, such as myosin-4, paramyosin and tropomyosin, were unique to L3-egg. Nearly 9% of proteins observed in L3-lung ES products are structural proteins that included cuticlin-1, cuticle collagen 12 and 13, but were present in lesser amount in L3-egg and L4. Of the 17 binding proteins that were identified, 82% of them were ATP-, ion-, carbohydrate and DNA-binding proteins. The glycosyl hydrolases belonging to family 31 (GH31) were the most frequently identified proteins in ES products.

Parasite Morphology

Adult Worm

A. suum has a cylindrical body, which is bilaterally symmetrical, unsegmented and covered externally by a cuticle (Fig. 1). The body cuticle is striated transversely and is thick. There are two different types of striations, narrow at the anterior end and wider at the posterior end. In the male,

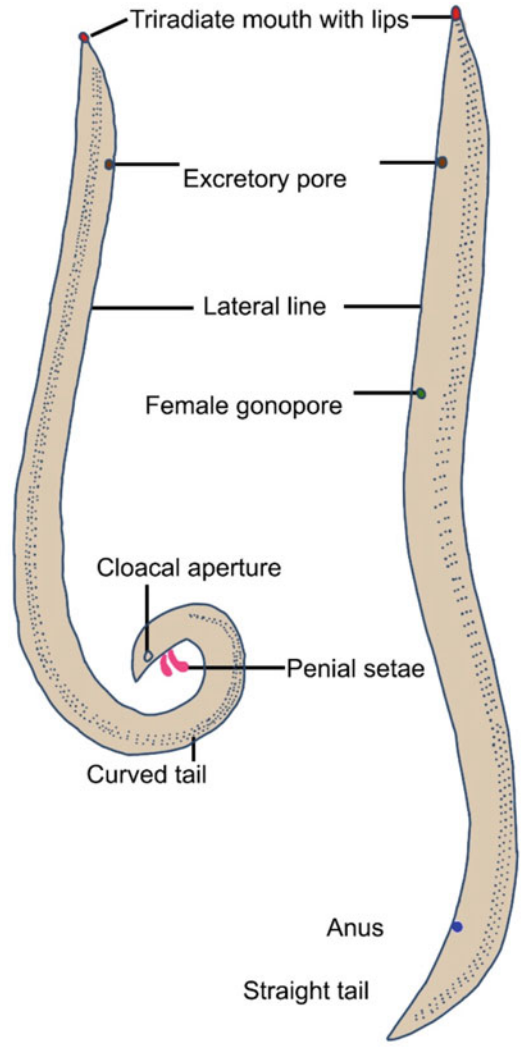

Fig. 1 Schematic diagram of adult male and female of *Ascaris suum*. Males have a curved posterior end and females have a straight tail

the wide stria at the posterior part of the body has a narrow wrinkling, which aids in bending.

A. suum males' body size ranges from 15 to 31 cm in length and 2–4 mm in width. The males have a pointed tail, with their posterior end curved towards the ventral side. They also have spicules that help them in mating.

The females are larger in size as compared to the males and are about 20–49 cm long and 3–6 mm wide. The vulva occupies about one-third of the anterior part of the body length.

The mouth cavity has three lips, one dorsal and two latero-ventral. The dorsal lip is provided with two labial papillae and the latero-ventral lips have only single labial papillae each. The variability of teeth has been studied by different authors. The teeth are pointed and conical in the young and gradually round off and become cylindrical in old parasites. It has been observed that the wearing of denticles increased with age, which appeared truncated from any angle. The average denticle size is related to the size and age of the worm and no detectable difference exists between males and females.

The caudal end in males contains a large number of papillae on the ventral side, which have been described as being constant or inconstant in numbers with irregular arrangement. The tip of the papillae terminates into a small crater, from which protrudes a tubular, funnel-shaped or knob-like protrusion. The end of the spicules is blunt and two crossing furrows are observed at higher magnification. In both males and females, the caudal end usually terminates in a knob-like structure, but in females, it has a pit in the middle. The caudal papillae in males are surrounded by numerous bacteria.

Eggs

A. suum eggs vary in size from 45 to 75 μm in length and 35 to 50 μm in diameter (Fig. 2). The egg is thick-shelled, rounded or elliptical with a brown sculptured surface. The unfertilized eggs are narrower and longer than the fertilized eggs, without the outer sculptured brown layer. A female *A. suum* can lay up to 2,00,000 to 1 million eggs per day.

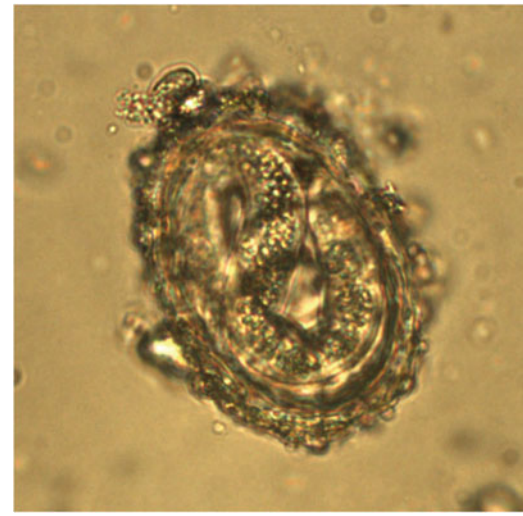

Fig. 2 Embryonated *Ascaris suum* egg with a visible infective L3 stage larva inside. (By Vlaminck J Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=45655455)

Cultivation of the Parasite

Cultivation of *A. suum* larvae has been carried out in synthetic media. The growth and survival of the larva were found to be optimum in RPMI 1640 media.

Laboratory Animals

Mouse has been used for experimental animal infections to understand the host response to *A. suum*.

Life Cycle of *Ascaris suum*

Host

Life cycle is completed in a single host (Fig. 3). Pig is the primary host. Infections are also seen in cattle.

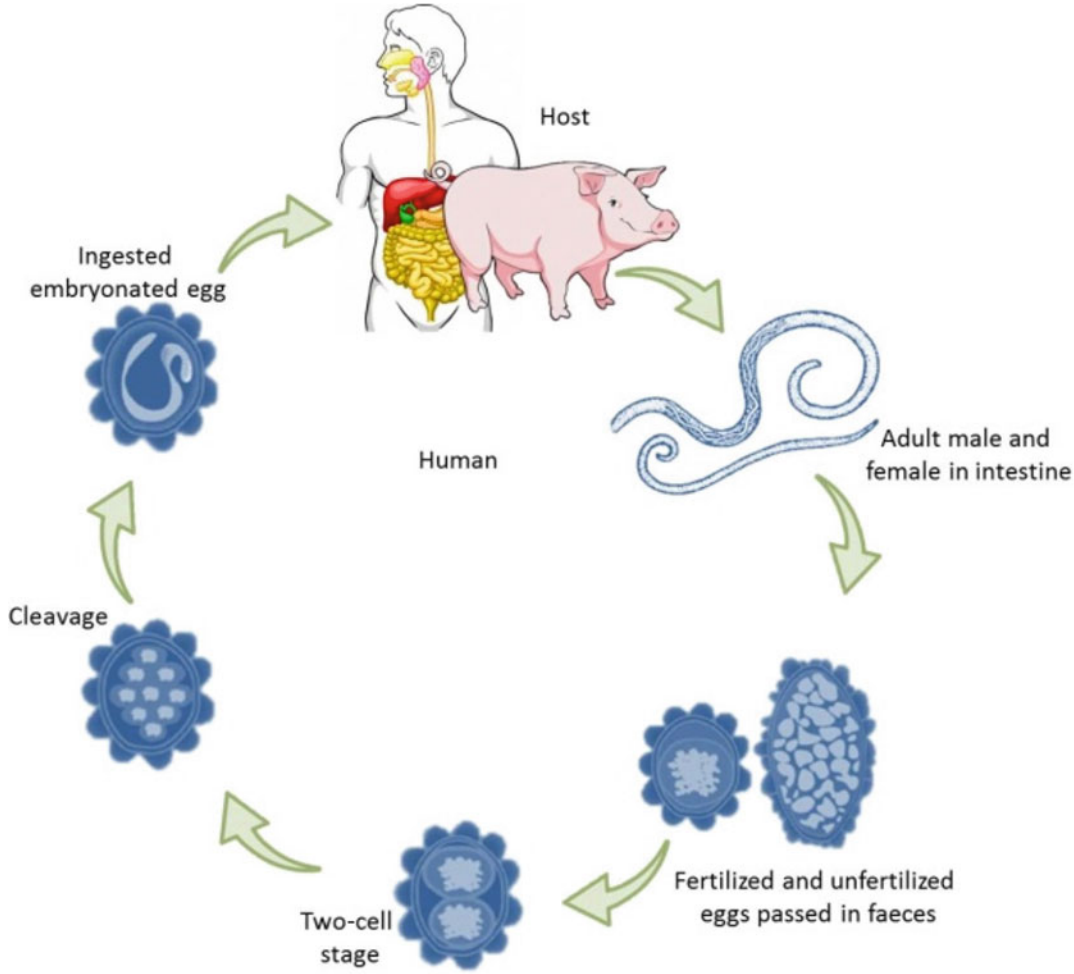

Fig. 3 Life cycle of *Ascaris suum*

Infective Stage

Embryonated *A. suum* eggs containing infective L3 larva are infective.

Transmission of Infection

Transmission to humans occurs on ingestion of *A. suum* eggs present in the contaminated soil. This occurs when people do not wash their hands properly after handling pig or pig manure or if they consume produce which has been fertilized with pig manure. As the eggs of *A. suum* are not present in an infected pig, it cannot get transmitted to humans by consumption of pork or pork-related products.

The life cycle of *A. suum* is direct; hence, there is no involvement of any intermediate hosts. Pigs get infected with embryonated eggs (with infective L3 larva inside) of *A. suum* orally that are present in the contaminated environment. Within the egg, the larva undergoes moulting twice, so the larva that emerges is a third-stage larva (L3) loosely covered by L2 cuticle.

After ingestion, the infective larva is released in the small intestine, which then penetrates the mucosa of the colon and caecum and then migrates to the liver. The L3 larvae are transported through the mesenteric blood vessels to the liver, where they obstruct the capillaries and subsequently destroy liver tissue, ultimately to reach the efferent blood vessels.

L3 larvae subsequently travel to the lungs through the blood stream and penetrate the capillaries to enter the alveoli, after which they pass through the respiratory tract and reach the pharynx. The larvae are then swallowed back to the small intestine. Most of the larvae are expelled from the intestine in faeces after 14–21 days following infection (*Self-cure reaction*). The remaining larvae transform into adults after moulting twice to L4 and L5 stages, and finally, becomes adults by 42–49 days post infection. Thus, it takes nearly, 6–8 weeks after ingestion of infective eggs by pigs to produce eggs by female worms.

Pathogenesis and Pathology

Both the damage of the organ by the migrating larvae and the immune reaction of the host to larval migration contribute to the pathogenesis of ascariasis. The pathology of organs in the infected host depends on the number of adult worms and the site of their location in the infected hosts.

In Humans

The migration of the larvae in the lungs manifests as clinical syndrome of massive pulmonary infiltrates, asthma and eosinophilia. The pulmonary lesions are caused not only by the larval migration but also by two types of antibody responses: IgE being responsible for urticaria and asthma, and IgM for the pulmonary infiltrate. The pulmonary eosinophilia due to the migration of the larvae through the lungs in the case of ascariasis is referred to as *Loeffler's syndrome*. It has been demonstrated that pig *Ascaris* is not capable of reaching the adult stage in humans, although intestinal obstruction due to *A. suum* has been reported as early as in 1977.

In Pigs

When the larvae penetrate the caecum and colon of the infected pig, marginal petechial bleeding is observed in the mucosa. The intestinal phase is associated with hypertrophy of tunica muscularis and decreased villous height. Migration of the L3 larva induces inflammation in the liver and the lesion is visible macroscopically as white stains (also called *milk spots*). These lesions heal and disappear in about 5–6 weeks' time but due to hardening of the liver, the organ becomes less suitable for human consumption. Migration of the larval stage in the lungs of the pigs also induces a haemorrhagic and inflammatory cell response and the pigs suffer from pneumonitis.

Immunology

The role of the humoral immune response against *A. suum* has been demonstrated experimentally in laboratory animals. Immunisation with As14, a 14 kDa surface protein of *A. suum*, in combination with Cholera B toxin, was found to cause a significant increase in IgE and IgG levels in the serum and mucosa. This was also found to be associated with a reduction in larval burden in the lungs in mice.

Multiple infections of mice to *A. suum* demonstrated a mixed Th2/Th17 immune response with production of higher levels of systemic cytokines when compared to those which were infected with a single parasite.

Pigs demonstrated a protective immunity in the gut after a long exposure to *Ascaris*. This prevented the incoming larvae to penetrate the intestinal tissue and thus prevented migration. It was observed that pigs were protected due to the presence of increased numbers of eosinophils in the gut, which facilitated the killing of the larvae.

Infection in Humans

A. suum infections in humans are often asymptomatic. In symptomatic cases, the clinical

symptoms of *A. suum* infections maybe related to the migration of the larvae, total number of adult worms present and their location in the infected host. Adult *A. suum* rarely causes any clinical symptoms in infected humans.

Ascariasis caused by *A. lumbricoides*, a human species, is a common cause for Loeffler's syndrome globally. Loeffler's syndrome is an immune-mediated type I hypersensitivity reaction caused by the migration of the *A. lumbricoides* larvae to the lung tissue. A report from the USA has documented a case of Loeffler's syndrome due to *A. suum* in an 8-year-old child, and it has suggested that pigs were the source of the infection. Visceral larva migrans (VLM), affecting the viscera, caused by the migrating larvae of A. *suum* has also been documented recently. In most of these cases, the lungs were involved. Encephalopathy associated with VLM due to *A. suum* and intestinal obstruction due to *A. suum* have also been reported.

Infection in Animals

Although, *A. suum* is a common parasitic infection of pigs, the pigs suffer less from clinical disease. The clinical manifestations depend upon the stage of development of the parasite and also on the burden of the parasite. Pigs may suffer from frequent cough during the migratory phase of the larvae in the lungs. The migration of the larvae to the liver causes severe damage, characterised by the presence of *milk spots* and hardening of the general texture, making it less suitable for consumption. The presence of worms in the intestine also causes diarrhoea and a reduction in weight gain of the pigs. Intestinal obstruction is rare.

Epidemiology and Public Health

A. suum infection in pigs has been reported as an important source of human infection in many parts of the world, including Maine, China, Japan and Denmark (Table 1). Several cases of ascariasis among children have been reported due to their close contact with pigs or pig manure. The prevalence of *A. suum* infection in pigs depends on a variety of factors, including type of pig rearing system, housing and management practices, the age of the pigs and also the season.

Studies using molecular markers have been carried out to understand the molecular epidemiology of *Ascaris*. The transmission dynamics of *Ascaris* in people and pigs has been studied using mitochondrial and microsatellite markers. *Ascaris* worm samples were obtained from humans and pigs from different geographical regions, including the UK, Denmark, Uganda, Kenya, Zambia, Bangladesh, Nepal, Zanzibar, the Philippines, Tanzania, Guatemala and various countries. On analysing the *cox1* sequences, 75 different haplotypes of *Ascaris*. H1 and H3 haplotypes were found in *Ascaris* from humans, whereas haplotypes H7, H28, H52 and H65 were found abundantly in *Ascaris* from pigs, with haplotype H65 unique to both of them. It was observed that *Ascaris* from humans in Europe clustered with pig *Ascaris* instead of human *Ascaris*, providing evidence that Ascaris infection is zoonotic in developed countries.

In order to reveal the source of the human *Ascaris* infections, *Ascaris* worms obtained from humans and from pigs in Denmark and also from some developing countries were compared by analysing a specific region of nuclear rDNA, the internal transcribed spacer region. Results of the study showed domestic pigs as the source of Ascaris infections in all the Danish patients.

Table 1 Distribution of *Ascaris suum* in humans

Species	Distribution	Intermediate host	Definitive hosts
Ascaris suum	Maine, China, Japan, Denmark	None	Pigs, cattle

Diagnosis

A variety of diagnostic methods are available for laboratory diagnosis of infections caused by *A. suum* (Table 2).

In Humans

Microscopy

Although microscopy of stool can demonstrate *Ascaris* eggs, it cannot differentiate between eggs of *A. lumbricodes* and *A. suum*. Hence, specific diagnosis of *A. suum* infection by stool microscopy is difficult. Furthermore, adult worms cannot be detected in the stool because larvae of *A. suum* do not develop from eggs in the human intestine.

Serodiagnosis

A. suum antigen-based immunoblot assay has been found to be useful for the diagnosis of visceral larva migrans in humans. ELISA using a haemoglobin antigen of *A. suum* is available for diagnosis of *A. suum* infection both in humans and in pigs.

Molecular Diagnosis

PCR is useful to detect *Ascaris* eggs but cannot differentiate between *A. lumbricodes* and *A. suum*. Tests based on other genetic markers (mitochondrial genome) are being evaluated in epidemiological studies, even though the differentiation between the two species, A. *lumbricodes* and *A. suum*, is very minimal.

In Pigs

Milk spots in the liver or the presence of adult worms in the intestine suggests VLM due to *A. suum*. Microscopy of stool is useful to demonstrate the presence of eggs of *Ascaris* in the stool. ELISA using a haemoglobin antigen of *A. suum* is available for diagnosis of *A. suum* infection in pigs.

Treatment

The World Health Organization (WHO) recommends the use of albendazole, mebendazole, levamisole and pyrantel pamoate for the treatment of ascariasis. Corticosteroids may be administered in the management of severe cases of Loeffler's syndrome. Treatment with

Table 2 Diagnostic techniques to detect ascariasis in humans

Approaches	Techniques	Targets for detection	Remarks for application
Direct microscopy	Stool examination	Detection of eggs	*Limitation*: Cannot differentiate between eggs of *A. lumbricoides* from *A. suum*
Immunodiagnostics	Antigen detection by enzyme-linked immunosorbent assay (ELISA)	*Ascaris suum* haemoglobin antigen	It has higher sensitivity than microscopy and has low cross-reactivity with *Trichuris suis.*
Molecular assays	Real-time PCR (qPCR)	Nuclear first internal transcribed spacer region (ITS1)	*Limitation*: the mitochondria of *A. lumbriciodes* and *A. suum* vary by only 1.9% and differentiation between the two may not be possible

albendazole or mebendazole for eradicating adult worms is delayed until pulmonary symptoms have cleared.

Prevention and Control

Zoonotic transmission of *Ascaris* from pigs has highlighted the need for efficient control of such infections among pig populations. The preventive measures include avoidance of contact with soil contaminated with pig manure, washing hands with soap and water after handling pigs or pig manures, and properly washing/peeling/cooking of vegetables and fruits before consuming.

Vaccine efficacy study using crude extracts of adult worms, adult worm cuticle and extract of infective *A. suum* L3 larvae showed promising results contributing to protective immunity in an experimental mice model. *A. suum* enolase and yeast-expressed rAs16 formulated with ISA720 or alum have also been demonstrated to be potential vaccine candidates against ascariasis.

Case Study

A worm in the stool of an adult was found. Treatment with niclosamide and a laxative was given and parasitological stool examinations were performed over a 3-day period. The parasite was identified as a female *Ascaris* spp. and the patient was treated with mebendazole for 3 days. After 8 days of the first antihelmenthic treatment, another *Ascaris* spp. was passed out with the stool, which was identified to be a male worm. The patient was further treated with mebendazole in combination with an osmotic laxative. The blood tests, chest X-ray and sonography of the abdomen appeared to be normal. By applying PCR-RFLP, the worm from the patient was found to be an intermediate of both the species, *A. suum* and *A. lumbricoides*. *A. suum* was found in the pigs of the patient's farms. Cross-infestation from the pigs was concluded as the source of transmission.

1. How can we differentiate between *A. lumbricoides* and *A. suum*?
2. How to conduct a survey to find out the true prevalence of *A. suum* in a given geographical area?
3. In which situation *A. suum* infection in humans can become life-threatening?

Research Questions

1. Is *A. suum* really causing human disease? What is the actual pathogenic potential of *A. suum?*
2. What is the exact burden of *A. suum* among humans worldwide?
3. Are *A. lumbriciodes* and *A. suum* the same or different?

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Dioctophymiasis

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Learning Objectives

1. To know that *Dioctophyma* is one of the few parasites which can primarily affect the kidneys.
2. To understand the importance of surgical intervention in the absence of effective chemotherapy.

Introduction

Dioctophyma renale, the giant kidney worm, is one of the largest parasitic nematodes of mink, wolves, dogs, cats and other carnivorous mammals. The disease caused by *D. renale* is known as dioctophymiasis. Human infections by *D. renale* are rare, and often infection is acquired by eating raw or undercooked fish or frog infected with the larva of *D. renale*. Diagnosis of dioctophymiasis may be achieved by demonstration of the characteristic eggs or worm in urine or on stained tissue sections of affected organ(s). The most effective preventive measures include non-consumption of raw or undercooked fish or other possible paratenic hosts and use of clean water for consumption.

History

Discovery of *Dioctophyma* eggs has been reported in human coprolites from a Neolithic site of Arbon-Bleiche 3 (Switzerland). This is the oldest record of dioctophymiasis reported in archaeological material dated from 3384–3370 BC. *D. renale* has been known since 1583. Back in the sixteenth century, it was known as the Red scourge. Goeze first described this worm in a dog's kidney in 1782. The complete account of the life cycle of the parasite, was described by Woodhead in 1945. The International Commission on Zoological Nomenclature (1989) decided in favor of the nomenclature as *Dioctophyme renale*, as described by Tollitt in the year 1987.

Taxonomy

D. renale (Goeze 1782) belongs to the Dioctophymatidae family, which includes three genera, *Dioctophyme, Eustrongylides* and *Hystrichis*. Taxonomically, it is classified under Kingdom, Animalia; Phylum, Nematoda; Class, Enoplea; Order, Dioctophymatida; Family, Dioctophymidae and Genus, *Dioctophyme*.

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Genomics and Proteomics

Various lipid-binding proteins produced by *D. renale* have been identified as major antigens. These are structurally different from host proteins and have been incriminated to play a plausible role in certain functions such as energy store distribution, cell-to-cell signalling and interfere with hosts' immune system. A possible haem-containing hemoglobin, the P17 protein of 16.6 kDa, has been identified and this protein possibly carries oxygen, making the adult worm bright red. Another 44.46 kDa protein (P44) from the pseudocelomic fluid of the adult worm has a possible role of lipid distribution within the nematode.

Parasite Morphology

Adult Worm

The adult worm of *D. renale* is one of the largest nematode. It is bright red in colour, covered with cuticle and three or more outer non-cellular layers secreted by the epidermis. The worm characteristically tapers at both ends. Like any other nematode, the female worms are larger than the males and measure up to 103 cm in length, with a diameter of 0.5–1.2 cm. The males measure between 20 and 40 cm in length and 5–6 mm in diameter. The males have a bell-shaped conspicuous copulatory bursa without papillae or supporting rays (Fig. 1).

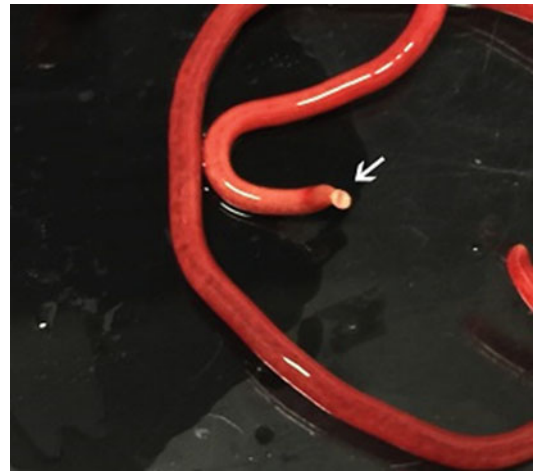

Fig. 1 Adult male worm of *Dioctophyma renale*, showing a bell-shaped conspicuous copulatory bursa. Image courtesy of DPDx, Centers for Disease Control and Prevention (https://www.cdc.gov/dpdx/dioctophymiasis/index.html)

Eggs

The eggs of *D. renale* are oval to elliptical in shape, and transparent to yellow, with bipolar plugs. They are enclosed in a thick roughened shell. Their size can vary from 60–80 µm in length to 39–46 µm in width (Fig. 2).

Larvae

D. renale larvae are 6–10 mm long and 0.1–0.2 mm in diameter. The vulvar primordium of the third-stage female larva is situated near the intestinal-esophageal junction and is morphologically differentiated from that of *Eustrongylides*, where it is located near the anal aperture.

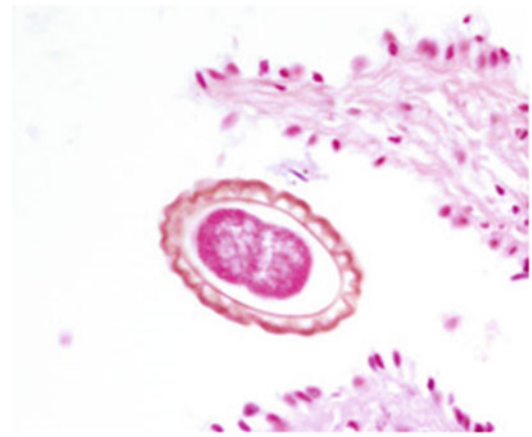

Fig. 2 *Dioctophyma renale* egg, stained with hematoxylin and eosin. Image courtesy of DPDx, Centers for Disease Control and Prevention (https://www.cdc.gov/dpdx/dioctophymiasis/index.html)

Cultivation of the Parasite

Several techniques have been used and evaluated for cultivation of various helminths with varied success. However, current literature on cultivation of *D. renale* is limited. Mace et al. studied the effect of temperature on embryogenesis by incubating the eggs in a 0.1% formalin solution. They also studied the development of various larval stages in oligochaetes, frogs and mink. Pedrassani and colleagues studied the development of *D. renale* eggs and the effect of temperature on their further development into first-stage larvae by incubating the *D. renale* eggs in mineral water (pH ranging from 7.2 to 7.7) for 90 days at 15 °C, 20 °C and 26 °C. They concluded that embryogenesis of *D. renale* eggs can be studied in mineral water (pH 7.2) and also in purified milliQ water along with 0.1% formalin (pH 7.0) at a temperature of 26 °C.

Laboratory Animals

Animal models have not been well established in human dioctophymiasis. In a study by Mace and Anderson in 1975, mink were infected by *D. renale* L3 larva and transmission and effects were studied. In another study by Abdel-Hakeem et al., the authors have reported *D. renale* infection in BALB− mice which were being screened for intestinal parasites. Application and evaluation of appropriate animal models for human dioctophymiasis still needs to be explored and this may help in understanding both pathogenesis and therapeutic modalities.

Life Cycle of *Dioctophyma renale*

Hosts

D. renale mostly requires two intermediate hosts to complete its complex life cycle (Fig. 3). The first intermediate host is an invertebrate aquatic oligochaete worm (e.g. *Lumbriculus variegatus*). Frogs or fish may consume the first intermediate host and act as paratenic hosts. Humans, however, are the incidental host.

Infective Stage

L3 larva is the infective stage.

Transmission of Infection

Dioctophyma adult worm is oviparous and produces eggs in the definitive host. These eggs are unembryonated and are shed in the urine of the definitive host to reach the external environment. The first-stage (L1) larva starts developing inside the egg and takes about 4 weeks. The eggs lose viability upon desiccation by exposure to direct sun light. Upon ingestion by oligochaete worms, frogs or fish, the first intermediate hosts, these eggs hatch inside the host's digestive tract. The larva undergoes two moultings and matures into infective L3 larva within a period of 2–3 months. The infected first intermediate hosts may be consumed by frogs or fish, which act as paratenic hosts. The L3 larvae invade and encyst in various tissues of the paratenic host. The definitive hosts like dogs and cats may acquire the infection after eating infected paratenic hosts with the encysted L3 larvae. Occasionally, hosts may get infected by consuming the infected intermediate hosts. The larvae then pass through the stomach wall of the definitive host. They ultimately reach the kidney following their migration across tissues. It may take upto 2 years for *D. renale* to complete its life cycle and a pre-patent period of approximately 5 months period has been described.

Humans, an incidental host, acquire the infection after consuming raw or undercooked paratenic hosts containing infective larvae. The larvae migrate aberrantly and end up being encapsulated as subcutaneous nodules without any further development.

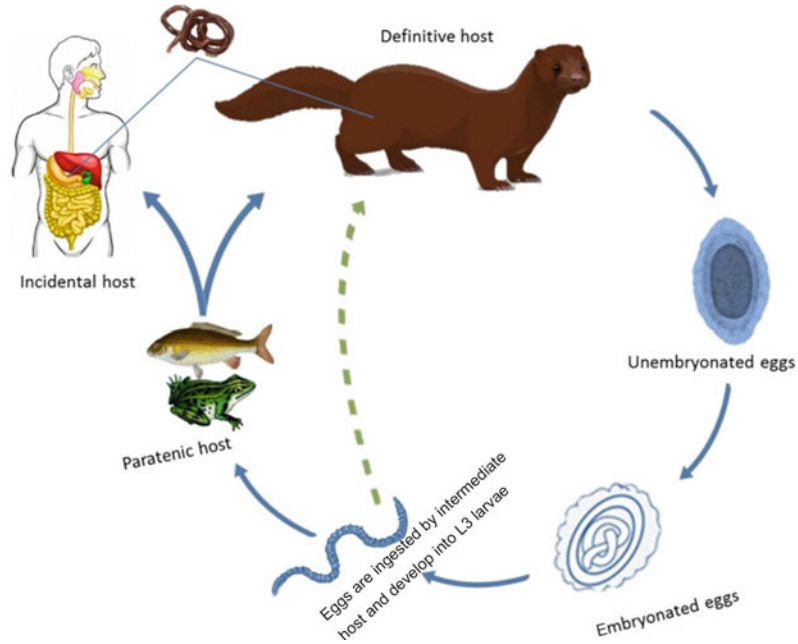

Fig. 3 Life cycle of *Dioctophyma* spp.

Pathogenesis and Pathology

The cuticle protects the nematode inside the digestive tract of animals and allows to invade the wall of the digestive tract and subsequent migration to various regions. The adult worm resides in the kidneys of the definitive host but its possible location inside the definitive host depends on the site of penetration of the larva in the digestive tract. Various hypotheses have been suggested for the migration of the larva within the definitive host. These are: (a) if the larva passes through the duodenum wall, it can mature in the right kidney; (b) if it penetrates through the greater curvature of the stomach, maturation in left kidney is more likely; and (c) if it passes through the lesser curvature, lobes of the liver may be affected.

Once the adult worm reaches the target organ, it gives rise to strong inflammatory reactions, mostly inside the renal parenchyma. This leads to the destruction of the renal parenchyma and distention of the renal capsule. This may result in a gradual loss of function of the affected kidney. On the other hand, the unaffected kidney may show compensatory hypertrophy (hydronephrosis). The migration and movements of the worm cause severe loin pain mimicking renal colic. The presence of worms inside the body cavity may result in peritonitis and formation of adhesions.

D. renale eggs are shed in the urine of the definitive hosts. They develop in water, where their thickness and roughness facilitate in their survival. The wavy character of the egg's surface increases the surface area, which reflects possible adaption for areas with low dissolved oxygen. While the depressions and projections on the egg's surface help them to attach to plant surfaces, thereby increasing their chance of being ingested by the intermediate aquatic oligochaete host.

Immunology

There is paucity of information regarding the immunological response due to *D. renale* infection. The P44 protein in the pseudocelomic fluid has been incriminated to stimulate the host for both inflammatory and local tissue responses. Antigens derived from the oesophagus of the adult *D. renale* worm have also been evaluated for detection of anti-*D. renale* antibodies in dogs.

Identification of other antigens specific to *D. renale* would contribute in the development of better serological diagnostic modalities and can also aid in identifying potential therapeutic modalities.

Infection in Humans

The clinical presentation of *D. renale* infestations in humans is of a varied nature. Although the majority of the cases reported so far have renal involvement, cases of ectopic parasitism involving subcutaneous tissues and the retroperitoneal cavity have also been reported.

More than 80% of the cases of human dioctophymiasis have renal involvement. The infected individual may present with non-specific signs and symptoms, including loin pain, abdominal pain, fever, weight loss, urine retention, haematuria and pyuria. Loin pain and haematuria are the most common presenting symptoms observed in around 59.5% of the cases. Severe pain is attributed to the irritation caused by the adult worms migrating through the ureters. In extreme cases fatality has also been noted due to renal failure, sepsis or coexisting comorbid conditions.

Infection in Animals

D. renale infection has been reported in dogs and other canines, swine, cattle and horses. The adult worm is most commonly found in the renal pelvis of the infected host. The right kidney is affected more often than the left kidney, being anatomically proximal to the duodenum. Unilateral renal involvement is more commonly reported. It progressively damages the renal parenchyma and may show evidence of atrophy and ureteral obstruction, resulting in renal failure. Peri-glomerular fibrosis, renal tubular fibrosis, infiltration of interstitial tissue by connective tissue, renal calculi and necrotic tissue calcification have been observed in mink. Hydronephrosis may lead to the blockage of ureters or renal pelvis by adult worms. The encysted worm may also be seen in the abdominal cavity, subcutaneous tissues, mesenteric lymph nodes and other organs such as the uterus and ovary. If the worm enters the peritoneal cavity, signs and symptoms of peritonitis may be observed. In many cases, affected animals may remain asymptomatic.

Epidemiology and Public Health

D. renale has a worldwide distribution in mammalian species, especially in the temperate regions of the world. The parasite is known to infect a wide range of mammalian hosts, including dogs, wolfs, mink, horse, swine, long- and short-tailed weasel, ferrets, river otters, racoons and other wild carnivores. Brazil has reported a maximum number of dioctophymatosis cases in domestic dogs. In Brazilian wildlife, adult worms have also been observed in coati, little grison, maned wolf, two-toed sloth and neotropical river otter. Fresh-water fish and frogs are the common paratenic hosts. *D. renale* larva has also been reported in snakes, freshwater turtles and fish, which emphasizes the transmission of the nematode in various host species involved in the food web, especially in the wildlife. The possibility of such perpetuation of the life cycle in urban areas cannot be ruled out.

Transmission to humans is possible but rare. Sporadic human cases have been reported in over ten countries, including China, Australia, Greece, India, Indonesia, Iran, Japan, Thailand, the USA and Yugoslavia (Table 1). The maximum number of cases has been reported from China, which may be attributed to the consumption of raw and poorly cooked fish or frogs. Three cases have been reported from India till date. In India, the first human case was reported from Telangana in the year 2014. The patient presented with high-grade fever, passage of worms in urine along with haematuria. The second case was reported from Bareilly as an incidental finding. In 2016, a case was reported from Muzaffarnagar. The patient presented with urinary retention, hydronephrosis, haematuria and passage of worm in urine.

Table 1 Distribution of *Dioctophyma renale*

Species	Distribution	First intermediate hosts	Second intermediate hosts	Definitive hosts
Dioctophyma renale	Temperate regions (human cases reported from China, Australia, Greece, India, Indonesia, Iran, Japan, Thailand, the USA, Yugoslavia)	Invertebrate aquatic oligochaete worm (e.g. *Lumbriculus variegatus*)	Frogs, fish	Carnivores and mustelids (mink, wolves, dogs, cats); humans (incidental host)

Diagnosis

Human dioctophymiasis being a rare disease makes the diagnosis more challenging. Suggestive history of consumption of undercooked or raw fish and imaging findings such as enlarged or calcified kidneys raise suspicion in the infected individual. Urinalysis may reveal pyuria or proteinuria (Table 2).

Microscopy

Currently, the diagnosis of dioctophymiasis has mostly been made by the direct demonstration of *D. renale* eggs in urine by microcopy (Fig. 2). At times, the adult worm, may be spontaneously expelled and demonstrated in the urine. The adult worm may also be found in the abdominal cavity during surgical procedures like laparotomy. Gross examination of the morphological features helps in achieving the diagnosis.

Diagnosis may also be established on histopathological examination of stained tissue sections where cross-sections of the larva or adult worm or egg of *D. renale* surrounded by granulomatous reaction may be demonstrated.

Serodiagnosis

An indirect enzyme-linked immunosorbent assay (ELISA) had been evaluated for detecting anti-*D. renale* antibodies in animal sera based on soluble antigen derived from the oesophagus of adult *D. renale*. The reported specificity and sensitivity of the assay were 93.8% and 92.3%, respectively. Since not all infected hosts excrete *D. renale* eggs in urine, detection of antibodies, along with other radiological and clinical features, maybe helpful in the diagnosis of dioctophymiasis.

Molecular Diagnosis

Molecular identification of *D. renale* by PCR, followed by sequencing of gene targets, including the small-subunit ribosomal DNA and the mitochondrial cytochrome c oxidase subunit 1, has been described.

Treatment

Currently, there are no effective management protocol and therapeutic approach for the treatment of dioctophymiasis in humans. The worms can be surgically removed and nephrectomy may be performed in severe cases. In a case from Yugoslavia, ivermectin was used for the treatment of a patient with *D. renale* infestation who remained asymptomatic during the six-year follow-up. Treatment with albendazole has also been tried. Use of other anti-helminthic agents for the treatment of dioctophymiasis has not been extensively evaluated.

Table 2 Diagnostic methods for dioctophymiasis

Diagnostic approaches	Methods and sample	Targets	Remarks
Direct microscopy	Urine microscopy Histopathology	Eggs in urine Eggs/worm sections in tissues	Low sensitivity Invasive
Gross examination	Adults worms expelled in urine/ found in body cavities during surgeries	Adult worms	Adult worms not always expelled in urine
Immunodiagnosis	Antibody detection (indirect ELISA)	Soluble antigen derived from the oesophagus of adult *D. renale*	Evaluated in animals only. Described in experimental studies only
Molecular assays	PCR assay and sequencing	Small-subunit ribosomal DNA Mitochondrial cytochrome c oxidase subunit 1 gene	Not cost-effective

Prevention and Control

Dioctophymiasis carries public heath importance for its zoonotic significance. Human infections result following ingestion of undercooked paratenic hosts. So the most effective method of prevention includes avoiding consumption of raw or undercooked fish or other possible paratenic hosts. Public health measures to promote usage of clean and healthy food and water are also important. In addition, infection in cats and dogs may be avoided by preventing them from scavenging on fishing industry waste and byproducts.

Case Study

A 32-year-old male patient was admitted with complains of urine retention for 1 day. He also gave history of passing blood in the urine for the past 5 days and right loin pain for over an year. On general physical examination, pallor and tachycardia were noted. His laboratory investigations revealed leukocytosis and haemoglobin 8.0 gm/dL. The patient was catheterized and urine analysis revealed the presence of albumin in traces, 12–15 RBCs/hpf and numerous pus cells. The patient passed a red-coloured worm in his urine which was morphologically identified as *D. renale*. Further examination of urine sample did not reveal presence of any other parasitic element including eggs/ova or cysts.

Questions

1. What are the other parasites which can cause urinary problems?
2. How can you identify the worm?
3. What are the treatment options for this condition?

Research Questions

1. Which technique should be adopted or developed for the diagnosis and screening of Dioctophyma infections?
2. What should be the drug of choice in the treatment of dioctophymiasis?
3. How to improve our understanding regarding the epidemiology of human dioctophymiasis?

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Angiostrongyliasis

Vinay Khanna

Learning Objectives

1. To understand the important message that *Angiostrongylus* can directly affect the brain.
2. To gain information that anti-helmintic agents should not be used alone.

Introduction

Angiostrongylus spp., which belongs to genus *Parastrongylus*, with the nickname *rat lungworm*, is a tissue nematode, which mainly affects rodents. *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis* are the two species that cause infection in humans. *A. cantonensis* causes eosinophilic meningitis. *A. costaricensis* causes an eosinophilic inflammation of the intestinal tract that mimics appendicitis. *Angiostrongylus vasorum*, also called the *French heartworm* or canine lungworm, resides in the pulmonary artery and right side of the heart in dogs. Angiostrongylus infection primarily occurs in Asia-Pacific countries and is also being documented in the Caribbean regions.

History

A. cantonensis, a zoonotic parasite, was first discovered in rats by Xintao Chen from China in 1935. Medical significance of *A. cantonensis* was first described by Beaver and Rosen (1964) in patients with eosinophilic meningitis. Mackerass and Sanders (1955) were first to describe the life cycle and transmission of the worm in rats and established snails and slugs as the intermediate hosts. In 1965, Wallace and Rosen first conducted an epidemiological surveillance of *A. cantonensis* in Hawaiian and Society Islands rats. They also described the first case of eosinophilic meningitis caused by this parasite.

Genomics and Proteomics

The protein diversity of *A. cantonensis* has been demonstrated by SDS-PAGE. Peptide spots are detected by tandem mass spectrometry. Two-dimensional differential gel electrophoresis (2-D DIGE) is a useful method in the study of proteomic changes. The MALDI-PSD MS techniques have been used to characterize the differentially expressed proteins spots of various stages of *Angiostrongylus* species. Other proteomic studies on nematodes have focused

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on analyzing gender- and/or species-specific antigens. The most abundant proteins detected by these techniques in *Angiostrongylus* species extracts include cytoskeleton-associated proteins such as actin, myosin light chain, alpha tubulin, tropomyosin, and collagen. These proteins play important roles in maintaining the form and integrity of nematodes. Additional proteins included cytochrome *c* oxidase, ATP synthesis, enolase, glutamine synthetase, ammonia glutamate ligase, methionine adenosyltransferase, and ABC transporter. Enolase is a glycolytic protein that has traditionally been limited to the cytosol. The proteomic study also found proteins which interfere directly with the host effector mechanism. Some are antioxidant proteins, including peroxiredoxin, thioredoxin, tumor protein, and dehydrogenase aldehyde. They detoxify reactive oxygen species, which could otherwise harm the host. Other noteworthy proteins are As37 and cyclophilin members of the immunoglobulin family. The latter are folding helper enzymes that belong to the peptidyl prolyl cis–trans isomerase class. The systematic profiling of proteins contributes to our understanding of the parasite's physiology. Most proteomic experiments have been performed on *Caenorhabditis elegans*, a free-living soil nematode, which is a convenient model for in-vivo studies. One of the main limitations to performing such studies in *Angiostrongylus* worm is the scarcity of genomic information available, which may hamper faster progress in this area. *Angiostrongylus* species have been identified by molecular differentiation and phylogenetic trees based on small-subunit internal ribosomal DNA sequences transcribed spacer 2 (ITS-2), cytochrome c mitochondrial oxidase subunit (COI), and 66 kDa protein gene of *A. cantonensis*.

Parasite Morphology

Adult Worm

A. cantonensis adult worms are filariform, with the body tapering toward both ends (Fig. 1). They have three outer protective collagen layers. The males are 15–25 mm long and 0.25–0.35 mm wide, while females measure 18–35 mm × 0.28–0.5 mm. Males have a copulatory bursa at the posterior end, and female worms have the appearance of a *barber pole*, as white uterine tubules spiral around the blood-filled intestine, creating a red-and-white spiral (Fig. 1). At the base of the oral cavity, there are minute triangular teeth. The ovaries are present posteriorly. The vulva appears as a transverse slit situated at the posterior end. Microscopically, spicules are present, which are slender, striated and of equal length. There is a gubernacular present. *A. costaricensis* males measure about 20 mm and females 30–40 mm in length. The cephalic ends of the spicules are blunt, and the caudal tips are pointed, and ovoid eggs are laid in mesenteric arterioles. The caudal bursa of the adult male is an apparatus used for mating with females and helps to distinguish between genera and subgenera of Angiostrongylidae.

Eggs

Angiostrongylus unembryonated eggs are laid in pulmonary arteries of infected hosts and are oval,

Fig. 1 *Angiostrongylus cantonensis*. Adult male (left) and female (right) (copulatory bursa is at the posterior end of the male and a "barber pole" appearance in the female) (courtesy: CDC)

thin-shelled and transparent. The eggs are not normally excreted out in the feces but remain sequestered in tissues. One female may lay up to 15,000 eggs per day. These eggs can be seen in biopsied samples of the intestinal tissue, where the eggs are engulfed within giant cells and/or form granulomas.

Larvae

The first-stage *Angiostrongylus* larva (L1) measures 0.27–0.30 mm in length and has a notched tail. The second-stage larva (L2) measures 0.42–0.47 mm in size, surrounded by the first cast skin, and molts again to get enclosed by two skin sheaths. The third-stage larva (L3) measures 0.42–0.49 mm in size and remains in the two cast skins before skin sheath is digested in rats to release the larva. The fourth-stage larvae have a pointed tail and are only present in the definitive host. The esophagus-intestine junction characteristically separates the larval body into an anterior section with a few refractive granules and a posterior section with dense granules. The third-stage (L3) larvae are the infective stage for mammalian hosts.

Cultivation of the Parasite

Uga et.al (1982) cultured eggs of *A. cantonensis* in NCTC 109 medium supplemented with an equal volume of rat, horse, fetal calf, calf, or bovine serum at 37 °C. They observed that the eggs were 8- to 16-cell stage on the first day; on the third day, the 32-cell stage developed; and the eggs were fully embryonated on the fifth day, with hatching of larvae by the eighth day. In the inactivated rat serum, 64% eggs got embryonated, but only 10% got hatched. Male and female adult worms obtained from the lungs of rats were cultured on NCTC 109 with an equal volume of horse serum. The eggs obtained became embryonated within 30 min.

Hata and Kojirna (1990) isolated the first stage of larvae from Wistar rats that were experimentally infected. The fecal material obtained from the rat was washed with Chernin's balanced salt solution (CBSS) containing an antibiotic solution, followed by centrifugation at 1000 RPM for 1 min. Culture tubes containing 5 mL of media and larvae were further cultured at 27 °C in 5% CO_2–95% air or 5% CO_2–95% N2. In their experiment, a CBSS-based solution containing 10% L-15 (GIBCO), 10% tryptose bottom (TPB), 20% fetal serum, and 26 mM sodium bicarbonate was the best culture medium for development to the third-stage larvae. The first-stage larva's initial development, as seen in the expansion of the large intestine, was followed by the emergence of many nutrient granules and formation of a curved worm. The second-stage larva (L2) was characterized by ensheathment. On 50th day, the third-stage larva (L3) developed and shed its sheath and eventually formed the adult worm. The larvae were also cultured in RPMI 1640 medium supplemented with the calf serum. About 30% of the worms developed from the third stage, however, died slowly. The fourth-stage larvae obtained from the brain of infected rats were cultured in Waymouth-defined medium (MB 752/1), of which about 74% grew into adult worms, though did not survive for long.

Adult worms of *A. costaricensis* were made to shed eggs in Hank's salt solution at 37 ° C incubated for 3 h. The eggs cultured in Ham's f-12 nutrient mixture under 8% CO_2–92% air became embryonated after 5 days of culture. The first-stage larvae hatched by the tenth day. These first-stage larvae were fed into the snail intermediate host, where they developed into third-stage larvae.

Cultivation of larvae: The third-stage larvae (L3) were cultured in Waymouth culture media. After 28 days of incubation, the larvae grew into adult worms. Addition of mouse red blood cells enhanced the development of the larvae. Hata et al. found that the addition of choline and tryptophan was required for the optimum growth of these nematodes.

Laboratory Animals

Mature Wistar rats (*Rattus norvegicus*) are used in experimental infection by *Angiostrongylus* spp. The nematode causes pulmonary, vascular, and

cardiac changes in the infected rats. It also causes confluent granulomas (often centered on egg nests) and fibrotic nodules in the lungs. Wild rats are also used for experimental infection by *Angiostrongylus* spp.

Life Cycle of *Angiostrongylus* spp.

Hosts

Common rats of genus *Rattus* are the definitive host of *A. cantonensis*. The intermediate host is terrestrial mollusks such as slugs and snails. The land snails *Achatina fulica* and *Pomacea canaliculata* are the common intermediate hosts. Crabs, shrimp, prawns, and snails-eating lizards are the paratenic hosts.

Infective Stage

L3 larva is the infective stage.

Transmission of Infection

Angiostrongylus adults residing in pulmonary arteries of infected rats lay the eggs. These eggs hatch in pulmonary capillaries to give rise to first-stage larvae, which subsequently are excreted out in the feces of the infected rodents. The first-stage larvae are ingested by mollusks, an intermediate host, in which they develop after two molts into the infective third-stage larvae in about 15–20 days.

Cerebral angiostrongyliasis caused by *A. cantonensis* is acquired by ingestion of a mollusk carrying the L3 larvae, which are the infective-stage larvae (Fig. 2). The larvae are released into the digestive tract and move through the hepatic portal system and the lungs to the nervous system, where they undergo two molts. Young worms gradually move to pulmonary arteries through the cerebral vein. On the other hand, in abdominal angiostrongyliasis, the larvae are deposited in the feces of the rodent and are consumed by a mollusk, in which the L3 stage of development takes place. After ingestion of mollusks by rats or ingestion of contaminated vegetation, the L3 larvae migrate via lymphatics. After two molts, the larvae migrate to ileocecal arteries, where they mature into adult worms to lay eggs in the intestinal wall.

Humans are the accidental hosts. They acquire the infection on ingestion of mollusks, shrimps, and crabs infected with the L3 larvae. The larvae then migrate to the brain parenchyma, rarely to the lungs or eyes. In the brain parenchyma, the larvae cause increased levels of eosinophils, leading to eosinophilic meningitis and encephalomyelitis in about 2 weeks.

The life cycle of *A. costaricensis* is similar to that of *A. cantonensis* except that adult worms get lodged in mesenteric arteries of the cecum of the definitive host. Slugs (*Vaginulus plebeius)* and terrestrial snails (*Bradybaena similaris)* are the intermediate hosts. The L3 stage larvae are released into the mucus of the intermediate host. The L3-stage larvae that penetrate the cotton rat (*Sigmodon hispidus*) get trapped inside granulomas, which are formed in the intestinal wall and get released in the feces. Humans acquire infection, which occurs after accidental ingestion of infective slugs or vegetables and salads contaminated by the mucous secretion of these slugs or snails. The L3 larvae eventually form adult worms inside mesenteric blood vessels of infected humans. These adult worms cause arthritis, thrombosis, infarction, and hemorrhages. Eggs present in capillaries cause severe inflammatory reactions. The degraded eggs present in the appendix and terminal ileum cause eosinophilic granulomatous reactions.

Pathogenesis and Pathology

An eosinophilic CSF pleocytosis is the characteristic feature of *A. cantonensis* infection in humans, which occurs as a result of larvae migration and larval death in brain tissue. Very rarely, the lungs are also affected. The tracks and microcavities observed in the brain and spinal cord are the structural damage caused by the movement of the larvae in the tissue. Focal lesions in the brain are generally absent, which may help to differentiate neural

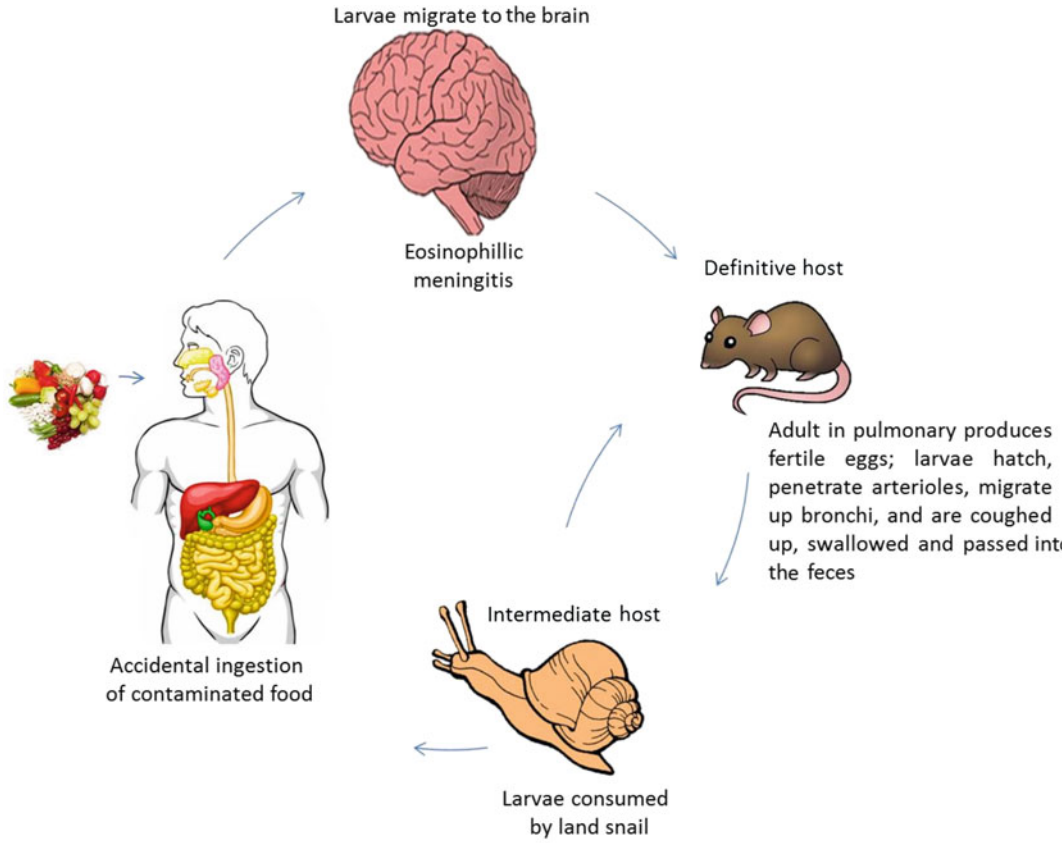

Fig. 2 Life cycle of *Angiostrongylus* spp.

angiostrongyliasis from neurocysticercosis and gnathostomiasis. In infected rodents, *A. cantonensis* infection is limited to the central nervous system and lungs. The migrating larvae cause inflammatory reactions in these organs. Usually, leptomeninges are involved in granuloma formation and hemorrhage around the dead larvae.

Human *A. costaricensis* infection is restricted to the intestines and mesentery. Arteritis, thrombosis, infarction, and gastrointestinal hemorrhage may occur due to the presence of adult parasites in these arteries. Degeneration of eggs causes severe inflammatory reaction in the intestinal wall, which results in an eosinophilic granuloma. The terminal ileum and appendix are the most common sites that are involved in the infected rodents such as *Sigmodon hispidus, S. angouya*, *Oligoryzomys fulvescens,* and *Rattus rattus*. *A. costaricensis* adult worm is found in the ileocecal branch of the cranial artery and subserosal arteries of the cecum. Macroscopically, there is perivascular edema and thinning of the cecal wall. Histological changes include subserosal edema, atrophy of mesenteric fat, and enlargement of ileocecal lymph nodes. Minimal inflammatory reaction against eggs or larvae is observed.

Immunology

Eosinophilic meningitis is the most common presentation of *A. Cantonensis* infection in humans. Initial response to invading larvae occurs in leptomeninges and local lymph nodes. Immune cells at these sites recognize the parasite with the help of pattern recognition receptors (PRRs). These receptors include pathogen-associated molecular pattern (PAMP) and endogenous stress

signals termed as danger-associated molecular patterns (DAMP). Antihelminthic mechanisms are seen in various immune cells such as eosinophils, activated macrophages, basophils and IgE and IgM antibody response. Eosinophils cause the release of proteases, which initiate attack parasites with mediators such as NO and H_2O_2, contributing to the pathogenesis of eosinophilic meningitis.

Infection in Humans

Meningitis is the most common clinical presentation of angiostrongyliasis caused by *A. cantonensis*. Projectile vomiting, headache, photophobia and neck stiffness and fever are the typical presentations. Paraesthesia lasting for many months is typically noted in extremities. Cranial nerves palsies are uncommon, except for the involvement of abducent and facial nerves. Most of the cases recover without serious complications. Death occurs with the involvement of critical areas of the brain.

A. costaricensis infection typically involves the small intestine and its blood vessels. The presentation mimics that of acute appendicitis. Larvae enter mesenteric arteries to cause arteritis, thrombosis, infarction, and hemorrhage. Degenerative eggs in capillaries cause an increased response from eosinophils, which may cause eosinophilic vasculitis. Most of the cases resolve on their own, but in some cases, death due to complications such as intestinal obstruction and perforation has also been reported.

Infection in Animals

A. vasorum causes canine angiostrongyliasis in dogs, the symptoms of which depend on the load of the parasite. Migration of the larvae in the brain and other tissues causes cervical and lumbar pain, paresis of the hind limbs, and intracranial and subdural bleeding. Uveitis is also a noted presentation. Infected rodents do not suffer from the disease.

Epidemiology and Public Health

A. cantonensis infection been documented beyond the Indo-Pacific zone, in Madagascar, Cuba, Egypt, Puerto Rico, New Orleans, Louisiana, and Port Harcourt, Nigeria, and India (Fig. 3). The majority of human infections were reported in Taiwan, Thailand, and China. Amazonian apple snail (*Pomacea canaliculata*) introduced in Taiwan and China during the 1980s contributed significantly to the spread of the disease (Table 1). The ingestion of *Pila pickled snails* was linked with cases of eosinophilic meningitis in Thailand. Other cases were related to the consumption of raw *Achatina fulica* snails.

It is postulated that *A. cantonensis* infection is not always associated with the consumption of mollusks, snails, or slugs but even may occur among vegetarians (Table 2). This is due to the larvae, which remain viable in the slime of snails or slugs and cause infection if contaminated unwashed vegetable materials are ingested. In Thailand, India, and Sri Lanka, uncooked monitor lizards and frogs have been recognized as important sources of infection. An infected mollusk may inadvertently be ingested by cold-blooded paratenic hosts such as freshwater shrimps or crabs. Larvae remain viable in these paratenic hosts and, if ingested, can cause infection in humans.

A. costaricensis infection is a major public health problem in South America and North America. In South America, particularly Costa Rica, *A. costaricensis* affects 12/100,000 persons with about 500–600 new cases every year. Most of the cases have been reported in children, with a higher incidence in boys than in girls. Due to low sanitary standards, thousands of people are infected, especially in slum areas of Venezuela, Colombia, and Brazil. *A. costaricensis* infection has been reported among primates such as

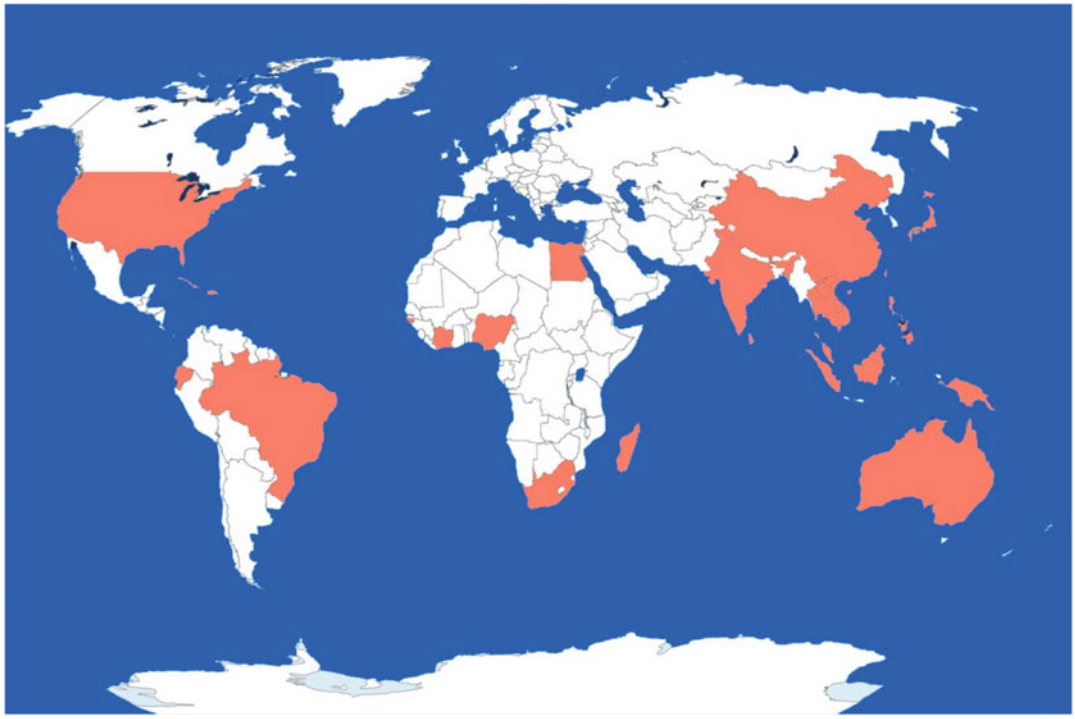

Fig. 3 Countries where *Angiostrongylus cantonensis* has been detected in naturally infected hosts. Shaded countries are those in which *A. cantonensis* was identified by screening naturally infected animals or where humans have acquired infections. Unshaded countries include those that are yet to find evidence of *A. cantonensis* or countries where studies examining potential hosts for *A. cantonensis* infection have not been carried out

Table 1 Epidemiological data of *Angiostrongylus costaricensis* in humans

Countries	Number of cases (%)
Costa Rica	198 (89.6)
Brazil	6 (2.7)
USA	4 (1.8)
Spain	2 (0.9)
Guadeloupe	2 (0.9)
Venezuela	1 (0.5)
France	1 (0.5)
Dominican Republic	1 (0.5)
Honduras	1 (0.5)
Panama	1 (0.5)
Zaire	1 (0.5)
Martinique	1 (0.5)
No data	2 (0.9)

Table 2 Epidemiology of *Angiostrongylus* species

Species	Hosts	Site	Intermediate hosts
Angiostrongylus vasorum	Dog, fox	Heart, pulmonary vessels	Slugs and snails
Angiostrongylus cantonensis	Rat, human	Pulmonary artery (rat), meninges (humans)	Slugs and snails
Angiostrongylus cantonensis	Rat, human	Ileocecal arteries (rat), intestines (humans)	Slugs and snails

Table 3 Diagnostic modalities in angiostrongyliasis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	CSF wet mount/anterior eye chamber, histopathology of intestinal tissues	Eggs/larval forms in intestinal tissues; third-stage larvae in CSF; eosinophilia	Not very sensitive. Specificity is very high
In vitro cultivation	Continuous-flow culture system	Transformation of larval form to adult.	Complicated procedure; live larva needed.
Serology	ELISA, indirect fluorescent antibody; immunological rapid dot immunogold filtration assay	The purified proteins such as 29 kD, 31 kD, and 32 kD are specific	The sensitivity and specificity to detect human serum IgG4 using these antigens were found to be 75% and 95%, respectively
Molecular diagnosis	Real-time PCR assay/ conventional PCR/LAMP	ITS-1 and ITS-2 rRNA/A. cantonensis gene sequence encoding a 66 kDa protein/18S rRNA gene	Species and genotype identification. No cross-reactivity with DNA from *Clonorchis sinensis* and *Gnathostoma spinigerum*
Other laboratory tests	Hematological investigations/imaging studies	Leukocytosis and eosinophilia. In cerebral ancylostomiasis, hyper-intense lesions in the punctate areas of the brain, abdominal angiostrongyliasis, small-bowel thickening	Detected in chronic infection

opossum and racoons in various zoos in North America.

Diagnosis

Diagnosis of angiostrongyliasis is based on clinical and laboratory findings (Table 3). Differential diagnosis of angiostrongyliasis needs to be considered in areas where the disease is endemic.

Microscopy

A conclusive diagnosis of *A. cantonensis* would require finding the larvae in the CSF or in the anterior eye chamber, though it is a rare finding. Eosinophilic meningitis is suggested with the presence of more than 10% of eosinophils in the CSF. In a wide range of conditions, low CSF eosinophils may be found, but finding more or $\geq$10% eosinophils in the total CSF leukocyte count suggests cerebral angiostrongyliasis. Typically, 100–5000 leukocytes/mL are present in the CSF, 10–90% of which are eosinophils. The CSF protein remains elevated, but the glucose is normal or slightly reduced. Worm recovery from the CSF is uncommon.

Definitive diagnosis of abdominal angiostrongyliasis is made by demonstrating parasitic larvae or its eggs in tissue (Fig. 4). Clinically, patients are with symptoms of acute appendicitis. The condition is associated with raised peripheral blood eosinophilia in about 30–80% patients. The worm is usually present in mesenteric arteries and the intestinal wall, where it causes

inflammatory reaction, thrombosis, infarction, and occlusion of arteries. There may be increased spasticity and bowel edema. Other parasitic diseases which may cause similar presentations are anisakiasis and *Enterobius vermicularis* infection. A stool examination is warranted to rule out these parasites.

Serodiagnosis

ELISA employing purified antigens from both adult and young *A. cantonensis* worms has been used for the detection of antibodies in the serum and CSF. Infected individuals show an elevated level of IgG, IgA, IgM, and IgE levels, with higher levels of IgM and IgE, in the serum as compared to the CSF. A dot-blot ELISA using blood dried on a filter paper has proven to be convenient for handling field samples for epidemiological surveys. The purified *A. cantonensis* proteins such as 29 kD, 31 kD, and 32 kD have also been used with success. The sensitivity and specificity to detect human serum IgG4 using these antigens were found to be 75% and 95%, respectively.

Molecular Diagnosis

Molecular methods have been evaluated for the detection of *A. cantonensis* in invertebrate hosts. A conventional nucleic acid amplification test (NAAT) has been developed that amplified a 1134 bp fragment from the parasite's 18S rRNA gene, in addition to a real-time PCR assay (TaqMan) targeting the internal transcribed spacer-1 (ITS-1). But, this molecular assay is yet to be validated for clinical use.

Other Tests

Imaging methods are frequently used in the diagnosis of angiostrongyliasis. Imaging studies can be useful for the differential diagnosis of the disease from other parasitic diseases, such as cysticercosis, paragonimiasis, gnathostomiasis, and schistosomiasis.

Computed tomography (CT) scan of the head is usually non-specific in cases of cerebral angiostrongyliasis. Magnetic resonance imaging (MRI) with contrast, however, may reveal abnormal enhancing lesions in the brain, especially hyper-intense T2 signal lesions in the punctate areas of the brain. The CT images in the case of abdominal angiostrongyliasis may depict multiple ill-defined non-specific hypoattenuating lesions in the liver parenchyma with small-bowel wall thickening.

Treatment

Treatment of eosinophilic meningitis is based mainly on supportive therapy. Both albendazole and mebendazole have been evaluated for the treatment of the condition. Albendazole is administered in a dosage of 15 mg/kg/day in two divided doses for 14 days. It is also well tolerated and achieves higher concentration in the CNS as compared to mebendazole. Randomized trials have shown that combining anthelmintics with corticosteroids significantly reduced the symptoms of eosinophilic meningitis. Anthelmintic drugs are not effective in the treatment of abdominal angiostrongyliasis. Acute symptoms that are uncomplicated resolve spontaneously.

Case Study

A 45-year-old man had a 5-day history of headache, nausea, vomiting, diarrhea, insomnia, and photophobia. He was a cleaner on a ship yard. The patient used to eat shrimps and freshwater prawns. When asked for more information, he mentioned a number of rats in the ship that contaminate the food. On clinical suspicion of meningitis, lumbar puncture was done: CSF cytology showed CSF: 1067 cells/mL, 25% eosinophils, glucose 2.6 mmol/L, and protein 0.54 g/L. CT head showed suspected vascular occlusion in the

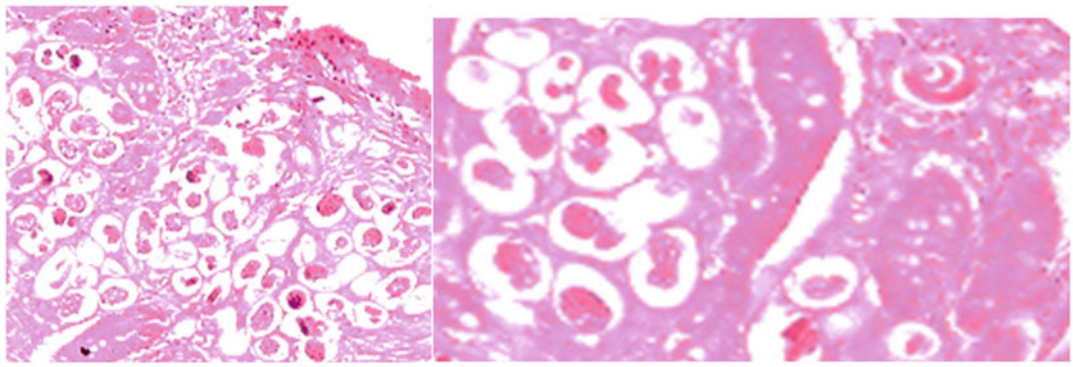

Fig. 4 Thin-shelled *Angiostrongylus* eggs in the intestinal tissue stained with H&E (left). A magnified view of thin-shelled *Angiostrongylus* eggs with different larval stages (right) (courtesy: CDC)

supply area of the right middle cerebral artery (MCA).

(a) What is the likely diagnosis?
(b) What could be the causative agent in this case?
(c) How will you diagnose the condition in laboratory?
(d) What is the treatment of choice in this case?

Research Questions

1. What are the alternate and lesser known routes of transmission of *Angiostrongylus* in humans?
2. What antigens can be used to develop a sensitive and specific immunological test for diagnosis of *Angiostrongylus* in CSF samples?
3. Which drug can be used for a radical treatment of angiostrongylosis?

Further Readings

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Trichostrongyliasis

Vinay Khanna

Learning Objectives

1. To understand the importance of resistance of the larva to environmental conditions and to survive for long periods.
2. To learn that the parasites can cause malnutrition and anemia in the infected individual.

Introduction

The nematode, *Trichostrongylus* spp., is primarily a parasite of herbivorous animals with worldwide distribution. More than 30 species of *Trichostrongylus* are known to cause infections in both humans and animals, the most common being *Trichostrongylus orientalis, Trichostrongylus colubriformis, Trichostrongylus axei, Trichostrongylus affinis, Trichostrongylus sigmodontis,* and *Trichostrongylus tenuis.* The importance of *Trichostrongylus* nematodes lies in their role in causing substantial production losses in farm animals, through reduction in weight, meat, and/or milk production. *Trichostrongylus* spp. are ubiquitous parasite and are common among livestocks such as goats, cattle, pigs, horses, and poultry. Infection among wild herbivorous animals such as deer, antelopes, camels, monkeys, and wild boars has also been reported. Human trichostrongyliasis, caused by *T. orientalis, T. colubriformis, and T. axei,* are most prevalent in the Middle East, Asia, and Africa.

History

Giles in 1892 described *Strongylus colubriformis* from the intestine of sheep in Shillong (Assam) and Sanawar (Punjab) regions of India. Railliet in 1893 described *Strongylus instabilis* from the intestine of European sheep, and Looss in 1895 described *Strongylus subtilis* from the gut of man and later of the sheep in Egypt, while Ijima has identified it as far East as Japan. Looss introduced the term genus *Trichostrongylus* for these nematodes and allied forms. In 1930s, Walter E. Collengae has made a note on the life cycle of *T. tenuis,* while investigating the cause of "*grouse disease*" in partridge birds. The fecal contents of infected partridge grew larvae of *Trichostrongylus*.

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Taxonomy

Trichostrongylus spp. belongs to the order Strongylida that contains four suborders, namely Ancylostomatina, Strongylina, Trichostrongylina, and Metastrongylina. The Strongylina and the Ancylostomatina have a well-developed buccal capsule and are distinguished from the Trichostrongylina and the Metastrongylina, which have reduced or absent buccal capsule. Based on morphological characters and their presumed evolution, trichostrongyloids are divided into 14 families and 24 subfamilies. The genus *Trichostrongylus* has been placed under family Trichostrongyloidea; the genus *Trichostrongylus* consists of more the 30 species, of which more than 10 species are known to cause human infections. The important *Trichostrongylus* species causing human infections include *T. axei*, *T. tenuis, T. colubriformis*, *Trichostrongylus longispicularis*, *Trichostrongylus retortaeformis*, *Trichostrongylus capricola*, and *Trichostrongylus vitrinus*.

Genomics and Proteomics

The genome sizes of *Trichostrongylus* spp. are estimated to contain 53–59 Mb genomes as established using flow cytometry. As a tool for knocking down the expression of individual genes post-transcriptionally, RNA interference (RNAi) knockout technique has been widely used to study the cellular function of genes in *Trichostrongylus* spp. The RNA interference (RNAi) is a process by which dsRNA induces sequence-specific gene silencing by targeting mRNA for degradation. RNAi knockdown techniques have been successfully used for *T. colubriformis* and *T. vitrines*. Expressed sequence tags (ESTs) are short (200–500 nucleotides) DNA sequences that can be used to identify a gene that is being expressed in a cell at a particular time. Expressed sequence tags (ESTs) are available for *Trichostrongylus* spp. at dbEST GenBank. The transcriptome (complete set of RNA transcripts) studies of adult *T. colubriformis* have been described that has identified molecules, which played pivotal roles in essential biological processes in this parasite.

The Parasite Morphology

Adult Worm

Sexes are separate, and the males are generally smaller than the females. Adult *Trichostrongylus* are small, whitish, and hair-like, and measure less than 1 cm in length. Mouth part does not contain buccal capsule. They have characteristics such as excretory notch in the esophageal region and the copulatory bursa and spicule at tail end. The gubernaculum is bent anteriorly at right angles. Adult males have a bilobed copulatory bursa and two spicules and measure between 3.8 and 8.2 mm. Brown-colored paired spicules are characteristic features of male worms (Fig. 1). Females are larger and generally measure between 4.9 and 9.8 mm in length. They are slender and pink colored with a posterior vulva. The mouth is unarmed.

DNA-based technology is used for species identification, particularly for female worms of morphologically indistinguishable *Trichostrongylus* species. Definitive identification of the species is made by means of internal transcribed spacer (ITS) region ribosomal gene sequence of *Trichostrongylus* spp. These molecular techniques are very useful in understanding the distribution, speciation, and prevalence of *Trichostrongylus* spp. in the endemic area.

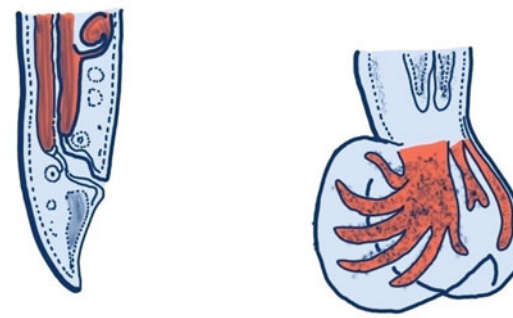

Fig. 1 Posterior end of female and male adult worm of *Trichostrongylus* spp. Note the pointed tail in female worm and presence of a bursa and spicules in male worm

Larva

The third-stage strongylid larvae (L3) are the infective stage of the parasite, which measure between 622 and 796 μm in length. The head is tapered, and the tail sheath is short. The tail may end in one or two tuberosities. Hatched ensheathed larvae of *Trichostrongylus* spp. need to be differentiated from rhabditiform larvae of both hookworm and *Strongyloides stercoralis*. *S. stercoralis* larva has a short buccal cavity, whereas both hookworm and *Trichostrongylus* have a long buccal cavity. *Trichostrongylus* larvae, however, have a distinctive bead-like swelling at the end of its tail (Fig. 2).

Eggs

Trichostrongylus spp. eggs are large, elongated, and thin shelled (73–95 by 40–50 μm) and are tapered at one end. The eggs are covered with transparent hyaline shell with inner membrane frequently wrinkled. They are passed in the stool in the advanced cleavage stage (16–32 morula stage). It is important to differentiate *Trichostrongylus* eggs from that of hookworm eggs. Their survival optimally depends on moist, shady, warm wet soil, where larvae get hatched from eggs within 1 to 2 days and are remarkably resistant to desiccation and cold (Fig. 3).

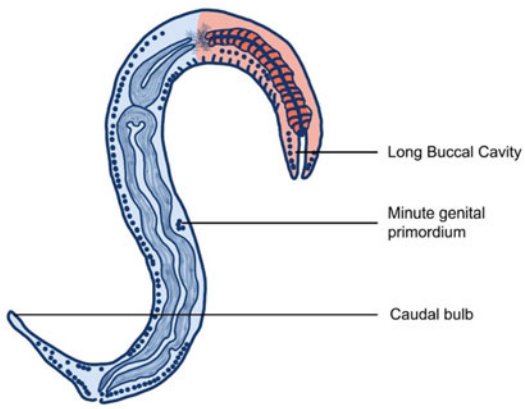

Fig. 2 Third-stage rhabditiform larvae of *Trichostrongylus* spp.

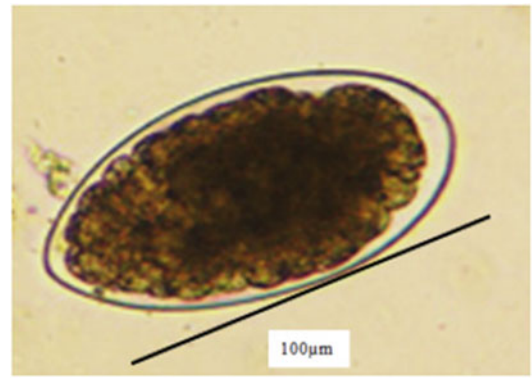

Fig. 3 Egg of *Trichostrongylus* spp. tapered at one end

Cultivation of Parasites

Two techniques are widely used for the fecal culture of *Trichostrongylus* larvae from eggs.

In first technique, feces are placed in a jar with a lid and kept in the dark place at a temperature of 21–24 °C. The loosely attached lid is lined with moist filter paper. After 7–10 days of incubation, the jar is filled with water and allowed to stand for 2–3 h. The water containing larvae is concentrated using sedimentation technique. The larval suspension is then cleaned and further concentrated by Baermann apparatus and killed with Lugol's iodine and examined microscopically. In the second method, the feces are spread on the middle of filter paper placed in a moistened Petri dish. After incubating at 21–24 °C for 7–10 days, the dish is flooded with water and the larvae are harvested.

Trichostrongylus can be grown in bacteria-free media from hatched larvae in the absence of animal tissue extract or serum. Approximately, 50% of fourth-stage larvae of *T. colubriformis* molt to the young adult stage in 7–10 days when cultured in a complex medium. The media used for the culture contain enzymatically hydrolyzed casein, yeast, phosphatidylcholine, and minerals including salt solution, sterol, and iron porphyrin. Infective larval production has been shown to be possible in peptone instead of casein hydrolysate but to a very limited degree, as it is one of the requirements for identifying free-living stage of the parasite. Successful cultivation of

T. colubriformis larvae from hatched first- to third-stage has been achieved in media containing NCTC 135, chick embryo extract, fetal calf serum, and either lactalbumin hydrolysate or freshly prepared baker's yeast extract. Cultured third-stage larvae, when injected in guinea pigs resulted in severe infection and also used for the species identification.

Laboratory Animals

Many animals are being used for various animal experimentation studies in laboratories. Gerbils are most frequently used for *T. colubriformis* and for many other nematodes such as *S. stercoralis, Ostertagia Circumcincta, Haemonchus contortus, Nematospiroides dubius,* and *Wuchereria bancrofti*. Gerbils are rat-like animals covered with bushy fur till the tail end. These animals are commonly found in China and Mangolia. These animals are also used to study pathogenesis and drug resistances in these nematodes.

The other rodent Woodchuck, which is large, dig burrowing having thick body, short legs, long claws, and big flathead with almost no neck, short hairy tail are also implicated to carry these nematodes. They are found in the eastern and midwestern USA. They harbor *Trichostrongylus* spp., *Baylisascaris laevis, Baylisascaris columnaris, Capillaria hepatica, Citrullinema bifurcatum,* and *Strongyloides* spp. These rodents are used to study the life cycle of these parasites.

Life Cycle of *Trichostrongylus* spp.

Hosts

Definitive hosts include mammalian herbivore such as rabbits, sheep, cattle, and rodents; humans are incidental host.

Various species of *Trichostrongylus* are related to their hosts as they help in the survival and infection of the parasite. For example, *T. tenuis* infects game birds (grouse, partridge, pheasant). *T. affinis* and *T. sigmodontis* infect cottontail rats and hispid cotton rats, respectively, while *T. retortaeformis* mainly affects *Oryctolagus cuniculus,* European rabbits. Humans are the accidental hosts.

Infective Stage

The infective stage, L3 larvae, is enclosed in the cuticle sheath of L2 larvae, which serves to prevent larval desiccation. The sheath not only protects the larva from harsh environment but also helps in its survival. Development and survival of the larvae depend on ambient temperature, with faster development of larvae occurs at higher temperatures whereas longer survival seen at lower temperature. Sufficient moisture in environment makes it possible for the larvae to spread from the animal feces onto the vegetation, from where they are ingested by grazing herbivores, thus facilitating its spread.

Transmission of Infection

Humans are the accidental host and acquire infection either by ingesting contaminated vegetables containing infectious larvae or by larval (L3) penetration of the skin (Fig. 4). Larvae mature to adults in the small intestine, where they gets embed in the mucosa and causes severe inflammation. Animals get infection while grazing the vegetation contaminated with larvae. Fully embryonated eggs and infective larvae are very resistant to both cold and dry climate. During a dry season, eggs get accumulated on animals, and during rainy season, mass hatching and development of larvae is seen, which finally results in spread of the disease to humans and animals.

The lifecycle has two distinct phases: (1) within the host and (2) the free-living stage, where the parasites are developing in the environment.

Direct life cycle: Definitive host passes unembryonated eggs in the faces. Under optimum

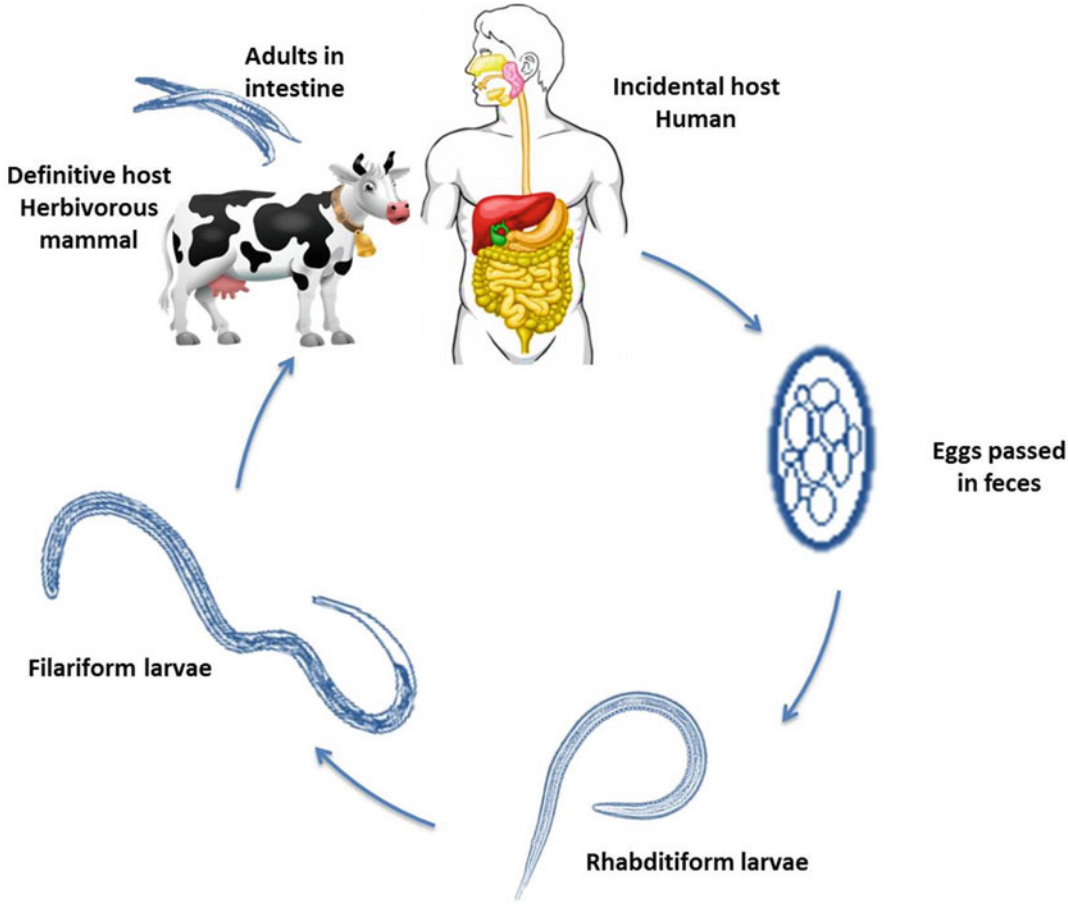

Fig. 4 Life cycle of *Trichostrongylus* spp.

conditions, rhabditiform larvae hatch in about 7–10 days and become infective filariform (third-stage) larvae after 5–10 days. The L3 larva gets ingested by the definitive host or by man. Humans acquire infection through ingestion of raw or undercooked contaminated leafy vegetables. Rarely, infection can occur through larval penetration of the skin. When ingested, these larvae exsheath under low oxygen tension, high CO_2 levels and rise of body temperature. The larvae penetrate the small intestinal mucosa. Within a short time, the larva molts to form L4. Underneath the intestinal epithelium, the larvae develop into the fifth-stage L5 in 2 weeks. Later L5 larvae releases exsheathing fluid containing enzyme leucine aminopeptidase that results in dissolution of its sheath. It then wriggles free from the sheath and develops into sexually mature male and female worms. The females are fertilized by the males, and subsequently, the gravid female lays eggs, which are passed out in the feces. Under normal development, the worms mature and begin producing eggs in around 3 weeks. If not, the worm enters *hypobiosis*, term used for arrested development, that occurs due to cessation of metabolic activities. Immune cells fail to recognize the hypobiotic worms, which give survival advantage to the parasite. The time taken for the development until maturity is known as the prepatent period. It normally takes 2–3 weeks for adults worms to produce eggs or larvae.

Free-living stage: Eggs that pass out of the host contain 16–32 blastomeres. These eggs

hatch in damp soil in 3–4 days, giving rise to L1 larvae. The L1 larvae undergo two molts and are transformed to L3 larvae, the infective stage, in the soil.

Pathogenesis and Pathology

Trichostrongylus larvae mature and develop in the small intestinal mucosa to form adult worms. The adult worms are buried underneath the duodenum and jejunum mucosa and rupture to liberate the young worms. A large number of these young worms cause considerable trauma, mucosal desquamation, edema, and hemorrhage in the affected tissue. They cause villous atrophy, distortion, flattening, or reduction in the area available for absorption. Plasma proteins are lost into the lumen leading to hypoalbuminemia and hypoproteinemia. In heavy infections, diarrhea has also been reported. Like hookworm infection, some patients pass small amounts of blood in the stools, especially if the egg count is between 100 and 400 eggs/g in feces. Eosinophilia is very common in this disease.

Degree of infestation in ruminants such as cattle has been classified as light if larval (L3) counts are less than 1000 L3/kg of feces, moderate when the counts exceed 1000 L3/kg, while values of over 5000 L3/kg of feces are considered as severe. Trichostrongylosis in ruminants is also known to impair the absorption of proteins and iron, and minerals such as calcium and phosphorus; the calcium and phosphorus deficiency often leads to osteoporosis in animals. Equine hypertrophic gastritis, caused by *T. axei,* is characterized by thickened rugae, as a result of gastric gland hyperplasia. This occurs due to chronic retention of gastric fluid and reflux of intestinal bile.

Immunology

The majority of immunological studies on *Trichostrongylus* spp. is performed using rodent model in standard laboratory setting. Observations from natural and experimental infections have shown that lower level of *Trichostrongylus* infection is consistent with longer survival rates. Intestinal T cells secrete IL-5, a potent eosinophil chemoattractant and activator in response to *T. colubriformis.* Correlation between elevated IgE, decreased fecal egg count and survival of adult have been reported in *T. colubriformis* infection in sheeps. Mucosal mast cells and eosinophils play an important role in maintaining immunity against Trichostrongylus in these herbivores.

Experimental infections of the rat with *Nippostrongylus brasiliensis* have shown that the adults may be stunted in size, and in some cases, these adult worms are killed and expelled automatically from the animal. The infection stimulates a slower immune response in herbivores, which causes development of immunity in these animals. Studies in sheep have indicated that Th2 responses are necessary to reduce these nematodes.

Infection in Humans

The clinical manifestations of *Trichostrongylus* infection in humans depend on the worm load. Low-intensity infections with *Trichostrongylus* may be difficult to distinguish from malnutrition.

Most patients are asymptomatic. Patients infected with large number of worms may lead to abdominal pain, diarrhea, and anemia. Malabsorption and wasting can ensue if mucosal damage is severe. There may be hemorrhage, edema, and desquamation of intestinal villi. Heavy infection may also cause biliary obstruction. Severe anemia may occur due to loss of blood, which is similarly seen in other nematodes such as hookworm.

Infection in Animals

Trichostrongylus spp. affects the abomasum and small intestine of animals causing parasitic gastroenteritis (PGE). Different species of parasites causes varied manifestations in different animals, for example in horses, *T. axei* chronic gastritis,

typhlitis, or hypertrophic gastritis, characterized by thickening of stomach rugae. *T. colubriformis* and *T. vitrinus* cause diarrhea (*black scour*) and weight loss in sheep and cattle. Severe enteritis results in hemorrhagic diarrhea, weight loss, and death in animals associated with high worm load. Some ruminants such as cattle and sheep also exhibit weight loss, cachexia, and metabolic disorders.

Epidemiology and Public Health

Trichostrongyliasis has worldwide distribution with reported cases from Iran, Iraq, India, Egypt, Indonesia, Australia, USA, Japan, Korea, China, Taiwan, Russia, Chile, Peru, and Brazil (Table 1). It is predominant in the area of poor hygiene, especially where animal/human feces are used as a fertilizer, which leads to contamination of vegetables and water. The important human species, which are known to cause human trichostrongyliasis, include *T. orientalis, T. axei, T. colubriformis, T. capricola*, *T. probolurus, and T. vitrines.* In Asia, *T. orientalis* have been documented from endemic regions such as Mainland China, Japan, and Korea. In Middle East, *T. colubriformis* has been commonly been reported. Highest rate of infections is seen in Iran (up to 70%) due to multiple *Trichostrongylus* species. The use of animal manure as fertilizer in farming communities is the main contributing factor of the spread of disease to humans. The contamination of farm vegetables and water is common in rural setting, due to poor sanitary conditions. Close human–animal interaction with one another is also another important reason of the spread of the disease.

Trichostrongylus has been responsible for considerable morbidity and mortality in ruminants. These nematodes are known to contaminate their environment through their biotic potential, in which the parasites are able to multiply and develop both in intermediate and in definitive hosts. In temperate regions, ability of larvae to survive in the cold and dry environment causes increase in number of animal cases for example, during spring season, which coincides with animals grazing out has high incidence of infection. At the advent of rains, a large numbers of apparently desiccated larvae become hydrated and active. These larvae rapidly multiply on vegetation. Many animals such as cattle, sheep, and goat gets infected while grazing through contaminated vegetation. Seasonal hypobiosis which occurs as a result of arrested development of larvae in host also contributes to disease as it leads to increase parasitic burden when conditions becomes optimal.

Diagnosis

Various modalities are used for the optimal diagnosis of trichostrongyliasis, in both humans and in animals (Table 2).

Human Trichostrongyliasis

Microscopy

Microscopic examination of stool specimens for parasite eggs is currently a routine laboratory practice for the detection of *Trichostrongylus* eggs. Stool concentration techniques may be needed, particularly in the setting of light infection. Primarily, identification of eggs, larvae, and adult worms is done using microscopy. The eggs are large, elongated, thin shelled (75–95 by 40–50 μm) and are tapered at one end. They are covered with transparent hyaline shell with inner membrane, which is frequently wrinkled. It is important to differentiate *Trichostrongylus* eggs from those of hookworm eggs. When excreted along with stool, *Trichostrongylus* eggs are relatively larger and are in the advanced cleavage stage (16–32 morula stage), while hookworm eggs are smaller (56–75 by 36–40 μm) and in 4- to 16-morula stage when excreted.

The microscopy is helpful in identifying characteristic *Trichostrongylus* larva. Hatched ensheathed larvae of *Trichostrongylus* spp. have to be differentiated from rhabditiform larvae of both hookworm and *S. stercoralis*. *Trichostrongylus* larvae have a distinctive bead-

Table 1 Distribution of *Trichostrongylus* species of importance in humans

Species	Distribution	Definitive host
Trichostrongylus orientalis	Mainland China, Korea, Japan, Indonesia, Loa, and Russia	Cattle, sheep, donkeys, goats, deer, rabbits, and humans
Trichostrongylus axei	Worldwide	Cattle, sheep, goat, deer, horse, donkey, pig, and occasionally human
Trichostrongylus colubriformis	Tropical and subtropical regions, mainly in Middle East	Sheep, goat, cattle, camel, and occasionally pig and human
Trichostrongylus vitrinus	Temperate regions mainly Australia	Sheep, goat, deer, camel, and occasionally pig and human
Trichostrongylus longispicularis	Australia, America, and parts of Europe	Cattle, goat, and sheep
Trichostrongylus tenuis	North America, Asia, and Europe	Game birds (grouse, partridge, and pheasant), chicken, duck, goose, Turkey, emu, and humans
Trichostrongylus retortaeformis	British Isles, Europe, and Australia	Rabbits and occasionally humans

like swelling at the tail end. Other differentiating features include long buccal cavity in hookworm and *Trichostrongylus* larvae, whereas *S. stercoralis* larvae have a short buccal cavity.

Microscopic identification of adult male worms is a reliable method to differentiate various species of *Trichostrongylus*. Adult *Trichostrongylus* are small, whitish, hair-like, and less than 1 cm in length. They have characteristics bursa, spicules, and a boat-shaped gubernaculum. Adult males are smaller than the females. Adult *Trichostrongylus* spp. are much smaller than those of hookworms, but their eggs are usually large.

Diagnosis of Animal Trichostrongyliasis

The diagnosis of *Trichostrongylus* spp. infection in animal is based upon the recovery of adult worms from the abomasum or small intestine. The eggs are narrower and little longer as compared to those of hookworm or *S. stercoralis*. Fecal egg count of $\geq$200 eggs per gram is considered to be significant worm burden, and specific anthelmintic treatment is recommended in these cases. Differentiation of Trichostrongylus from other genera of Trichostrongylidae family genus such as *Ostertagia, Haemonchus, Cooperia,* and *Oesophagostomum* is based on their L3 larvae

Table 2 Various diagnostic approaches in trichostrongyliasis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Routine stool wet mount/duodenal aspirate	Eggs, larvae, and adult worms	It is important to differentiate hookworm eggs from *Trichostrongylus* spp. Larvae may be mistaken for *Strongyloides*
In vitro cultivation	Continuous-flow culture system	Transformation of larval form to adult	Complicated procedure; live larva needed
Serology	Antibody-based ELISA	Coproantigen is used	The test shows high cross-reactivity
Molecular diagnosis	RFLP-PCR	ITS-1 and ITS-2 regions of rRNA	Species and genotype identification
Additional laboratory tests	Blood biochemistry and hematological investigations	Elevated serum levels of pepsinogen and trypsin. Elevated leukocytosis and eosinophilia	Useful for diagnosis in animals

Table 3 Identification of *Trichostrongylus* species based upon size, spicules, and gubernaculum

Species	Identification features
Trichostrongylus orientalis	Males are around 3.5–4.5 mm and females 5–7 mm in length. The spicules are light brown, with characteristic shape; one slightly bigger than the other
Trichostrongylus axei	Measured length in males 3.2–6.2 mm and in females 4–8 mm. The male spicules are dissimilar and unequal in length
Trichostrongylus colubriformis	Males measure around 4.5–5.5 mm and females 5.5–7.5 mm in length. Spicules are slightly irregular in length, with a structure similar to that of a small boat with a thick outcrop that closes the root proximally. The gubernaculum is seen laterally, in the shape of an oblique curve with two bends
Trichostrongylus vitrinus	Males are 4.2–6.2 mm and females 5–8 mm in length. Spicules are small and straight with sharply tapering at extremities
Trichostrongylus longispicularis	Males are around 5.6 mm in length. The spicules are brown, unbranched, and slightly thicker at proximal end. The gubernaculums are light brown in color, with simple shape
Trichostrongylus tenuis	Males are around 5.2–6.5 mm and females 7–9 mm in length. The spicules are curved distally
Trichostrongylus retortaeformis	Length is 6.8–8.4 mm and 9.6–10.4 mm for males and females, respectively, with characteristic thin transverse and longitudinal grooves. The spicules are short
Trichostrongylus probolorus	Males are 4.3–5.55 in length. Spicules are larger than those of other *Trichostrongylus* species. It is dark brown, with two triangular projections on the ventral side. The two spicules are roughly the same in length. The gubernaculum is a glossy dark brown one
Trichostrongylus capricola	Males are 4.3–4.9 in length. The spicules are thicker at the proximal end, and the distal ends of the spicules are less pointed. The gubernaculum is light brown

morphology. *Trichostrongylus* larvae are short sized (700–750 μm), with a rounded head and short tail sheath, which bears one or two tuberosities (Table 3).

Serodiagnosis

Various immunoassays including ELISA, using crude and purified antigens of *T. colubriformis,* have been developed to demonstrate *Trichostrongylus*-specific antibodies for the diagnosis of human trichostrongyliasis. However, till date no serodiagnostic tests are available commercially. Antibody-based ELISA is available to detect serum IgG antibodies for the diagnosis of animal trichostrongyliasis. Currently, diagnostic assays for the quantitative detection of *Trichostrongylus* coproantigens in the feces of infected animals have been evaluated. Nevertheless, the coproantigen assay has limitations due to cross-reactivity and fecal components interfering with the test reactivity.

Molecular Diagnosis

The molecular methods such as PCR are useful for differentiating various *Trichostrongylus* species. These tests also helps in the analysis of genetic variations and phylogenetic relationships between different species. The molecular tests are based on determining the ribosomal DNA sequences internal transcribed spacer (ITS1 and ITS2) regions. The multiplex RT-PCR has also been developed, which has shown better sensitivity and specificity as compared with conventional PCR.

Other Tests

Plasma Pepsinogen Assay: The estimation of circulating pepsinogen in serum is of value in the diagnosis of gastrointestinal infection in animals. “High serum pepsinogen level indicates an elevated Trichostrongylus spp. infection in animals”?

In this test, serum or plasma sample is acidified at pH 2.0, thus activating the inactive zymogen, pepsinogen to the active proteolytic enzyme, pepsin. The activated pepsin then reacts with a protein substrate (usually bovine serum albumin), and then enzyme concentration is measured. The tyrosine released from the protein substrate is determined by the appearance of blue color, which is formed after adding phenolic compounds that react with the pepsin. In normal or uninfected animal, the tyrosine level ranges less than 1.0 IU, while in moderate infection, the level ranges from 1.0 to 2.0 IU, and in highly infected animals, the tyrosine level typically reaches up to 3.0 IU, sometimes as much as 10.0 IU or more. This test is not standardized for diagnosis in human infections.

The postmortem diagnosis of trichostrongylosis in animals is employed to determine the intensity of infection and to assess anthelmintic efficacy in the infected animals.

Treatment

Treatment of human trichostrongylosis is by giving mebendazole, 100 mg twice daily for three consecutive days, or albendazole in a single dose of 400 mg given empty stomach. Pyrantel pamoate is also used in a single dosage of 11 mg/kg orally; maximum recommended dose is 1 gm. Ivermectin is a potent, broad antiparasitic spectrum at low doses in humans. It is active against many immature nematodes including hypobiotic larvae, in a dosage of 200 μg/kg daily for 1–2 days.

Numerous broad-spectrum anthelmintics such as albendazole, fenbendazole, mebendazole, levamisole, and several macrocyclic lactones (e.g., ivermectin) are used for the treatment of animal trichostrongylosis. These are effective against both adult worms and larvae. Fenbendazole is given in a dose of 10–20 mg/kg orally and repeated after 10–14 days. Albendazole is given in a dosage of 10 mg/kg orally. The ivermectin is given in a dose of 0.2–0.4 mg/kg orally and repeated after 10–14 days. The excessive and indiscriminate use of anthelmintics is the biggest drawback as it has led to resistance in sheep, goats, and cattle. Attempts to develop effective vaccines to circumvent resistance problems have largely been unsuccessful till date.

Prevention and Control

Improved sanitation, personal hygiene, and adequate nutrition as well as avoidance of raw vegetables in endemic areas often prevents *Trichostrongylus* infection in humans.

Infective *Trichostrongylus* larvae are resistant to cold and dryness. They survive on pasture for up to 6 months. Livestock exposed to these worms often develop natural resistance. Such resistant animals may continue shedding eggs that contaminate their surrounding environment and infects other livestocks.

Control of trichostrongylosis in animals depends on regular deworming of animals and proper pasture management. Numerous broad-spectrum anthelmintics (albendazole, ivermectin, pyrantel pamoate, etc.) effective against adult worms and larvae have been used widely in controlling trichostrongylosis in herbivores. Other preventive measures include rotational grazing of animals and reducing contamination of pastures by reducing exposure of livestock to other infected pasture. Maintaining adequate nutrition also reduces infection among animals. It has been established that a well-fed animal are resistant to these parasites.

Case Study

A 52-year-old man living in a rural area presented with chronic gastric pain. He was diagnosed as a case of chronic gastritis and was on treatment with proton pump inhibitors for long term. The patient condition did not improve with this therapy. Recently, patient started noticing mucus and blood while passing stool. The blood examination was done, and eosinophil count was found to be high. The stool examination is done, which showed helminthic eggs. The patient was put on albendazole, and he improved clinically in 2 weeks.

1. What are the likely causative agents in this case?
2. What are the different *Trichostrongylus* species that cause infections in humans?
3. What additional laboratory tests are required to differentiate species of *Trichostrongylus*?
4. How to prevent this infection in humans?

Research Questions

1. Which animal model can be ideal to study immunological responses in *Trichostrongylus* infection?
2. How to improve the microscopic techniques for better identification of the egg and larva of *Trichostrongylus* spp.?

Further Readings

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Toxocariosis

V. C. Rayulu and Manigandan Lejeune

Learning Objectives

1. To understand the importance of this parasite is not only to cause visceral larva migrans, but also as an agent for ocular and neuro-toxocariosis.
2. To highlight the critical role played by serology in the diagnosis of the condition.

Introduction

Human toxocariosis is a soil-transmitted zoonosis primarily caused by *Toxocara canis* and probably to a lesser extent by *Toxocara cati*. Man's penchant for surrounding themselves with cats and dogs as companion animals along with rise in stray dog populations has ensured a worldwide distribution for toxocariosis. Two parasites of dogs that fall under this discussion are members of same nematode family: Toxocaridae, viz. *T. canis* and *T. cati* (syn. *Toxocara mystax*). Most humans infected by *T. canis* do not develop overt clinical disease as the larvae cannot establish patent infection in humans. *T. canis* infection in humans is manifested as syndromes, viz. covert toxocariosis (CT), neurological toxocariosis (NT), visceral larva migrans (VLM) and ocular larva migrans/ocular toxocariosis (OLM/OT).

History

Though the first description of *T. canis* was dated back to as early as 1782 by Werner, the parasite was reported with diverse names like *Belascaris marginata/Ascaris marginata/Ascaris lumbricus*, until the genus name was established by Stiles (1905). Earliest documented human case of toxocariosis was established by Wilder (1950), providing the first description of *ocular larva migrans* (OLM). Further, the term *visceral larva migrans* (VLM) and its clinical syndrome were described by Beaver and colleagues (1952), who for the first-time recorded *T. canis* larvae in the liver biopsy of a human patient suffering from chronic eosinophilia. Neural form of toxocariosis came into limelight after the published report of Beautyman and Woolf (1951), who described the encapsulated larvae in the brain of a child. In 1970, Woodruff stated that this disease is more common in tropics as compared to temperate zones, and from an epidemiological point of

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view, the pioneering report of *T. canis* from the Indian subcontinent was from the parasite collection of the Zoological Survey of India (Baylis and Daubney 1922). Despite the high incidence of toxocariosis in dogs in India, the first human case of VLM was recorded after many decades in the late twentieth century by Singh and his associates in 1992.

Taxonomy

The genus *Toxocara* (Stiles 1905) belongs to the family Toxocaridae, order Ascaridida, class Chromadorea and phylum Nematoda.

The type species are *T. canis* (Werner 1782) and *T. cati* (Schrank 1788) Brumpt, 1927 (syn: *T. mystax*).

Genomics and Proteomics

The genome size of *T. canis* is about 300 MB. Approximately 20,000 coding genes were annotated in this species. When compared with closely related species *like Caenorhabditis elegans, Trichinella spiralis, Brugia malayi* and *Ascaris suum*, it was observed that the *T. canis* genes are more similar to *A. suum.* About 6000 genes were observed to be unique to this species when compared to the four other species.

The mitochondrial genome of *T. canis* is 14,309 bp. The mitochondrial genome encodes 12 protein-coding genes, 2 rRNA genes and 22 tRNA genes. All the mitochondrial genes are located on same strand and transcribed in the same direction. Phylogenetic analysis based on the protein-coding genes suggested that the *T. canis* is paraphyletic with cogeneric species *T. cati* and *Toxocara malayensis*. Transcriptomic investigations, till date, have mainly focused on the secretary–excretory protein of migrating larvae, which aimed at designing new diagnostic and intervention strategies. A total of 19 proteins are present in the secretary and excretory protein group in this species. Most of the gene ontology terms associated with the secretary and excretory proteins are related to binding functions like protein binding, inorganic binding and organic cyclic compound binding.

The Parasite Morphology

Adult Worms

Adult worms of *T. canis* occur in the small intestine of dogs and wild carnivores. The males are 10–12 cm long, and the females measure 12–18 cm. Three prominent lips at the cephalic end, with each lip bordered with dentigerous ridges, are a typical feature of both males and females. Obvious lateral hypodermal cords and prominent cervical alae in both sexes are also characteristics. Finger-like projection of the tail end of male is a diagnostic feature. The male has a single tubular testis and simple spicules without gubernaculum. In female, the ovaries are large and coil extensively leading to uteri, which may contain millions of eggs. The vulval opening is about one-third from the cephalic end typical of opisthodelphic presentation. Adult parasites of *T. cati* are smaller than *T. canis* with males 3–6 cm and females 4–10 cm long. The distinguishing feature of *T. cati,* cervical alae, is overly broad and striated. The major differentiating features of *T. canis* and *T. cati* worms are depicted in Table 1.

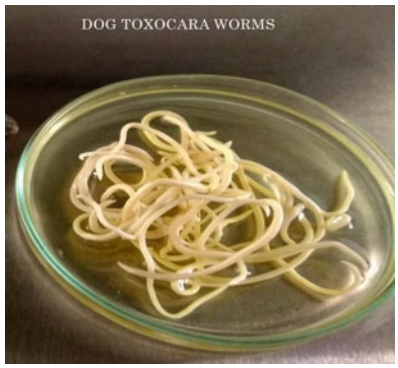
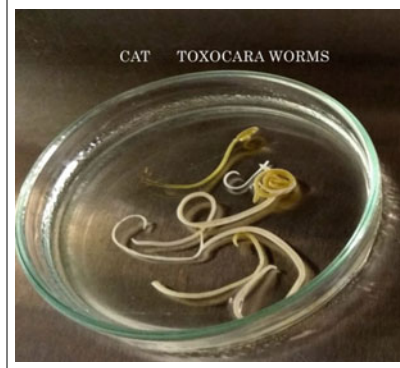
Eggs

Toxocara eggs are brownish and almost spherical. The eggs measure 75–90 μm. The eggs are embryonated when laid and have surficial pits. Eggs of *T. cati* are similar to that of *T. canis* but are smaller in size (65–75 μm). These eggs are very resistant to various weather and chemical conditions.

Larvae

The larvae of *T. canis* measure between 290 and 350 μm in length and 18–21 μm in width. The larvae of *T. cati* are somewhat smaller in width than *T. canis*.

Table 1 Differentiating features of *Toxocara canis* and *Toxocara cati*

Toxocara canis	*Toxocara cati (Toxocara mystax)*
Common name: Arrow-headed worm of dogs	Common name: Arrow-headed worm of cats
Worms are milky white in colour measuring about 10–18 cm	White worms with 3–10 cm in length
 *Courtesy:* Charitha 2019	 *Courtesy:* Charitha 2019

Cultivation of Parasites

In the mid-1970s, introduction of in vitro culture technique of *T. canis* second-stage larvae for prolonged periods of time and collection of excretory and secretory (ES) antigen created a breakthrough in the diagnosis of *Toxocara* larval migrans (TLM), which considerably improved the specificity, sensitivity and reproducibility of various serodiagnostic methods.

T. canis eggs collected by the dissection of female worms are incubated in 4% formal saline at 26 °C for 35 days to induce embryonation. Decortication of embryonated eggs is facilitated by 4% sodium hypochlorite solution and hatching initiated by incubating with RPMI 1640 medium. Live larvae for culture are separated from debris, dead larvae and unembryonated eggs using histopaque solution/Baermann apparatus. HEPES-buffered RPMI 1640 medium containing L glutamine is found to be good for larval culture in terms of better survival of larvae and higher yield of ES products. The larvae are usually incubated at a concentration of 10^3 larvae/ml and kept at 37 °C in CO_2 incubator with 5% CO_2. Collected medium having crude ES antigen is concentrated using PEG 20,000.

Laboratory Animals

Since *T. canis* has zoonotic importance, researchers attempted to induce *T. canis* infection in laboratory animals to study its migratory route and histopathology of tissues and to understand immune responses and systemic cytokine profiles during the infection. Mouse is the common model organism used for such studies. Studies were attempted to infect *T. canis* in wild and farm minks to understand if minks could be a definitive host for the parasite and observed that the *T. canis* do infect minks, and infected minks developed IgG response to the parasite. The cockroaches and earthworms were also experimentally infected, suggesting that they could possibly serve as transport host or paratenic host.

Life Cycle of *Toxocara* Spp.

Hosts

Dogs and cats are definitive hosts of *T. canis* and *T. cati*, respectively. Humans and other mammals, such as foxes and wolves, are accidental hosts.

Infective Stage

Eggs with juvenile larva (L_2).

Transmission of Infection

The predominant mode of *Toxocara* transmission in humans is through the ingestion of embryonated eggs from soil or from consuming contaminated raw vegetables. Dog's hair coat contaminated with infective eggs is another new mode of transmission identified in recent studies.

Development in Canid Host

The life history of *T. canis* was studied by Sprent (1958). *T. canis* adopts a complex life cycle pattern. Infection of dogs can occur by four ways (Fig. 1): (**a**) orally by the ingestion of larvated eggs; (b) prenatal/intrauterine infection, the most relevant mode in dogs; (c) trans-mammary/lactogenic infection and (d) eating paratenic host. After per oral ingestion, the hatched larvae penetrate small intestinal mucosa to initiate the extra-intestinal route of larval migration, which is dependent on the age of the definitive host (canids). In younger dogs (<3 month), the larvae follow ascaroid type of migration with somatic and tracheal routes of migration (liver–lungs–trachea–gut). In older dogs (>3 months), toxocaroid type of migration becomes conspicuous with somatic migration followed by hypobiosis and formation of eosinophilic granulomas around dormant larvae in skeletal muscles and various organs. In bitches, larvae resume their migration activity due to (prolactin) hormonal changes and bring about intrauterine and lactogenic infection in puppies. Thus, pups are much more common source of infection than adult dogs in age-dependent variations of the cycle. Worms mature as adults in the lumen of the small intestine with a lapse of 60–90 days from the time of initial larval hatch. Mating occurs to produce fertilized but non-embryonated eggs, which are excreted in faeces. Embryonation is completed within a week in an ambient environment or is prolonged due to lower temperatures (Figs. 2 and 3). Life cycle of *T. cati* (*T. mystax*) is similar to *T. canis* except that prenatal infection does not occur, and lactogenic route is the most relevant transmission in cats.

However, in non-essential paratenic host (earthworms, ants and birds) no development occurs, and larvae carry out somatic migration and remain dormant in their organs and muscles, but they sustain the parasite through space and time until the definitive host ingests. In lactogenic mode and paratenic host route of entry into

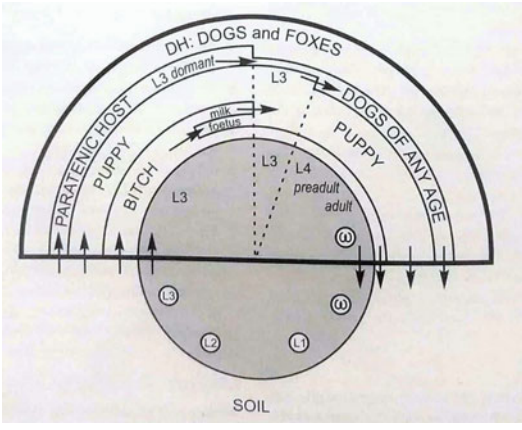


Fig. 1 Transmission pathways of *Toxocara canis* in definitive hosts (*adapted from* Tibor Kassai 1999)

Fig. 2 Unembryonated eggs of *Toxocara* spp. (100×) are *dark brown* in colour, subglobular with thick pitted shell (*Courtesy:* Charitha 2019)

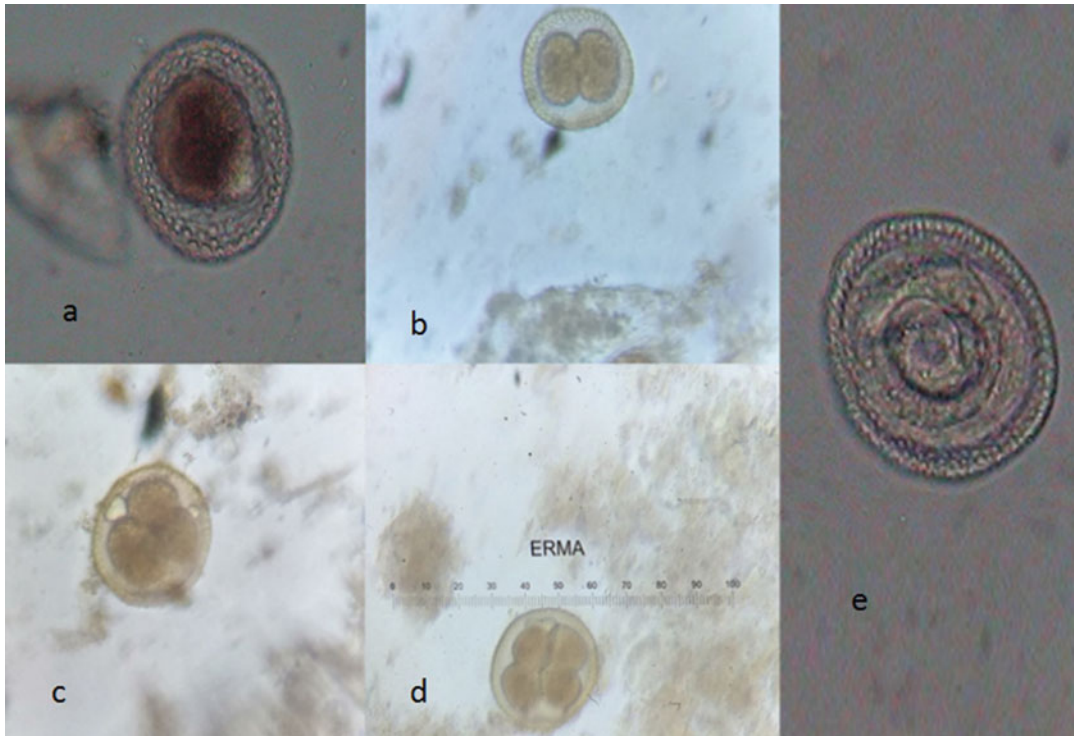


Fig. 3 Developmental stages of *Toxocara* spp. eggs in environmental samples (**a**) unembryonated stage, (**b**) two-celled stage, (**c**) three-celled stage, (**d**) four-celled stage and (**e**) infective egg with larvae (*Courtesy:* Charitha 2019)

definitive hosts (canids), larvae do not undergo somatic migration but establish patent infection in gut.

Development in Human Host

Man acts as aberrant host, and the parasites have an oral–faecal transmission, which is usually an outcome of accidental ingestion of the embryonated eggs from contaminated soil, raw vegetables and poor personal hygiene. After ingestion, the larvae follow the same somatic route of migration (Fig. 4) as for paratenic hosts and are carried away by bloodstream into a wide variety of organs and lodge for several years causing different clinical syndromes. The organs commonly involved are lungs, liver, heart and central nervous system including eyes as the most sensitive sites. Although CNS infection in humans is thought to be rare, *T. canis* larvae are neurotropic in experimental infections of primates, but experiments with *T. cati* reveals that the accumulation is restricted to the muscle (Graeff-Teixeira et al., 2009).

Pathogenesis and Pathology

The pathogenesis and clinical spectrum of toxocariosis in humans range from asymptomatic infection to debilitating injury. The disease manifestation is determined mainly by the parasite load during the initial insult, the anatomic sites of larval migration, the age of the host and the robustness of the inflammatory response that the host mounts to parasitic infection. Major pathological consequences in humans are associated

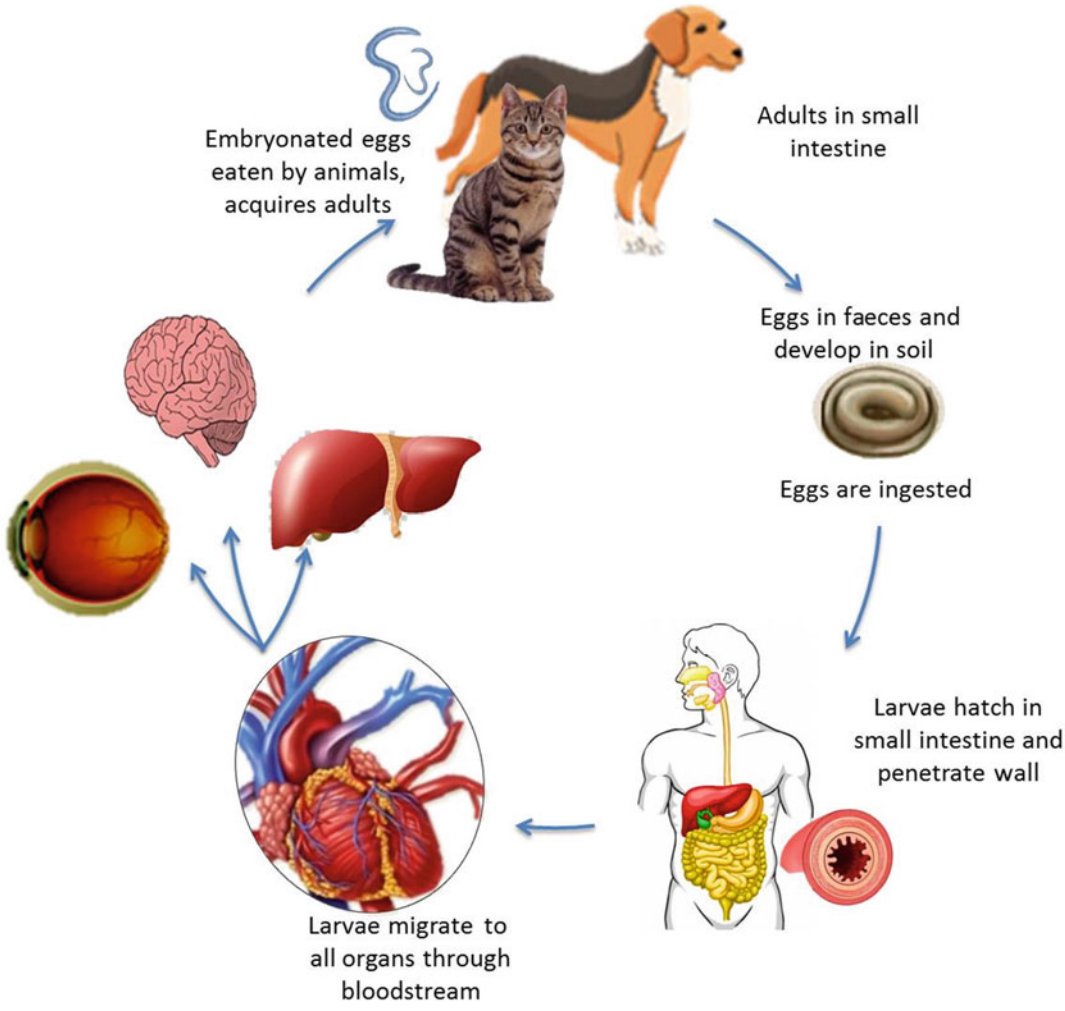


Fig. 4 Life cycle of *Toxocara canis* in human hosts

with host-mediated hypersensitive reactions towards juvenile migratory larvae (Fig. 5). Their death triggers the onset of acute immediate reactions followed by delayed hypersensitive responses. Eosinophilic granulomas resulted from inflammation heralds the symptomatic VLM reactions in patients. Likewise, granulomas can lead to peripheral retinochoroiditis, scleritis, chronic endophthalmitis and panuveitis, as observed in OT. The location of the larvae, the extent of eosinophilia and the severity of fibrotic granulomatous response decide the level of visual impairment of the host. Epidemiologic evidence suggests that *T. canis* has predilection to ocular disease (OT/OLM), which occurs exclusively in the absence of systemic involvement (VLM). Vice versa is also true; thus, two different manifestations as OLM and VLM are characteristic of *T. canis* infection. The chances of OLM are more among individuals who have not been previously sensitized, while VLM is associated with repeated waves of migrating larvae. Covert toxocariosis is seen mainly in children and common toxocariosis is seen predominantly in adults. Additionally, migrating larvae in the brain, predominantly in the white matter of cerebrum and cerebellum, and in the spinal cord, cause neural manifestations, which is categorized under neural toxocariosis (NT).

Fig. 5 *Toxocara canis* second-stage larva (@ olgaru79)

Infection in Humans

In general, human *Toxocara* infection elicits four clinical entities: Visceral larva migrans (VLM), Ocular toxocariasis/Ocular larva migrans (OT/OLM), Covert toxocariasis (CT, and Neurological toxocariasis (NT) depending on which organs are affected.

Visceral Larva Migrans (VLM)

The condition is reported more commonly from children between 2 and 7 years and is a consequence of repeated and high-intensity infection by *T. canis* larvae. The incubation period varies between weeks and months, which directly depends on the intensity of initial insult and the susceptibility of the patient.

The disease manifestation is associated with systemic migration of the larvae in the tissues of human viscera. The acute signs of VLM run with hepatic and pulmonary larval migration, and there are marked eosinophilia (>2000 cells/mm^3); leucocytosis and elevated IgM, IgG and IgE classes of immunoglobulins. Associated visible clinical signs are represented in Table 2. Further, implications of *Toxocara* as a contributing factor in skin disorders (prurigo and urticaria) and with eosinophilic arthritis have been recorded.

Ocular Larva Migrans/Ocular Toxocariosis (OLM/OT)

The condition typically occurs in children (>5 years) and young adults. There is a usual lag time of 4–10 years after the initial infection for OT to manifest. The most common sign is unilateral vision impairment. Heterotopia with or without macular detachment may accompany fibrotic granulomatous lesion. The most serious consequence is invasion of the retina, leading to granuloma formation, which occurs typically in the posterior pole. In response to the chronic presence of *Toxocara* larvae, host may suffer from diffuse unilateral subacute neuroretinitis (DUSN), bilateral distal symmetric sensory neuropathy (DSN) and choroidal neovascular membrane formation in the eye. In some cases, ocular infection may also be subclinical and can be detected only by fundoscopy.

Covert Toxocariosis (CT)

The condition often manifests as asymptomatic form or only has nonspecific/mild symptoms. This is corroborated through the fact that many serological surveys have identified high number of participants with seropositive results, whereas small proportion of them suffered from VLM and OLM. The case–control studies carried out in adults in France and in children in Ireland lead to new categorization of the clinical syndrome in to ‘common’ and ‘covert’ toxocariosis, respectively. Significant laboratory findings in common toxocariosis (adults) are eosinophilia, elevated levels of IgE and high titres of *Toxocara*-specific antibodies, whereas in covert form (children), there are moderate Toxocara-specific antibodies, with or without eosinophilia. Patients with these relatively mild forms of toxocariosis usually do not require treatment with anthelmintics.

Table 2 Manifestation of different clinical forms of toxocariosis in humans

Clinical form	Age susceptible	Organs involved	Associated signs	Treatment regime
Visceral larva migrans	2–7 years	Liver, lungs, heart and kidneys	Fever, abdominal pain, reduced appetite, diarrhoea, anorexia, weight loss, fatigue and hepatomegaly Respiratory symptoms: Cough, wheezing, dyspnoea, bronchospasm and asthma. Often, myalgia with eosinophilic polymyositis, myocarditis and nephritis	Albendazole (ABZ): 400 mg per os, twice a day for 5 days Mebendazole (MBZ): 100–200 mg, per os, twice a day for 5 days
Ocular toxocariasis/ Ocular larva migrans	5–10 years of age	Eye	Unilateral vision impairment complicated by strabismus and leucocoria. OLM can also cause diffuse endophthalmitis or papillitis, uveitis, cataract and secondary glaucomas	Corticosteroids in combination with ABZ: 200 mg twice a day for one month MBZ: 20–25 mg/kg/day 3 weeks
Covert toxocariasis	*Children*: Covert form *Adults*: Common form	No specific location	*In children*: Fever, headache, anorexia, nausea, behavioural disorder, insomnia, abdominal pain, pneumonia, coughing, wheezing, pruritus, skin rash and hepatomegaly *In adults*: Difficult breathing, skin rash, pruritus, weakness and abdominal pain	Treatment not needed
Neurological toxocariasis	Adults	Brain and spinal cord	Photophobia, epilepsy, seizures, dementia, depression, ataxia, rigor, headache, body ache, Para tetraparesis, dysaesthesia, urinary retention and faecal incontinence	ABZ: 400 mg per os, twice a day for 5 days MBZ: 100–200 mg per os, twice a day for 5 days

Neurological Toxocariosis (NT)

The condition characterized by clinical involvement of the nervous system is thought to be rare in human cases, although in experimental animals, the larvae frequently migrate to the brain. Central nervous system (CNS) toxocariosis was seen more frequently in adults as compared to children. The clinical spectrum of CNS toxocariosis is broad, causing various syndromes, viz. eosinophilic meningoencephalitis and meningitis, meningomyelitis or meningoencephalomyelitis, extramedullary space-occupying lesion, brain vasculitis, seizures and probably behaviour disorders. Uncommon presentations of CNS toxocariosis included spinal compression by an epidural abscess and cerebral vasculitis, documented in limited cases.

Toxocariosis in Animals

Heavy prenatal infection of *T. canis* may lead to death of puppies. Migrating larvae may cause pneumonia in newborn pups. Moderate infection with *T. canis* and *T. cati* yields potbellied condition, tucked up abdomen in affected pets along with intermittent diarrhoea, anaemia, unthriftiness and harsh hair coat. Severe intestinal obstruction due to balling up of worms leads to death.

Diagnosis

Diagnosis of Toxocariosis in Humans

The diversity of clinical conditions associated with different sites where *Toxocara* larvae can lodge makes it difficult to diagnose toxocariosis. Clinical manifestations coupled with peripheral elevated blood eosinophilia $>10{,}000$ cells/mm^3 (biochemical analysis) may facilitate in diagnosing the VLM infection to limited level as marked eosinophilia is absent in CT/OLM.

Microscopy

Thus, the gold standard test is biopsy and visual detection of the parasite in tissues, cerebrospinal fluid (CSF) and ocular fluid (OF). Nonetheless, this procedure is extremely invasive, insensitive and time consuming.

Serodiagnosis

A positive serological test resulting in addition to the detection of peripheral eosinophilia is suggestive of an ongoing *Toxocara* infection. The most utilized diagnostic serological test is the ELISA with TES-Ag (*Toxocara* excretory and secretory antigen) from second-stage *T. canis* larvae. A positive antigen ELISA for *Toxocara* can be further supplemented by a more specific Western blot assay by targeting lower molecular (24–35 kilo daltons) weight TES-Ag. ELISA testing for eosinophil cationic protein (ECP) that is released by activated eosinophils could be helpful. Despite the success of serological testing for TES antibodies in serum, these assays are of limited value to evaluate the progression of the diseases in CNS as antibodies are not detected in the CSF of NT patients. Recombinant *Toxocara* antigens have been developed to specifically improve the capabilities for diagnosing NT (Table 3). Serological tests, therefore, represent the least invasive and most sensitive approach to diagnosis, but these still require optimization and international standardization. Positive results need to be interpreted with caution in regions of endemic polyparasitism when equivocal symptoms are present.

Molecular Diagnosis

To overcome the serological drawbacks, the scientific community is looking forward to molecular techniques that have high diagnostic sensitivity and analytical specificity. More importantly, the turnaround time can be minimized with molecular assay. To date, molecular markers that confer detection specificity have been identified and tested, and the most useful of those are the genetic markers ITS-1 and ITS-2 of rDNA. Advances in PCR methodology have enabled accurate and faster identification while allowing phylogenetic analysis performed on the detected species with other ascarids. This technology is promising in detecting *Toxocara* from CSF in NT and for confirming larvae retrieved by biopsies in ocular larva migrans (OT).

Other Methods

Medical imaging techniques such as magnetic resonance imaging (MRI) and computed tomography (CT) can be used to detect granulomatous lesions caused due to *Toxocara* larvae in neural tissues, especially in NT patients. Cortical or subcortical, multifocal, circumscribed, homogeneously enhancing T2 hyperintense lesions, or a combination of circumscribed and diffuse changes have been described in MRI of NT patients. These lesions are hypodense on cerebral CT and hypointense on T1-weighted MRI images.

Ultrasound scan is usually performed to detect VLM lesions, which appear as multiple hyperechoic spots that are non-spherical and poorly defined, whereas VLM in liver appears as fluid-attenuating conglomerate lesions under ‘contrast-enhanced’ CT. On the other hand, hepatic VLM lesions can appear as either hypointense or hyperintense depending on the selection preference of T1-weighted (T1W) images or T2-weighted (T2W) images, respectively.

Imaging techniques to detect OT include fundus photography, fluorescein angiography, ophthalmic ultrasound and optical coherence tomography (OCT). These techniques aid detection and differentiation of eye granulomas caused by *Toxocara* migration from similar ocular conditions, such as retinoblastoma, toxoplasmosis, retinopathy and optic neuritis. However, these imaging features are only suggestive and may not be specific to NT/OT. Therefore, serum/CSF serology for *Toxocara* antibodies, and levels of eosinophils in the serum/CSF, along with clinical

Table 3 Common diagnostic methods for detecting human toxocariosis

Approaches	Methodology	Targets	Comments
Direct microscopy	Biopsy	Larval sections in tissues/fluids (CSF/OF)	**Gold standard test** *Drawback*: Invasive and insensitive
Laboratory tests	Blood biochemical analysis	Blood eosinophilia	Limited to VLM as marked eosinophilia is absent in CT/OLM
Immunodiagnostics	Antigen detection (sandwich ELISA)	Circulating TES antigen	Confirms active infection *Limitations:* Significant cross-reactivity with *Ascaris lumbricoides*
	Antibody (TES-ag-ELISA)	IgE, IgG antibodies	Good sensitivity and specificity *Limitations:* ELISA can be negative in the CSF of NT patients
	Recombinant antigens	rTES-30, rTES-26, TES-120	Recommended for the diagnosis of human toxocariasis Less cross-reactivity with other helminth infections in endemic regions
Molecular assays	RFLP, RAPD, PCR, qPCR, LAMP	ITS-1, ITS-2	High sensitivity and specificity *Limitations:* Require skilled personnel

or radiological assessments of post-anthelmintic treatment, is necessary to establish the diagnosis.

The neural larval migrans caused by *Baylisascaris procyonis* is an important parasitic disease that needs to be differentiated from toxocariosis, for effective management of the latter. OT/NT needs to be differentiated from various conditions including cancer such as retinoblastoma, parasitic infections such as angiostrongylosis, thelaziasis, trichinosis and cysticercosis, and bacterial and viral infections such as Lyme disease and cytomegaly.

Diagnosis of Toxocariosis in Animals

A characteristic clinical sign followed by laboratory confirmation is generally practised for the diagnosis of canine toxocariosis (Fig. 6). Various faecal examination methods, viz. direct smear, sedimentation, floatation, McMaster, and formol–ether concentration, are employed for the detection of eggs of *Toxocara* spp. Recently, PCR methodologies are increasingly being used to precisely amplify molecular markers specific for various members in the genus *Toxocara* and for other related nematodes. The internal transcribed spacer (ITS-1 and ITS-2) regions are a promising molecular target for the sensitive and specific identification of *T. canis, T. cati* and other ascarids.

Epidemiology and Public Health

Toxocariosis is an important neglected tropical helminth disease of zoonotic significance. *Toxocara canis* and *T. cati* are the most common parasites of domestic dogs and cats, particularly young ones. Contaminated soil plays a major role in the transmission of toxocariosis to humans. An undoubted source of infection is the immediate environment of a house in which there is a coexistence of human babies with the puppies or nursing bitch. Young children are at greater risk of infection because of their lifestyle and their playing environment, especially those with a

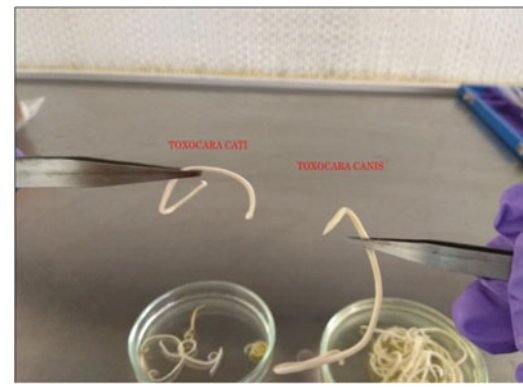


Fig. 6 Adult worms segregated from intestinal contents of dogs and cats during necropsy (*Courtesy:* Charitha 2019)

history of pica. In addition, the presence of parasitic form (egg with larva) in the soil is the most direct indicator displaying the local population's risk. To make matter worse, studies confirm outdoor parks in many urban and suburban areas frequented by people and their pets are heavily contaminated with infective eggs of *T. canis* and *T. cati*. Factors such as poverty, lack of personal hygiene, scarcity of potable water, abundance of stray animals and overcrowding are responsible for the rising prevalence of toxocariosis. Consensus is that the kids growing up in a poor community are vulnerable and end up with higher rate of seropositivity for toxocariosis than their counterparts raised in affluent neighbourhood. No wonder toxocariosis is the most prevalent human parasitic disease worldwide, with public perception of the health impact that this disease is low, and the true global burden is yet to be fully understood.

Treatment

There are two main goals while carrying out the treatment of toxocariosis in children: (a) to obtain a clinical resolution and (b) to reduce the level of larval migration to other organs, particularly the brain and eyes. The mainstay of toxocariosis therapy includes benzimidazole group of anthelmintics that include albendazole (ABZ), mebendazole (MBZ) and thiabendazole. ABZ is the first drug of choice administered as 400 mg twice a day for 5 days, while MBZ is the preferred second choice for treating VLM. Note that both ABZ and MBZ are administered with fatty foods orally to overcome their limitation of poor gastrointestinal absorption. No new drugs are currently in the pipeline, but continued efforts are on designing formulation for enhanced delivery of anthelmintic drugs. In this regard, various delivery strategies for ABZ such as chitosan encapsulation, polyethylene glycol (PEG) conjugation and stealth liposome are currently being tested. Anti-inflammatory drugs are the commonly used ancillary treatment, and corticosteroids have been used in cases of pulmonary toxocariosis and toxocariosis-associated cardiac diseases.

Prevention and Control

Prevention and control of toxocariosis requires a one health approach with collaboration of groups invested in protecting the health of humans, animals and the environment. Efforts to minimize indiscriminate deposition of dog and cat faeces in parks and play areas in urban centres remain the best control strategy. Kids and pet owners are encouraged to wash their hands after handling pets and when accessed playgrounds and parks, which should always be considered a place of high risk for soil contamination. Improved hygiene when preparing food can also help in avoiding toxocariosis. This sanitary education is a slow but essential process for public awareness and must involve both human health care and the control of stray dogs and cats.

Healthcare providers need to be aware of clinical manifestations of toxocariosis and educate their patients at risk, especially children, about avoiding exposure to potentially contaminated soil and preventing infection in their pets. Veterinarians' role is paramount in combating the spread of *Toxocara* infection, as they can educate pet owners and public the importance of this neglected disease. Regular faecal examinations of pet dogs and cats are recommended, and appropriate drug treatment is advised for controlling animal infection. Vaccine against toxocariosis is still at its infancy and remains a steep challenge due to lack of defined antigens that are immune protective.

Case Study

A 10-year-old boy suffering with blurred vision in the right eye and fever of unknown reasons for 3 months was admitted to the ophthalmology department of a referral hospital. An ophthalmoscopic examination revealed white masses in the superior peripheral retina of the right eye with cells in the vitreous. Fundus examination revealed tracks made by some larva. Patient had normal total leucocyte count but with elevated eosinophil count of 15%. Stool examination was negative for

ova, cyst or larva of any parasite. Specific antibodies to *Toxocara* ES antigens were detected in the serum and vitreous fluid collected from the patient by ELISA. (Adapted from Zibaei et al. 2014.)

1. What are the other parasites which can be observed by ophthalmoscopic examination?
2. What are the other organs which may be affected by this parasite?
3. Which treatment regimen should be prescribed for this patient?

Research Questions

1. What are the defined antigens which can be used for *Toxocara* immunoassays?
2. What vaccine can be developed against toxocariosis?
3. How to create public awareness on clinical manifestation of toxocariosis?

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Anisakiasis

Vibhor Tak

Learning Objectives

1. To have an idea about the various manifestations of the disease.
2. To understand the importance of endoscopy and its role in removal of the worm.

Introduction

Anisakiasis is an emerging seafood-borne zoonotic infection occurring in humans caused by the L3 larvae belonging to the family Anisakidae. Nearly 97% of infections are caused by *Anisakis* spp., especially *Anisakis simplex* sensu stricto and only 3% of infections are caused by other genera (*Pseudoterranova, Contracaecum, Hysterothylacium* and *Porrocaecum*). Human infection occurs due to consumption of raw, pickled, salted or smoked marine fish or squid containing L3 larvae of *Anisakis* spp. Gastric anisakiasis is the most common form of anisakiasis. Diagnosis of anisakiasis is challenging due to the lack of specificity of the symptoms. Endoscopy is the preferred technique for diagnosing acute gastric or intestinal anisakiasis.

History

Anisakis was detected in fish in the thirteenth century. In 1767, it was named as *Gordius marinus* by Linnaeus. In 1809, Rudolphi described larvae in fish and adults in porpoise, but he could not establish any relationship between the two. In 1845, Dujardin finally classified this parasite in the genus *Anisakis,* in which the commonest species is *A. simplex*. In 1950, Hitchcock while examining a stool specimen from an Inuit in Alaska observed a larva, possibly of *Anisakis*. The first symptomatic cases associated with the presence of *Anisakis* larvae were diagnosed in the Netherlands in 1955–59. Subsequently, the parasite was identified as a nematode belonging to *Anisakis* spp. causing infection in human beings, and the condition was termed as anisakiasis. After the initial discovery of the parasite, a large number of human anisakiasis cases have been reported from Japan and other countries.

Taxonomy

The genus *Anisakis* belongs to the phylum Nemathelminthes; class Chromadorea; order Rhabditida; superfamily Ascaridoidea; and

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family Anisakidae. An expert group on the standardized nomenclature of animal parasite diseases in 1988 recommended the use of the following terminology for the description of three different clinical conditions caused by nematodes belonging to family Anisakidae: (a) Anisakidosis, (b) Anisakiasis and (c) Pseudoterranovosis. Anisakidosis is the term used for the disease caused by any member of the family Anisakidae, whereas the infection caused by members of the genus *Anisakis* proper is known as Anisakiasis. Infections caused by members of the genus *Pseudoterranova* is termed as Pseudoterranovosis.

The genus *Anisakis*, based on the use of molecular markers (nuclear and mitochondrial regions), has been classified into nine species. These species include *A. simplex* sensu stricto, *Anisakis pegreffi*, *Anisakis berlandi*, *Anisakis ziphidarum*, *Anisakis nascettii*, *Anisakis paggiae*, *Anisakis brevispiculata*, *Anisakis physeteris* and *Anisakis typica*. Of these species, only *A. simplex* sensu stricto and *A. pegreffi* cause most infections in humans.

Genomics and Proteomics

The draft genome of *A. simplex* was the first to be elaborated by the parasite genomic group at the Wellcome Trust Sanger Institute, in the framework of the 50 Helminth Genomes project (PRJEB496 project). The genome is 126,869,778 bp long, and it has been predicted to contain 20,971 genes.

The first proteomic study was performed by comparing protein profiles of the pathogenic *A. simplex* complex (*A. simplex s.s., A. pegrefii* and their hybrid). This study was performed using 2D gel electrophoresis hybridized with pools of sera from *Anisakis* allergic patients, and a parallel Western blot was done. A differential expression of proteins study of *A. simplex* s.s. and *A. pegreffi*, performed by MALDI-TOF/TOF technique, has identified 28 different *Anisakis* proteins. Most of them were novel proteins, identified as potential new allergens produced by *Anisakis* spp.

The Parasite Morphology

Adult Worm

Anisakis adult worms are found in the stomach chambers of the cetacean mammals. Just like other nematodes, they are dioecious in nature. Female worms are longer than the males and measure 4.5–15 cm in length. Males are 3.5–7.2 cm long.

L3 Larvae

Initially, the L3 larvae of *A. simplex* were called as *Anisakis* type I larvae and L3 larvae of A. *physeteris* were called as *Anisakis* type II larvae. *A. simplex* larvae are slightly longer than *A. physeteris* larvae. L3 larvae are 1–3 cm long and 1 mm broad. The larvae have one dorsal and two sub-ventral lips orally. Excretory pore opens on the base of the sub-ventral lips. The oesophagus consists of two parts—proventriculus and ventriculus. The tail may or may not possess a mucron. They have a thick cuticle and possess Y-shaped large lateral cords that extend into the body cavity.

Eggs

The eggs are colourless, oval and measure 40×50 μm in size. The eggs are unembryonated when excreted in the faeces of the cetacean mammals.

Cultivation of Parasites

There is not much literature available regarding the cultivation of Anisakids in the laboratory. Culturing of parasites is a tedious, technically demanding and labour-intensive exercise; therefore, it is not a diagnostic modality in parasitology laboratories. *Anisakis* spp. are not routinely cultured in the laboratory.

Laboratory Animals

Wistar rats, mice, guinea pigs and rabbits are various laboratory animals that have been used to study the pathogenesis of anisakiasis. Experimental infection of parasites in fishes has been done in laboratories to understand the pathogenesis of Anisakiasis.

Life Cycle of *Anisakis* Spp.

Hosts

Dolphin, porpoise and whale are the definitive hosts for *Anisakis* spp. Other marine mammals like seal, fur seal, sea lions and walruses are definitive hosts for *Pseudoterranova* spp.

The planktonic or semi-planktonic crustaceans serve as first intermediate hosts, while fishes and squids are the intermediate or paratenic hosts. Till date, more than 200 species of fish and 40 cephalopod species have been reported as paratenic or intermediate hosts of *Anisakis*. These include fishes of great economical and commercial value such as cod, salmon, mackerel, herring, anchovy, sardine, hake, saithe, redfish, blue whiting and pouting. Herring, mackerel, cod, salmon and squid transmit *Anisakis* infection, whereas cod, flatfish, greenling, halibut and red snapper transmit *Pseudoterranova* infections.

Infective Stage

Third-stage larva (L3) of *Anisakis* spp. is the infective stage.

Transmission of Infection

The marine mammals such as dolphin, porpoise and whale, seals, walrus and sea lions acquire infection by predating on fish and squids that harbour the infective third-stage larvae (L3) of *Anisakis* spp. or *Pseudoterranova* spp. (Fig. 1). On ingestion, the larvae after being released in the stomach penetrate the gastric mucosa, and after two moults, they develop into male and female worms. The adult worms live in clusters with their anterior ends embedded in the gastric wall.

Males subsequently mate and fertilize the females while moving in the gastric chambers of the cetacean mammals. After fertilization of sexually mature females by the male worms, the gravid female passes the unembryonated eggs, which are released in the faeces of the hosts, which contaminate the marine environment. The eggs hatch to release free-swimming unsheathed larvae. These larvae undergo one or two moults before being ingested by intermediate hosts, and mostly small crustaceans like krill and copepods are the intermediate invertebrate hosts of the parasite. The infected crustaceans are then eaten by a wide variety of fishes and cephalopods such as squids, which act as transport or paratenic hosts.

Depending on the species of *Anisakis*, many different life cycle patterns may be observed at this stage. The infected fish may be directly eaten by dolphin, porpoise and whale, the definitive hosts. In these hosts, the third-stage larva (L3) moults to fourth-stage larva L4 and then develops and matures to the adult worms. Alternatively, the infected fish may be eaten by another fish, and this leads to the accumulation of a large number of Anisakis larvae during its passage in the natural food web. These are further consumed by new susceptible marine mammalian hosts, thus sustaining *Anisakis* life cycle in the marine environment.

Human infection occurs on consumption of raw, pickled, salted or smoked marine fish or squid containing L3 larvae of *Anisakis* spp. As humans are accidental hosts, no further development of L3 larvae takes place. However, the larval development may progress to L4 stage, but not to adult worm, in the infection caused by a few *Pseudoterranova* spp.

Pathogenesis and Pathology

Inflammation, ulcer and subsequently eosinophilic granulomas are the characteristic features of anisakiasis in humans. These lesions are

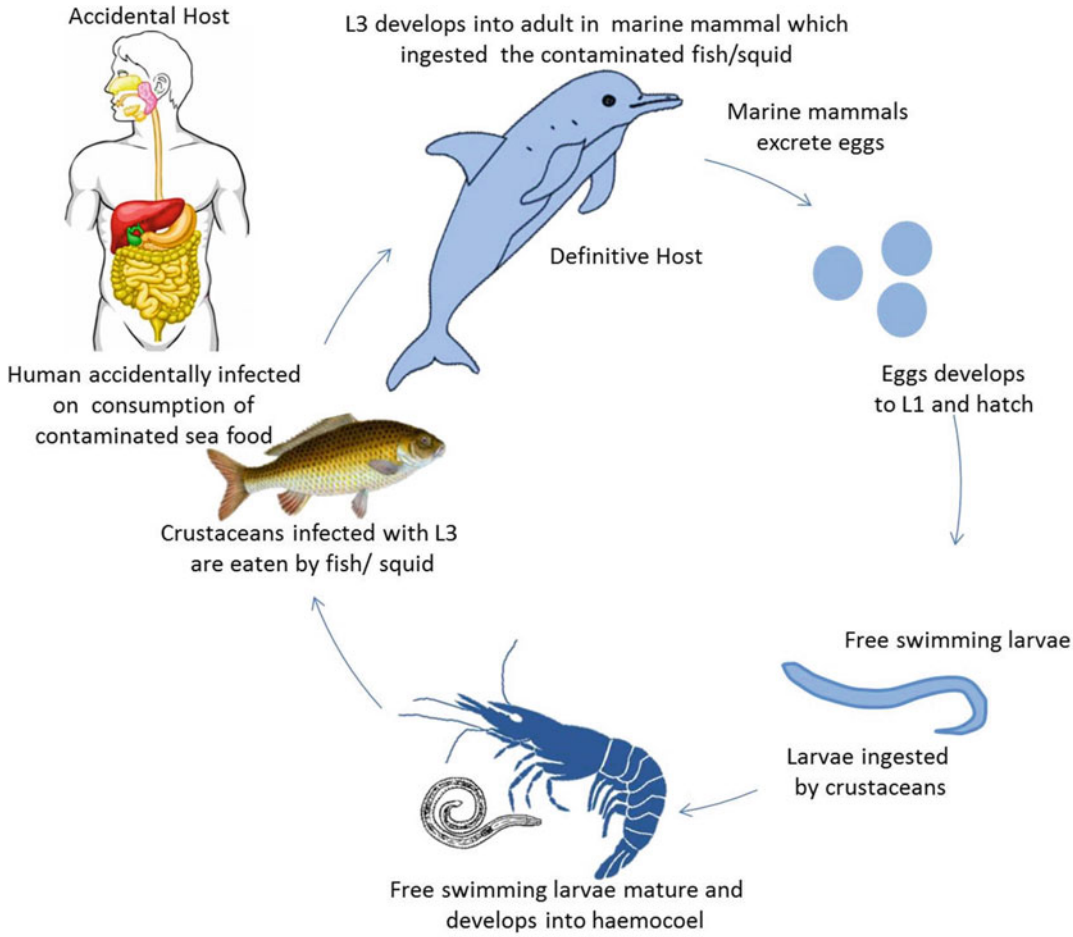

Fig. 1 Life cycle of *Anisakis* spp.

caused by L3 larvae at their site of attachment in the gastric/intestinal mucosa. The L3 larvae of *Anisakis* spp. on entering the host via contaminated fish or squid invade and attach to the stomach or intestinal mucosa, causing gastric or intestinal anisakiasis, respectively. The pathological changes in anisakiasis occur as a result of direct tissue injury caused by the larval invasion and complex interaction between the excretory–secretory substances produced by parasite and host's immune system.

The process of parasitic invasion and attachment to granuloma formation occur in four stages. First stage is the formation of eosinophilic phlegmon followed by the abscess formation in the second stage. Abscess formation is characterized by the presence of necrotic tissue around the larvae surrounded by a large number of eosinophils. The formation of abscess is seen more frequently in gastric anisakiasis. In the third stage, abscess–granuloma formation occurs months after the ingestion of the larva. In this stage, there are a few larval remnants, associated with abundant inflammatory infiltrates, predominantly eosinophils surrounded by giant cells. Granuloma formation occurs in the fourth or final stage. Granuloma formation is characterized by a gradual replacement of eosinophilic infiltrates with numerous lymphocytes, giant cells and significant collagen deposition.

The formation of ulcers in the stomach of the fish or squid and in the stomach chambers of

cetaceans and other marine mammals is the typical pathology.

Immunology

Both humoral and cellular responses are elicited in anisakiasis. Th2-mediated immune response against the invading *Anisakis* larvae leads to the production of IgE antibodies, eosinophils, mastocytosis, goblet cell hyperplasia cell and smooth muscle contraction, which helps in the expulsion of the nematode.

In both acute anisakiasis and chronic anisakiasis, the parasites are surrounded by intense eosinophilic infiltration. Eosinophils not only help in the effective destruction of larvae but also contribute to hypersensitivity reactions in response to the *Anisakis* infection. The infected hosts develop specific IgE antibodies to a range of allergenic proteins liberated by *Anisakis*. These antibodies then bind to receptors present on mast cells in the gastrointestinal tract and the skin, and circulating basophils in the blood. On subsequent contact with these allergens, these specific IgE antibodies mediate release of a wide range of inflammatory mediators including histamine and various cytokines resulting in clinical manifestations of allergy.

Infection in Humans

Humans are accidental hosts for anisakiasis. Majority of human infections occur by *A. simplex* and manifest as gastric anisakiasis (Table 1).

Gastric anisakiasis is the most common form of anisakiasis, in nearly three-fourth of cases. The attachment of L3 larva to the gastric mucosa gives rise to intense epigastric pain, nausea, vomiting, urticaria and diarrhoea. These symptoms develop 2–6 h after the ingestion of the food infected with larva. The symptoms persist till the larvae remain alive.

Intestinal anisakiasis appears 2–3 days after ingestion of the infected food. The patient presents with severe abdominal pain, which may be accompanied by nausea, vomiting and/or diarrhoea. Occasionally, a chronic form of intestinal anisakiasis develops, resulting in the formation of granulomas or abscesses.

Ectopic or extra-intestinal anisakiasis is rarely encountered. The condition occurs when the larva penetrates through the intestinal wall and migrates to other extra-intestinal sites.

Allergic anisakiasis or 'allergy to *Anisakis*' is a condition that occurs due to allergic response by the host's immune system to *Anisakis* larval allergens. The sensitization occurs on exposure of the host to allergens from both live and dead parasite larvae. Symptoms vary from urticaria, angio-oedema to frank anaphylaxis. They usually appear within the first hour after consuming the parasitized fish. It is estimated that in endemic countries up to 7% of general population may be sensitized against *Anisakis* allergens. Allergic manifestations are usually associated with *A. simplex* infection and occur due to consumption of pickled anchovies in Spain.

Gastro-allergic anisakiasis is a severe manifestation of IgE-mediated allergy, accompanied by gastric digestive symptoms and even chronic urticaria. Occupational anisakiasis caused by *A. simplex* has been observed amongst fishermen, fishmongers or other fish industry workers. These occur as a result of sensitization to *Anisakis* allergens larvae present in fish harbouring the larvae. Various allergic dermatitis, asthma and conjunctivitis are the typical manifestations of the condition.

Pseudoterranova spp. is less invasive and virulent species. The parasite, found in the upper oral cavity or in the oesophagus, causes the *Tingling throat* syndrome in patients.

Infection in Animals

Anisakis infection in fishes often produces ulcers in their gastrointestinal tract; otherwise, it does not significantly affect the host's health. *Anisakis* infection in marine mammals may cause immunosuppression resulting in systemic microbial infections and dermal manifestations.

Table 1 Distribution of Anisakid genera causing human infections

Genus	Distribution	First intermediate hosts	Second intermediate/ paratenic hosts	Definitive hosts
Anisakis simplex sensu stricto	Japan, Europe and countries in Atlantic Basin, Pacific Ocean and Alaskan coast	Small crustaceans	Herring, salmon, mackerel, cod and squid	Cetacean mammalian species like dolphins, porpoise and whales
Pseudoterranova spp.	Mainly seen in colder coastlines like Canada, the USA and other countries in North Atlantic, the Arctic and Antarctic oceans, Chile, Japan	Small crustaceans	Cod, halibut, flatfish, greenling, red snapper	Pinnipeds like seals, sea lions and walruses
Contracaecum spp.	The Arctic, Antarctic, northern Atlantic oceans, Japan, Chile	Small crustaceans	Salmon, stickleback, eelpout, flatfish, gobi fish	Bearded seal (*Erignathus barbatus*) and grey seal (*Halichoerus grypus*)

Epidemiology and Public Health

Anisakiasis is an emerging seafood-borne zoonotic disease, which largely remains underdiagnosed. Global incidence of anisakiasis is estimated at 0.32 cases per 1,00,000 population with cases occurring in more than 20 countries over the 5 continents. The majority of reported infections, about 90%, occur in Japan. Cases are also reported from Spain, France, Italy, the Netherlands, Germany, Croatia, South Korea, China, Brazil, Chile, Peru, etc. The infection is widely prevalent in countries, where the fish infected with larvae is consumed raw or undercooked. A wide variety of fish dishes and their consumption are associated with high risk of contracting anisakiasis. These fish dishes include *sushi* and *sashimi* (Japan), *bagoong* (Philippines), *pickled anchovies* and *sardines* (Spain), *smoked and salted herring* (the Netherlands), *ceviche* (South America), *gravlax* (Scandinavian countries), and *lomilomi* and *palu* (Hawaii).

Anisakis infection is widely prevalent in the marine environments involving numerous intermediate, paratenic and definitive hosts. The natural cycle of a particular anisakid species is dependent on various factors including the variety of intermediate, paratenic and definitive hosts available at different marine latitudes.

Diagnosis

Diagnosis of anisakiasis is challenging due to the lack of specificity of the symptoms. History of consumption of raw or undercooked fish or squid in the last 72 h prior to the onset of symptoms like acute epigastric or abdominal pain is a pointer to *Anisakis* infection. Endoscopy is the preferred technique for diagnosing acute gastric or intestinal anisakiasis (Table 2).

Microscopy

Demonstration of the larvae or their remnants in surgically removed histological sections of eosinophilic granulomas aids in the diagnosis of infections caused by *Anisakis* spp. (Fig. 2).

Serodiagnosis

Radio allergosorbent assay test (RAST) and ELISA are frequently used in the diagnosis of allergic anisakiasis. These tests detect specific IgE antibodies in serum against antigens of the parasite, e.g. *Ani s 1* and *Ani s 3*. Recombinant antigens viz. Anis1, Anis3, Anis5, Anis9 and

Table 2 Diagnostic methods for human anisakiasis

Diagnostic approaches	Methods	Targets	Remarks
Microscopy	Endoscopic/ surgical removal	Larvae or their sections in gastric or intestinal tissue	Most commonly used technique Both diagnostic and curative in nature
Immunodiagnostics	Skin prick tests	Crude extracts of *Anisakis* larvae	Rapidly detects allergic reactions Limitations: Low sensitivity and specificity Can lead to sensitization or even frank anaphylaxis
	ELISA	Various crude/excretory–secretory or recombinant antigens like Anis1, Anis5 and Anis7	Different classes of antibodies including specific IgE antibodies are detected
	Immunoblotting	Crude *Anisakis* extracts or recombinant antigens like Anis1, Anis3, Anis5, Anis9 and Anis10	Anisakis-specific IgE detection is performed
	Microarrays	ImmunoCAP ISAC	
Molecular assays	Real-time PCR, RFLP	ITS 1, ITS 2, mitochondrial cox 2	Highly sensitive and specific test *Limitations*: Technically demanding

Anis10 have been used in immunoblotting and microarray techniques like ImmunoCAP ISAC for detecting specific IgE antibodies in patients with anisakiasis.

Molecular Diagnosis

Real-time PCR using various targets like ITS 1, ITS 2 and mitochondrial cox 2 genes has been used for successful detection of *Anisakis* spp.

Fig. 2 L3 larva of an anisakid worm (Courtesy CDC)

Other Tests

Endoscopic examination not only helps in visualization of the parasitic larvae but also aids in removing them, thus avoiding unnecessary surgical interventions.

Skin prick test using L3 larval antigen is used for the diagnosis of allergic anisakiasis. World Health Organization (WHO) and International Union of Immunological Societies Allergen Nomenclature sub-committee have approved 14 different allergens derived from *A. simplex*. Recombinant allergens containing Anis1, Anis3 and Anis7 are commercially available for diagnostic testing. Anis1 is the most commonly used allergen.

Abdominal computed tomography (CT) reveals segmented severe submucosal oedema along with ascites and distended proximal bowel, a characteristic finding in gastrointestinal anisakiasis.

Treatment

Treatment of anisakiasis consists of endoscopic removal of the larvae found in the gastrointestinal tract. However, in cases of difficulty in visualizing and removing the larvae various anthelmintic drugs have been tried. Albendazole

is the most commonly used. Thiabendazole, flubendazole and ivermectin are the other anthelmintics used in the treatment of the condition. Allergic manifestations of anisakiasis need to be treated immediately.

Prevention and Control

Sushi and *sashimi* (Japan), *Lomilomi* (Hawaii), g*ravlax* (Scandinavian countries), *ceviche* (South America), *pickle anchovies* and *raw sardines* (Spain) and *salted herring* (the Netherlands) are the dishes implicated in the transmission of anisakiasis. The prevention of anisakiasis, therefore, is achieved best by avoiding the ingestion of live larvae of *Anisakis* through the consumption of the raw or undercooked fish or squid.

The US-FDA recommends adequately cooking the fish at a temperature of at least 63–74 °C before consumption. Alternatively, the fish may be frozen at −20 °C for a minimum duration of 168 h or the fish may be blast-frozen at −35 °C for about 15 h to kill all the infective larvae in the raw or uncooked fish. Treatment with vinegar or lemon juice, smoking, brining, pickling, marinating, etc., does not inactivate the larvae in raw or uncooked fish. Health education and raising awareness against anisakiasis are also important to prevent and control the infection.

Case Study

A 27-year-old Japanese man presents to the emergency in the midnight with acute-onset intense upper abdominal pain, nausea and vomiting. He is also having intense itching over the entire body and also complaints of respiratory distress. He gives a history of eating sushi at a friend's party about 5 h ago. The doctors suspect anisakiasis, and endoscopy is done, which reveals a hyperaemic ulcerative lesion in the stomach fundus region with small larvae attached to the lesion. The larvae are removed, and subsequently, patient's condition improves. The larvae removed are about 3 cm long and based on their morphological examination are identified as L3 larvae of *A. simplex* sensu stricto.

Questions

1. What is the importance of endoscopy in Anisakis infection?
2. What precautions are needed to prevent Anisakis infection?
3. What are the important parasites associated with seafood consumption?

Research Questions

1. Is there any relationship between genetic predisposition and allergic reactions in anisakiasis?
2. What is the role of Anisakis in gastric and colon cancer?
3. Can the development of allergen-specific therapy be helpful in anisakiasis?

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Gnathostomiasis

Rahul Garg, Aradhana Singh, and Tuhina Banerjee

Learning Objectives

1. To impart the knowledge that the wandering larva may prove dangerous if it reaches the brain or the eye.
2. To evaluate the inadequacy of ELISA in confirming the diagnosis and the usefulness of Western blot.

Introduction

Gnathostomiasis is a food-borne parasitic zoonotic infection caused by ingestion of the third-stage larva of the *Gnathostoma* nematode. The disease is most commonly found in Southeast Asia, Central and South America and parts of Africa. However, its geographical boundaries seem to be increasing owing to unlimited international travel. Man as an accidental host acquires the infection by ingestion of raw or undercooked freshwater fish, eels, cyclops and frogs containing the third-stage larva. Cats and dogs are the definitive hosts. The disease usually manifests as migratory swellings under the skin as creeping eruptions or invasive visceral lesions. A triad of migratory swellings marked eosinophilia, and history of travel to endemic regions often indicates possible infection by the nematode *Gnathostoma*.

History

The first human case of gnathostomiasis was reported by G.M.R. Levinson in 1889 in an infected woman from Thailand. Prior to this, the nematode had been described in the stomach of a young tiger in London Zoo (Richard Owen 1836) and stomach of a pig (Fedchenko 1872). The complete life cycle of the parasite was elucidated in 1937 by Prommas and Dangsavand.

Taxonomy

Gnathostoma spp. belong to the phylum Nemathelminthes (Rudolphi 1808), class Chromadorea (Linstow 1905), order Spirurida (Chitwood 1933), family Gnathostomatidae, subfamily, Gnathostomatinae and the genus *Gnathostoma* (Owen 1836).

The genus *Gnathostoma* is a member of the order Spirurida, which is one of the largest groups of nematodes. These groups of nematodes are known for their requirement of one or more

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intermediate hosts in their life cycles. The genus has 12 species, of which 4 are known to cause infections in humans: *Gnathostoma spinigerum* is the commonest species causing human diseases.

Genomics and Proteomics

The complete mitochondrial genome sequence of *G. spinigerum* has revealed a genome size of 14,079 bp containing 12 protein-coding genes (*cox1–3*, *nad1–6*, *nad4L*, *atp6* and *cytb*), 22 tRNA genes, 2 rRNA genes (*rrnL* and *rrnS*) and 2 non-coding (AT-rich) regions. All these genes were seen to be transcribed in the same direction. High A + T content was seen in the genome. However, a different gene arrangement pattern was seen in *G. spinigerum* as compared to other nematodes, in which a block of 12 genes has been relocated to 4 different locations. Phylogenetic analysis suggests its close relation with *Cucullanus robustus.*

The characterization of the excretory–secretory proteins (ESPs) of the infective third-stage larva has revealed the presence of 171 classical and 292 non-classical secretory proteins. Among these proteins in both the groups, 'molecular function' category comprising several protein kinases was the most abundant followed by 'cellular function' category comprising integral membrane proteins. A significant number of metalloproteases were also present of which a 24 kDa metalloprotease has previously been used for the diagnosis of human gnathostomiasis. Proteins with proteolytic metalloprotease activity, cell signalling regulatory kinases and phosphatase activity and metabolic regulatory functions involving glucose and lipid metabolism were significantly upregulated in the studied secretome. Further, analysis of ESPs of the infective stage larva with *G. spinigerum*-infected human sera and related helminthiases has suggested that the serine protease inhibitor named serpin could be used as a promising antigenic target for the development of immunodiagnostic methods for gnathostomiasis.

The Parasite Morphology

Adult Worm

The adult worm lives in the stomach or the oesophagus of the host. It has a cylindrical body of length varying from 25 to 54 mm in females. The males are shorter, and their length ranges from 11 to 25 mm.

The anterior end of the body possesses a round head bulb. The head contains concentric rows of hooks, 8 to 10 in number. The elongated mouth in the centre is surrounded by a pair of lips. The body is covered by cuticular spines of varying size and distribution. Based on the species, the genital organs are located at the caudal end of the worm. The male genitals comprise the papillae and a curve towards the ventral side with two spicules of unequal length. In females, a true vagina with double uterus containing eggs at various stages of development is seen. The eggs are discharged outside through a vulva, which is found located in the middle of the body.

Infective Larva

The third-stage larva (L3) is the infective form for definitive hosts such as the human (accidental host). *G. spinigerum* larvae usually measure up to 3–4 mm in length and 630 μm in diameter and are usually reddish-white. The head bulb contains four rows of cephalic hooklets, with approximately 45 hooklets per row. The body of the larva is covered with transverse rows of sharply pointed spines that diminish towards the posterior end of the worm. These rows of hooks enable the larva to position itself in the host tissues. This in turn is responsible for the mechanical damage to the host to some extent.

Eggs

Gnathostoma fertilized eggs are excreted along with stool of the definitive host. Eggs are oval, approximately 70 × 40 μm in size, and are yellow

or brown due to their direct contact with bile juice. The eggshell has either some small pits or a smooth surface, with one or two polar bulges.

The morphology of the different stages of *Gnathostoma* is depicted in Fig. 1.

Cultivation of Parasites

Cultivation of advanced third-stage larvae of *G. spinigerum* has been attempted in RPMI-1640 media containing various combinations of nutritive elements. Parasite survival rates in

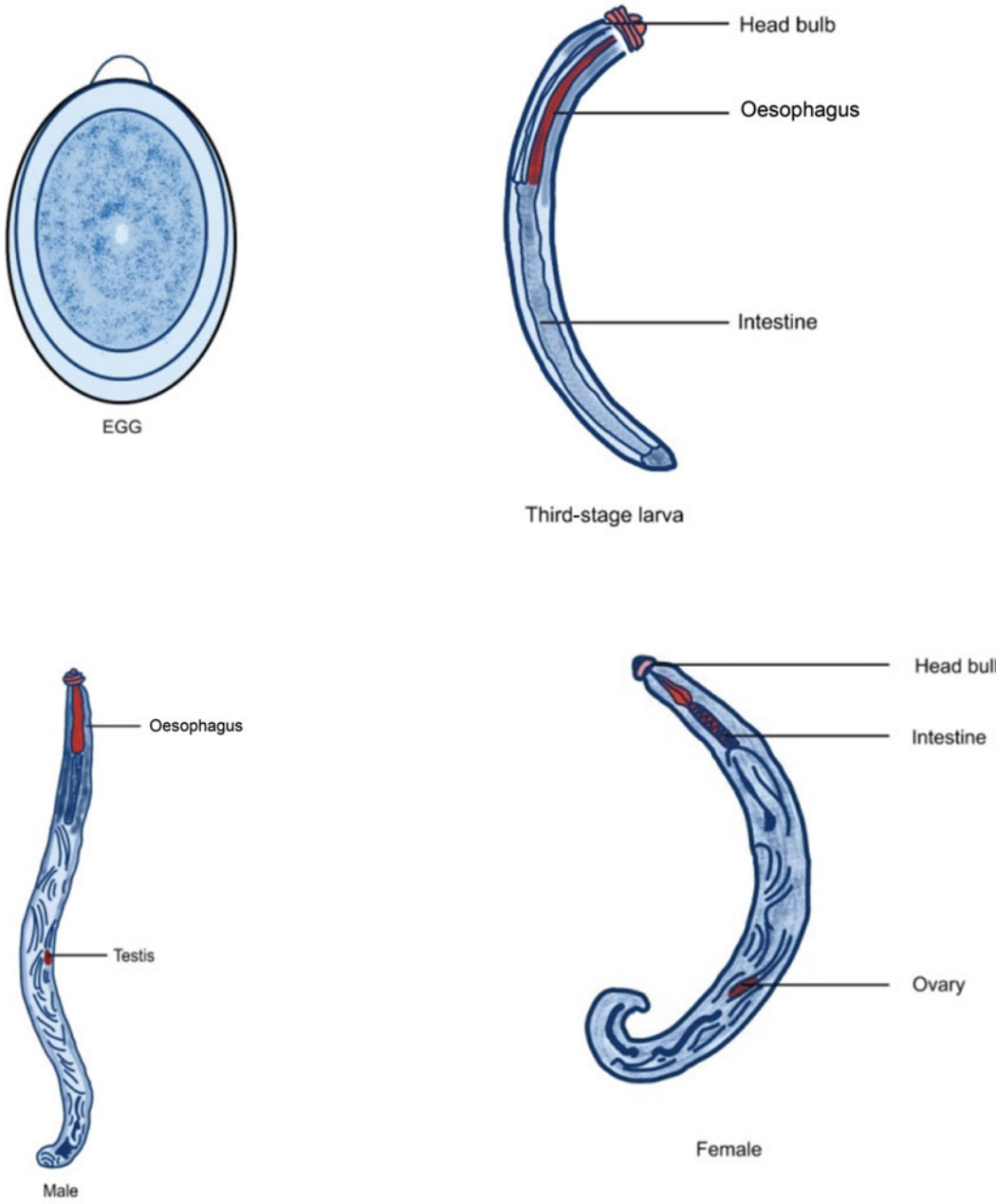

Fig. 1 Morphology of the different stages of *Gnathostoma* spp.

artificial media and larval development varied widely depending on the choice of the additives. While the most suitable medium for larval growth and development into the next stage was seen in media supplemented with 10% foetal calf serum, 1% dog serum and 0.25% dog haemolysate, survival of most of the larva was observed in media supplemented with sodium bicarbonate salt.

Laboratory Animals

Rodent models have been extensively used for studying larval migration, immune responses and drug susceptibilities against this parasite. As rodents are accidental hosts like humans, rodent models have been quite successful for studies on this parasite. The Swiss albino mice model has also been used for studies as larval encystment occurs in mice, and mice also acts as accidental hosts.

Life Cycle of *Gnathostoma* spp.

Hosts

The life cycle of *Gnathostoma* spp. comprises two stages namely the larval and adult worm. The definitive hosts are dogs, cats, tigers, leopards and other fish-eating mammals. In these hosts, the adult worm lies coiled up in the stomach wall, thus causing a tumour-like mass called nodules. Adult worms of a few species are found in the oesophagus or kidney. The cyclops or water fleas are the first intermediate hosts, whereas fish and amphibians are suitable second intermediate hosts. Man is the accidental definitive host.

Infective Stage

Advanced third-stage larvae (AL3) is the infective stage.

Transmission of Infection

Following ingestion of the water flea or copepod by a suitable second intermediate host (fish, amphibians), the EL3 migrates through the tissues of these hosts and encysts in their muscles. In these hosts, they develop further into AL3 and remain as infective larvae.

The infection is acquired by the definitive hosts such as cats, pigs, dogs and other wild hosts on ingestion of fish or other amphibians harbouring the advanced third-stage larvae (AL3) of the nematode, the infective stage of the parasite. The larvae are released in their gastrointestinal tract, following which they migrate to the liver and abdominal cavity. After about 4 weeks, they invade the gastric wall, in which they form tumour-like masses. The adult worms conjugate and produce unembryonated eggs. In the tumour-like mass, the eggs pass through a small opening and are finally excreted in the faeces. Thus, release of eggs into the environment occurs about 8–12 months after the initial ingestion of the infective third-stage larvae by the definitive host. The eggs contaminate freshwater bodies such as river, ponds and lakes in which they embryonate and nearly after 7 days of hatch release first-stage larvae (L1) in the water. The excysted larva is then ingested by the first intermediate host, which in most cases is a water flea or copepod (cyclops), inside which the larva moults twice to become early third-stage larvae (EL3). The second intermediate host may be ingested alternatively by a paratenic host such as snakes and birds. However, in these paratenic hosts the AL3 does not develop further but remain infective.

Humans acquire the infection by consuming raw or improperly cooked meat of the second intermediate hosts or paratenic hosts containing AL3. After entry, AL3 migrates in various tissues and may develop into immature adults without reproductive maturity. Their size varies from 2 mm to 2 cm depending on the species and the extent of development. However, two alternative routes of infection have been proposed. One is the ingestion of water containing infected copepods

instead of a second intermediate host. The other is by penetration of the skin by third-stage larvae from infected meat in the food handlers (Fig. 2).

Pathogenesis and Pathology

The exact pathogenesis of gnathostomiasis is uncertain. However, it is believed that the symptoms are caused by the combined effects of several factors like mechanical damage caused to the tissues and organs secondary to the larval migration, various excretory–secretory products of the parasite and the host's immunological response. The substances released contain different compounds, for example one compound similar to acetylcholine, a 'spreading factor' with hyaluronidase, a haemolytic substance and a proteolytic enzyme, which have been demonstrated in several studies. These substances result in the characteristic haemorrhagic tracks as seen in the subcutaneous tissues in patients, along with mechanical damage. These tracks can also be seen in the viscera or CNS at postmortem. These tracks are pathognomonic of this parasitic infection.

The human cycle starts with an enteric phase followed by perforation of the mucosa to invade other organs. The skin presentations wax and wane owing to the migration occurring in bouts. In addition, direct trauma and intense eosinophilia in the tissues cause severe tissue inflammation. Eosinophilia in subcutaneous tissue results in panniculitis, which is characteristic of the infection. This eosinophilic cellulitis is demonstrated as 'flame figures' on histopathology. Epidermal changes are rare.

Immunology

Monocytes are important in helminth infections, which act by complex interplay between the T cells, eosinophils, basophils and mast cells. They reach the sites of infection and execute their roles as immune cells by acting as precursors of macrophages and dendritic cells at the tissues sites and aid in antigen presentation in response

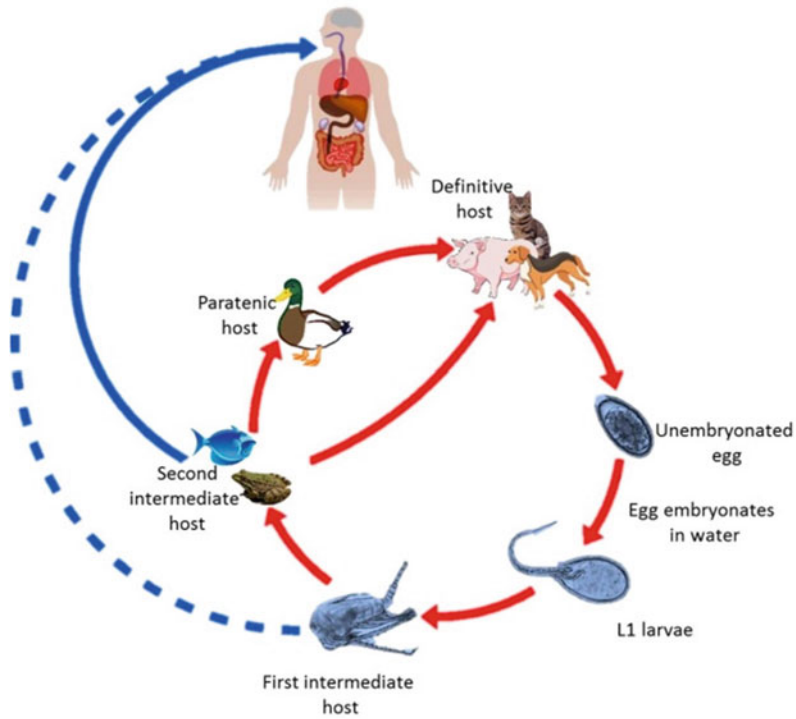

Fig. 2 Life cycle of *Gnathostoma* spp.

to helminth infections. Fc receptors are membrane glycoproteins with affinity for binding with the corresponding portion of the secreted antibodies. The FcγRI receptor expressed on antigen presenting cells (APC) is the human high-affinity receptor for IgG, and the upregulation of which by stimulation from cytokines like IFN-γ and IL-2 results in the cascade of reactions like phagocytosis, cytokine production and antibody-dependent cell-mediated cytotoxicity (ADCC). The role of monocytes in human gnathostomiasis is important. The excretory–secretory antigens of *Gnathostoma* affect the monocyte function by decreasing the expression of FcγRI receptor, thereby decreasing all the biological activities leading to phagocytosis.

Since gnathostomiasis is characterized by migration of the parasite within various tissues and organs in the human host, immune responses vary depending on the extent of invasion. Consequently, predominant rise of IgG4 has been reported in some cases, while rise of combined IgG1 and IgG2 responses has also been seen in other cases of infection.

Infection in Humans

The clinical manifestations of gnathostomiasis present a wide spectrum of infections in humans. These range from non-specific acute symptoms to a more common cutaneous form and a severe visceral form.

The patients within 24–48 h of ingestion of food infected with *Gnathostoma* spp. may present with non-specific symptoms like malaise, fever, generalized urticaria, anorexia, nausea, vomiting, diarrhoea and epigastric pain. These manifestations correspond to the phase of excystation of the larva and migration through the stomach or intestinal wall and the liver. These symptoms may persist for 2–3 weeks. Marked generalized eosinophilia usually develops at this stage, which can be as high as 50% of the total white blood cell count.

Cutaneous Gnathostomiasis

It is the most common manifestation, which is known by several local names like *Yangtze River's oedema* and *Shanghai's rheumatism* in China, tuao chid in Japan and *panniculitis nodular migratoria eosinofilica* in South America. The condition presents as swellings on the torso or upper limbs, which may be erythematous, pruritic, with or without pain. The swellings usually occur within 3–4 weeks after the ingestion of the larvae and last for about 1–2 weeks. Recurrence of the swellings may occur if the infection is not treated. The condition may also present as a creeping eruption simulating cutaneous larva migrans, and rarely as skin abscess, or a nodule.

Visceral Gnathostomiasis

Visceral gnathostomiasis or larva migrans is caused by migration of the larva in deeper tissues involving the pulmonary, gastrointestinal, genitourinary, ocular or the central nervous systems of the host.

The most serious manifestation of gnathostomiasis is associated with the CNS involvement. The acute onset of excruciating radicular pain with or without headache due to subarachnoid haemorrhage or eosinophilic meningitis is the hallmark of the CNS manifestations. The onset of manifestations depends on the migratory pathway of the parasite in the CNS. The parasite gains entry to the spinal cord along the nerve roots in cranial, cervical, thoracic or lumbar regions. This is marked by intense radicular pain or headache, which usually lasts for 5 days. The initial pain is followed by manifestations ranging from weakness to complete paralysis of one to four limbs based on the parasite's movement as it ascends through the spinal cord to the brain.

The CNS manifestations caused by *Gnathostoma* resembles that of *Angiostrongylus cantonensis* infection of the CNS, which is also a

highly prevalent parasite in Southeast Asia. However, on comparison, the acute nerve root pain, signs of spinal cord compression, and haemorrhagic or xanthochromic spinal fluid observed in gnathostomiasis are absent in *Angiostrongylus* infection. Being more invasive, the *Gnathostoma* larva produces more frequent focal neurological signs. In contrast, the *Angiostrongylus* larva, which is considerably smaller and usually multiple, more commonly causes a meningoencephalitis, which usually takes a non-fatal course.

Infection in Animals

Cats and dogs are the definitive hosts that acquire infection by ingestion of fish containing the third-stage larva. *Gnathostoma* infections in cats and dogs present as gastritis and loss of appetite. The abdominal nodules may burst open into the peritoneal cavity causing severe peritonitis and even infections. In pigs, massive infections cause severe gastritis and developmental delays. However, most of the cases are benign, and nodules are often noticed accidentally in the carnivores during postmortem.

Epidemiology and Public Health

Gnathostomiasis in humans is an emerging infectious disease with gradual spread to non-endemic areas. Changes in food habits have been implicated as one of the primary reasons for the global spread of infection. Though initially found in South Asian regions, currently, Japan accounts for maximum incidence of gnathostomiasis. Thailand, Cambodia, Laos, Malaysia, Myanmar, Indonesia, Philippines and Vietnam have also reported the infections. Though not as frequently as these countries, yet cases have also been seen in China, Sri Lanka and India (Fig. 3).

In South America, after the first case in 1970, cases are on rise with maximum reports being documented from Mexico, Ecuador and Peru. Surprisingly, as against the usual conditions of low socioeconomic status as one of the factors for this infection, cases in Peru have also been seen in high-class communities. Most of the cases in

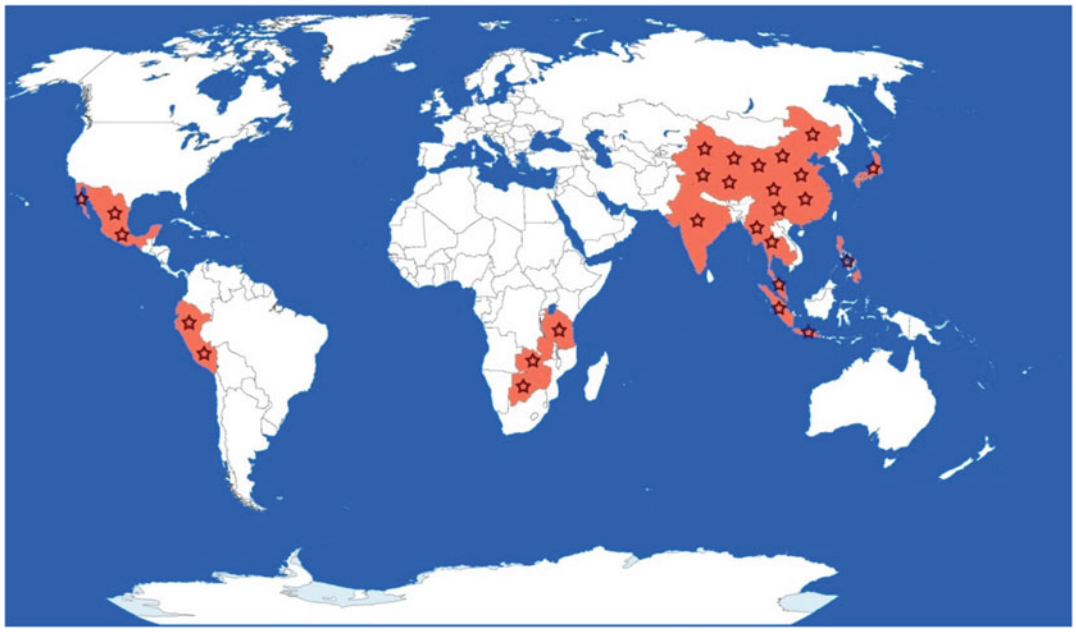

Fig. 3 World map showing the countries with acquisition of gnathostomiasis (indicated by *red star*)

developed countries have been linked with extensive intercontinental and intracontinental travel. Recently, imported and autochthonous cases have also been reported from Korea, Brazil and Colombia. The extensive development of pisciculture and business involving aquatic flora has been cited to be responsible for rise in these cases by infecting the flora with imported flora infested with the parasite or its larval forms.

Gnathostomiasis in animals is mainly found in wild and domestic cats and dogs. The species is prevalent in India, China, Japan and Southeast Asia. *Gnathostoma hispidum* is another species, which is found in wild and domestic pigs. The species is distributed in Europe, Asia and Australia. *Gnathostoma doloresi*, found in wild boars in parts of the Central and Eastern Europe, is the third species that can infect humans. *Gnathostoma nipponicum*, found in Japan and China, also cause human infections. Two human cases of *Gnathostoma malaysiae* infections have been reported from Myanmar but yet to be confirmed.

Data are inadequate on the wildlife reservoirs of the parasite. Characterization of all possible first and second intermediate hosts has also been one of the challenges to identify the possible reservoirs of this parasite. The major *Gnathostoma* species causing infections is listed in Table 1.

Diagnosis

Microscopy

The definitive diagnosis of *Gnathostoma* infection requires isolation and demonstration of the nematode. In cases of superficial pseudofuruncular skin lesions, dermatoscopy often allows the direct visualization of worms in the migratory swellings. Worms have also been isolated from incision drainage of other skin lesions like blisters on palms and hands. Biopsy of the skin lesions following induction therapy with ivermectin or albendazole increases the possibility of direct isolation and visualization of the worms. Under the light microscope, distinct head end with hooklets and oral opening confirms the identification of *Gnathostoma* (Fig. 4).

The isolation of larvae, the infective stage in humans, from the lesions they cause is often difficult, especially in migratory skin lesions. For this reason, most often the diagnosis of gnathostomiasis rests mainly on the four criteria, i.e. clinical presentation, epidemiological background, eosinophilia and supportive serological tests. The microscopic images of *Gnathostoma* are shown in Fig. 4.

Table 1 Major *Gnathostoma* species of importance for humans and animals

Gnathostoma species	Geographical occurrence	Infections in man	Infections in animals
Gnathostoma spinigerum	Southeast Asia, Japan, Australia, USA, Mexico	Cutaneous and visceral gnathostomiasis	Gastritis and lack of appetite in cats and dogs
Gnathostoma hispidum	Thailand, Japan, China, Korea, Mexico	Cutaneous gnathostomiasis, eosinophilic meningitis	Abdominal nodules leading to gastritis in pigs and wild boars
Gnathostoma doloresi	Southeast Asia, Australia	Cutaneous and visceral gnathostomiasis	Gastritis in wild boars
Gnathostoma malaysiae	Myanmar, Malaysia, Thailand	Cutaneous gnathostomiasis (unconfirmed cases)	Unknown
Gnathostoma nipponicum	Japan and China	Cutaneous and visceral gnathostomiasis	Unknown
Gnathostoma binucleatum	Mexico, North America	Cutaneous and visceral gnathostomiasis	Unknown

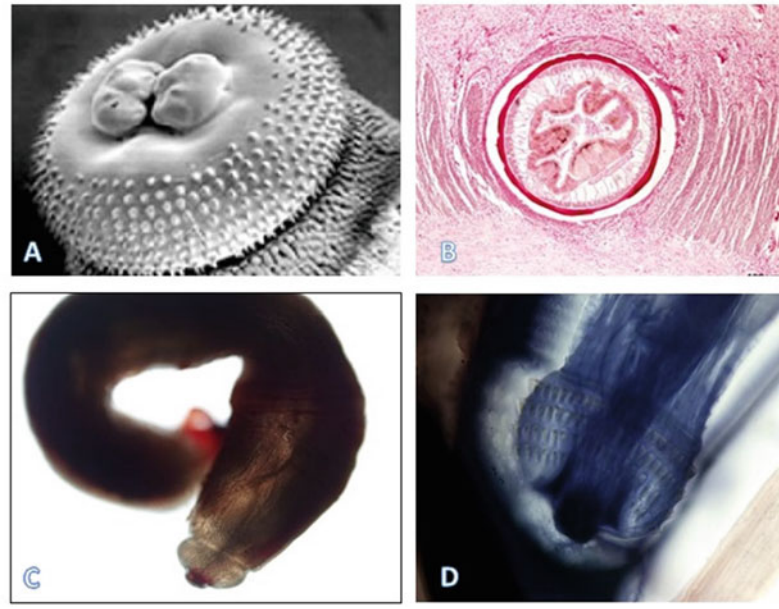

Fig. 4 Microscopic image showing (**a**) cephalic bulb of *Gnathostoma spinigerum* female worm through scanning electron microscope, (**b**) cross section of an immature *Gnathostoma spinigerum* by H&E staining, (**c**) *Gnathostoma spinigerum* immature male worm, (**d**) cephalic bulb of a *Gnathostoma spinigerum* larva by haematoxylin staining (adapted from CDC, https://www.cdc.gov/dpdx/gnathostomiasis/index.html)

Serodiagnosis

A positive serological test for IgG antibody, by indirect ELISA, using either crude or purified Gnathostome L3 larval antigen has shown suboptimal sensitivity (ranging from 59% to 87%) and specificity (ranging from 79% to 96%), showing cross-reactivity with *Paragonimus westermani, Toxocara canis, Anisakis* sp. and *Fasciola hepatica*. Currently, the immunoblot test, using L3 antigen of 24 kDa, is considered, as the test of choice for the diagnosis of gnathostomiasis (Fig. 5). The test has shown a high degree of specificity without demonstrating any false-positive reactions with sera from other parasitic infections.

Molecular Diagnosis

Molecular methods are very useful for the detection and identification of *Gnathostoma* spp. Primers against partial *cox1* gene and entire inter-transcriber sequence (ITS-2) region have been mostly used. As compared to the ITS1 region, the use of sequences in ITS2 region has been preferred for inter-species identification. The partial *cox1* gene shows the intraspecies variations. The use of molecular genetic information results in the accurate identification of species of the parasite. The important diagnostic methods have been summarized in Table 2.

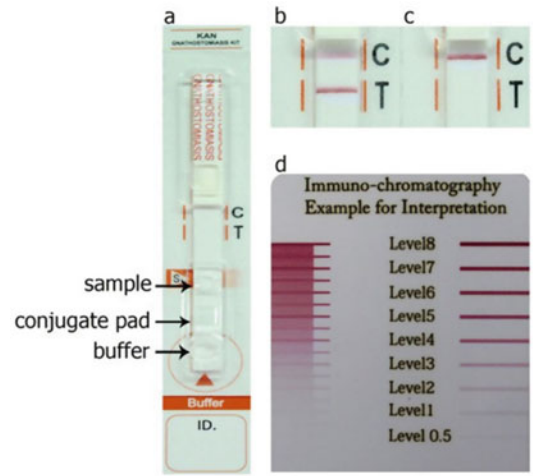

Fig. 5 The KAN gnathostomiasis kit showing (**a**) immunochromatographic (ICT) assay-based diagnostic test strip for human gnathostomiasis. (**b**) Representative images of ICT strips of positive result, (**c**) negative result, (**d**) guide for interpretation

Table 2 Diagnostic methods in gnathostomiasis

S. no.	Diagnostic method	Details
1.	Microscopy	• Distinct head end with hooklets and oral opening can be appreciated
2.	Morphological diagnosis	• Identification of the immature adult or larva after extraction or in biopsy specimen
3.	In vitro culture	• RPMI-1640 media most commonly used • Supplements used to promote growth include 10% foetal calf serum, 1% dog serum, 0.25% dog haemolysate, sodium bicarbonate salt
4.	Serology	• A specific 24 kDaL3 antigen is presently accepted as the test of choice
5.	Molecular diagnosis	• Identification at the species level • *cox1* gene and ITS2 region have been most frequently targeted

Other Methods

Peripheral eosinophilia and/or increased eosinophilia in the CSF, although not specific, may suggest the tentative diagnosis of the condition. During initial migration of the worm, peripheral eosinophilia is frequently present, with >50% of circulating leucocytes identified as eosinophils. CSF eosinophilia is also highly supportive of CNS disease caused by *G. spinigerum*, *A. cantonensis* or *Cysticercus cellulosae* in the countries of Southeast.

Treatment

Treatment of gnathostomiasis envisages multiple dosages of anthelmintic drugs, particularly the benzimidazole derivatives, such as albendazole and ivermectin. Albendazole administered at doses of 400 mg/day twice daily for 21 days is effective. The compound apparently stimulates outward migration of the larva and in turn facilitates surgical excision for removal of the larva. Similarly, ivermectin at 0.2 mg/kg stat dose or for two consecutive days or after 7 days also shows similar efficacy. However, the efficacy of ivermectin in the management of the secondary exacerbations of the cutaneous manifestations is still doubtful. A combination of albendazole 400 mg, three times daily for 3 weeks, and a single dose of ivermectin 0.2 mg/kg has also been reported as highly satisfactory with few episodes of relapses. More recently, trials using mebendazole have also shown high worm reduction rates varying from 82.8% to 96.4%. CNS involvement in gnathostomiasis simulates with that of neurocysticercosis; hence, the management of the condition encompasses the use of steroids prior to anthelmintic administration to treat oedema and local inflammation.

Prevention and Control

As 100% cure rate is not achievable with the available anthelmintic treatment options, the prevention of gnathostomiasis is of immense priority. Rapid freezing of fish at −20 °C and marinating fish are not effective methods for the destruction of larva inside the infected fish. Therefore, consumption of completely cooked food appears to be the only effective preventive measure for the infection. Increased public awareness for avoidance of eating raw uncooked fish and preference for cooked food, hence, is essential.

Case Study

A 29-year man presented with pain, redness and diminution of vision of right eye in the past 1 month. Patient reported mixed diet of fish and poultry. General examination revealed no oedema, pallor or organomegaly along with no history of migratory skin eruptions. Slit-lamp examination showed anterior uveitis and multiple iris atrophic patches with a live and motile worm on the anterior chamber of iris.

Routine urine and stool examination revealed no egg/larva/worm. All the other investigations including chest X-ray, brain MRI and ultrasonography B-scan of both the eyes were normal. The larva was removed surgically and identified as *G. spinigerum.*

Questions
1. What is the mode of infection?
2. What are the other zoonotic ocular parasites?

Research Questions

1. What may be the other wild reservoirs for gnathostomiasis other than the known one?
2. Which proteins are the highly specific antigens for immunodiagnosis?
3. What is the effective treatment regimen for gnathostomiasis?

Further readings

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Dirofilariasis

Sourav Maiti

Learning Objectives

1. To ascertain that as a zoonotic filarial infection, the worms in humans do not produce larval microfilarial forms.
2. To understand the pathology of dirofilariasis in the infected lungs in humans.

Introduction

Dirofilariasis is an arbohelminthic disease of zoonotic importance, caused by *Dirofilaria* spp. *Dirofilaria immitis* and *Dirofilaria repens* are the most well-known species, with a diverse impact on both human health and veterinary health. While *D. immitis* is responsible for canine heartworm disease and human pulmonary dirofilariasis over wide geographic regions, *D. repens* typically causes subcutaneous dirofilariasis in both. *Dirofilaria* sp. is a also potential health concern for travellers also.

History

Amato Lusitano was probably the first to describe a girl with worms in her eyes in 1566, possibly *D. repens*. However, the first human dirofilariasis case was documented by De Magelhaes in 1887 from the left ventricle of a Brazilian boy on post-mortem examination. Subsequently, several reports originated from Europe regarding human ocular and subcutaneous infections. Canine heartworms were first discovered in 1856 on the south-east coast of the USA. Ercolani pointed out the fact that in microfilaraemic dogs, worms may be found in subcutaneous tissues apart from the heart. Grassi showed experimental transmission of parasites to mosquitoes in 1900. In the next 10 years, Demiaszkiewicz (2014) first described the nematode and named it *Dirofilaria repens*. Years later, the larval development in vector mosquitoes was published. In 1921, infection in cats was recognized. It was not until 1952 that human infection by *D. immitis* was documented in the USA. *Wolbachia* sp., an endosymbiont bacteria residing in filarial worms, was first discovered as bacterium-like bodies in *D. immitis* by electron microscopy in the 1970s. Later, in 1995 16S rDNA-based phylogeny of *Wolbachia* sp. was published from Italy during ongoing studies on *D. immitis*.

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Taxonomy

The genus *Dirofilaria* belongs to subfamily Dirofilariinae; family Onchocercidae; superfamily Filarioidea; suborder Spirurina; order Rhabditida; class Chromadorea and phylum Nematoda.

The genus *Dirofilaria* is divided into two subgenera: *Dirofilaria* (including *D. immitis*) and *Nochtiella* (more than 20 species including *D. repens*, *Dirofilaria striata*, *Dirofilaria subdermata*, *Dirofilaria sudanensis*, *Dirofilaria tawila*, *Dirofilaria tenuis* and *Dirofilaria ursi*).

Phylogenetic analysis of cytochrome c oxidase 1 gene is utilized for the identification of *Dirofilaria* sp. Studies revealed low genetic variability among *D. immitis* isolates over several countries compared to *D. repens,* which shows high intra-species variability. Recently, a *D. repens*-like filarial worm has been described as *Candidatus Dirofilaria hongkongensis* based on ITS-1 sequence difference. Yilmaz E et al. studied complete mitochondrial genomes of *D. repens* and this proposed new member and found their sequences clustered together as a common sister group to *D. immitis*. Their study strengthens the hypothesis that *C. D. hongkongensis* might be an independent species. That study also described microfilaria from Thailand, which could be another cryptic species *Candidatus Dirofilaria* sp. 'Thailand II' or a divergent population of *C. D. hongkongensis*.

Genomics and Proteomics

High-throughput Illumina technology has delineated *D. immitis* genome with assembly size of 78.16 MB with GC content of 28.3%. Interestingly, sequence analysis of *D. immitis* isolated from different countries showed very little (0.04%) genetic variation. Also, it harbours neither DNA transposons nor any active retrotransposon. Differential presence of home and nucleotide synthesis pathways possibly points out the metabolic mutualism between *D. immitis* and *Wolbachia* sp., endosymbiont bacteria. Very recently, *D. repens* genome was analysed revealing 17% larger size (99.59 MB) with fewer overlapping consensus regions of DNA or contigs (916 versus 11,654) and 0.7% lower GC content. Fewer proteins could be predicted compared to *D. immitis* (11,262 versus 12,344) as the protein-coding sequence was shorter (15.5% versus 18%). The *D. repens* genome contains a larger number of exons per gene (7 versus 5) than *D. immitis* does, exons being slightly shorter (136 bp versus 142 bp). This difference could be meaningful to explain their biological difference. Of identified proteins, 1.8% were dissimilar to *D. immitis,* but a few of them were biologically similar to *Loa loa*.

The genomic similarity of *D. repens* to *Loa loa* was also reflected in proteomic studies. Significant enrichment of *D. repens* proteins is in stark contrast to those of *D. immitis*. *D. immitis* proteins get clustered with complete nematodal proteome and included 3199 proteins (31% of total proteome) unique to *D. immitis*, a proportion similar to *B. malayi* (27%). Interestingly, 850 proteins are uniquely shared by both of them. Myosin-like antigen OVT1 in *O. volvulus* has two homologues in *D. immitis*, particularly in third- and fourth-stage larvae. Mass spectrometry data group the protein moieties in major four categories. Of these, two groups contain enzymes for anaerobic glycolysis and for the redox reactions and detoxification. Another group consists of actin-1, actin-2 and other molecules involved in motility. Heat shock proteins (HSP70, p27, etc.) belong to the fourth category. Overall, a significant proportion of *D. immitis* proteins are collagenase-susceptible acidic polypeptides ranging from 82 kDa to >200 kDa. A 35 kDa polypeptide has been identified as an immunodominant surface antigen in third-stage larvae (L3). Although abundant glycosylated molecules are found in the extract, these are not exposed on the surface of the intact worm.

The Parasite Morphology

Adult Worm

The adult worm is long, thin, cylindrical and whitish in colour.

Dirofilaria immitis: Adult female worms measure 230–310 mm in length and 1.0–1.3 mm in thickness. The terminal oral aperture lacks lips but is surrounded by 6 small median papillae and 2 lateral papillae. An anal opening is located subterminally at the obtuse caudal end. The vulva opens posterior to the oesophago-intestinal junction. These are ovoviviparous. Adult males are smaller and thinner, measuring 120–200 mm in length and 0.7–0.9 mm in thickness. A spirally coiled tail-end harbouring two lateral alae characterizes the male adult *D. immitis*. The cloacal opening is located near the caudal end (0.13 mm proximally). Three groups of papillae are located on the ventral side around it. The cuticle is smooth with a striated ventral surface of the last coil of the caudal end (Fig. 1).

Dirofilaria repens: *D. repens* adults are smaller and stubbier than *D. immitis*. The cuticle contains the characteristic striations. Adult females measure 100–170 mm in length and 4.6–6.3 mm in thickness. Females are ovo-viviparous. The vulval opening is encircled by slightly projecting labia and is situated 1.84–1.92 mm from the cephalic end. The tail tip is obtuse and curves slightly to the ventral side. Adult males are 50–70 mm long and 3.7–4.5 mm thick. The ventrally curved caudal end bears 2 lateral alae and oblong pedunculate papillae.

Microfilariae

Dirofilaria microfilariae are devoid of a sheath unlike other microfilariae including *Acanthocheilonema dracunculoides* and *Cercopithifilaria grassii* found in infected dogs and cats (Fig. 2). Microfilariae of *D. immitis* are slightly shorter and thinner than those of *D. repens*. Microfilariae of *D. immitis* measure 290–330 μm in length and 5–7 μm in width, whereas those belonging to *D. repens* are 300–360 μm long and 6–8 μm thick. These can be differentiated by looking at the cephalic end, which is pointed in the former and obtuse in the latter. Also, the former has a pointed straight tail compared to a filiform/umbrella handle-like tail in *D. repens*. Sometimes non-sheathed microfilariae of *Acanthocheilonema reconditum* may be confused with these but the cephalic hook-like structure distinguishes it. Histochemical staining reveals two acid phosphatase activity spots in *D. immitis* microfilariae corresponding to the anal and excretory pores. Only one such spot is visible in *D. repens* microfilariae (anal pore), while *Acanthocheilonema* sp. shows spots over the whole microfilaria body. *D. striata* microfilariae measure 299 μm × 5–6.5 μm and are characterized by two prominent nuclei separated from the main body of the nuclear column within the cephalic space. *D. tenuis* microfilariae are longest, measuring 361–379 μm in length with a thickness of 7 μm.

Cultivation of Parasites

In vitro cultivation of filarial worms is difficult due to poor survival and developmental arrest. Attempts have been made to maintain *D. immitis* adult worms in a variety of media to extrude microfilariae. Sawyer and Weinstein (1963) first described successful development of microfilariae of *D. immitis* to the sausage-shaped late first-stage larva after inoculating host erythrocytes in a serum-supplemented chemically defined media NCTC 109. Insect mediums MM/MK and MM/VP_{12} improved survival up to 7 days without any development.

Laboratory Animals

Ferrets (*Mustela putorius*) have been utilized as the hosts for heartworm research. Upon a

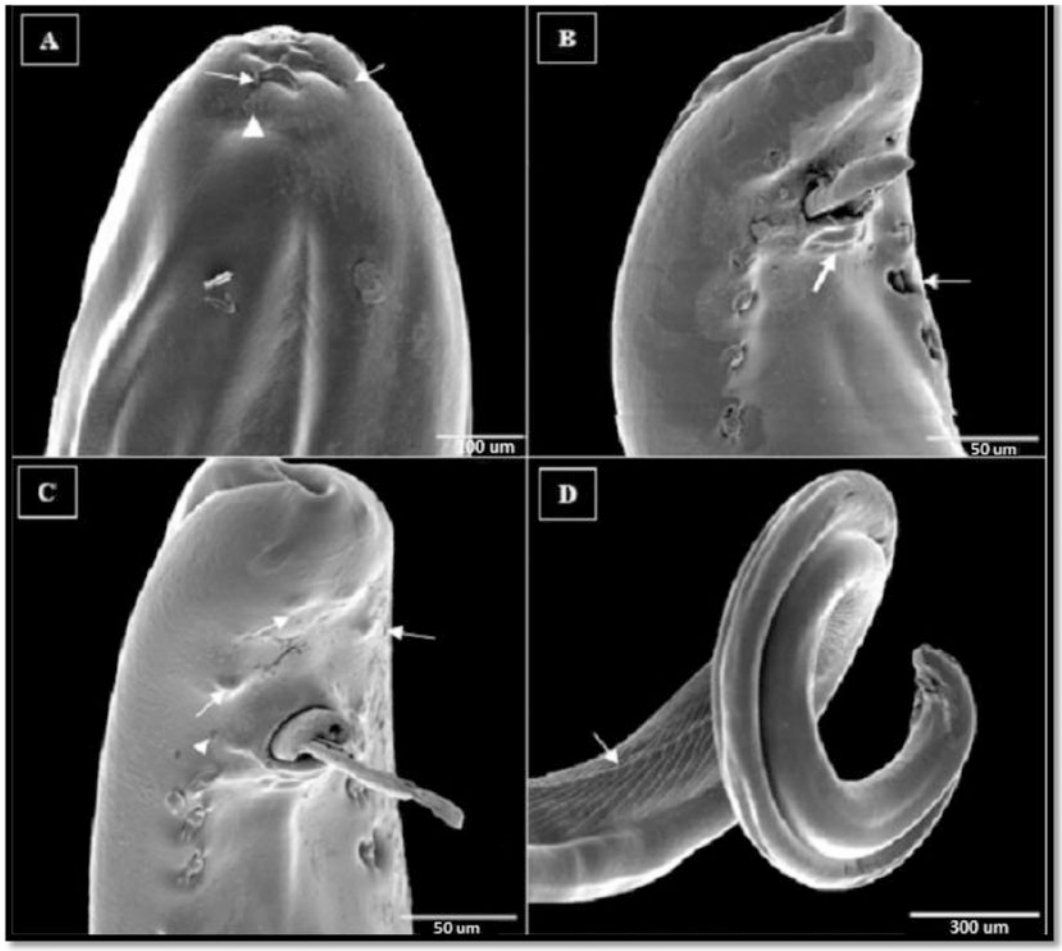

Fig. 1 Scanning electron micrographs of male *Dirofilaria immitis* showing (**a**) cephalic end with oral aperture details; (**b**), (**c**) and (**d**) showing posterior part ventral view showing papillae, anus, cloacal bumps, spicules and longitudinal striations. Image reproduced with permission from AR Meamar. Citation: Iranian Journal of Parasitology. 2020;15(1):57–66

successful infection, the adult worms are found mainly in the heart chambers and the associated veins and pulmonary arteries. Vena cava syndrome occurs commonly. Disease manifestation is similar to dogs but with a faster progression. The low parasitic load may cause death by pulmonary embolism. BALB/c mice have been utilized as animal models for immunological studies involving *Dirofilaria immitis*.

Life Cycle of *Dirofilaria* spp.

Hosts

Definitive Hosts

A variety of mammals including carnivores, primates and dogs are definitive hosts. Humans are the incidental hosts (Fig. 3).

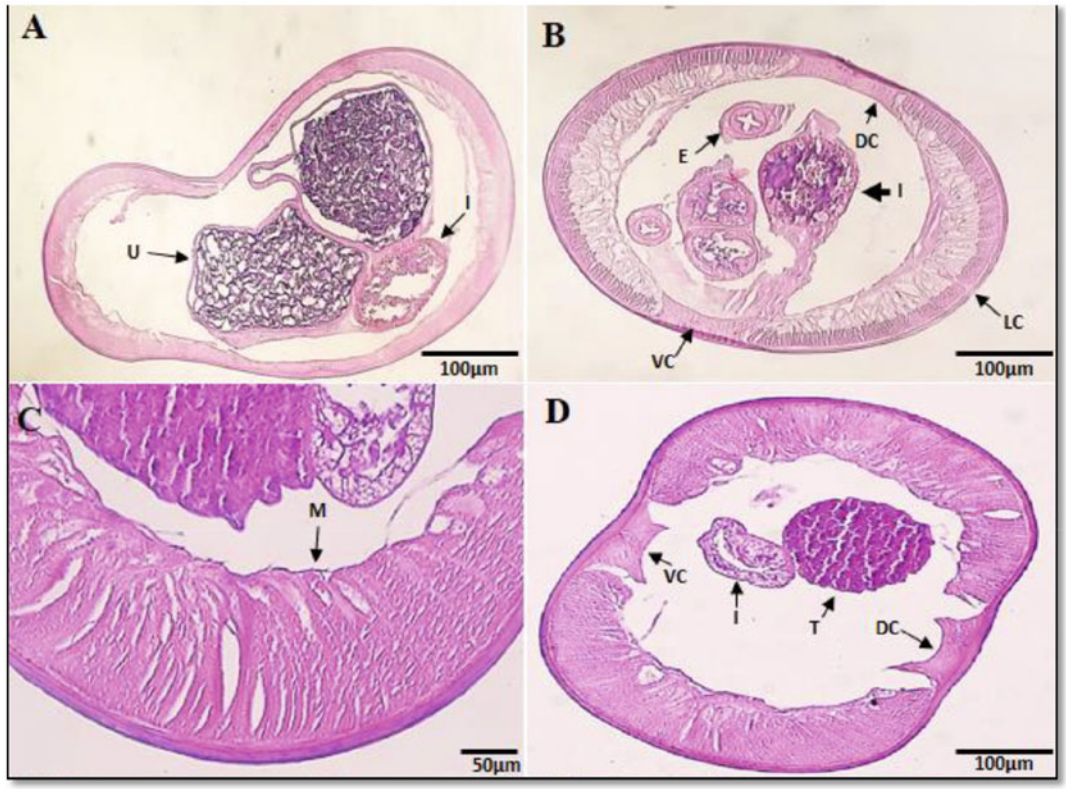

Fig. 2 Adult *Dirofilaria immitis* cross-sectional anatomy (**a**–**d**, **h** & **e**): (**a**) female (**b**) male. *DC* dorsal cord, *E* oesophagus, *I* intestine, *LC* lateral cord, *M* tail end muscle layer, *T* testis, *VC* ventral cord, *U* uterus. Image reproduced with permission from AR Meamar. Citation: Iranian Journal of Parasitology. 2020;15(1):57–66

Intermediate Hosts/Vectors

Aedes, *Anopheles*, *Culex*, *Culiseta* and *Mansonia* sp. are the arthropod mosquito vectors. The most important intermediate hosts are those species without the buccopharyngeal armature that damages the microfilarial cuticle.

Infective Stage

Third-stage larvae (L3) are the infective stage.

Transmission of Infection

A typical sylvatic life cycle involving carnivorous mammals is common. All the species depend on an arthropod vector (mosquito) to be infected by infective third-stage larvae (L3) during a blood meal from mammals. These L3 larvae invade on their own into the soft tissues of skin. Several reach the muscle sheaths too. These are the locations for moulting and maturing, which takes around 4 months. Thereafter, migration to the heart begins. Studies suggest that in six months, they fully mature into sexually competent adults and mate inside the pulmonary arteries. After mating, the female becomes gravid and begins to release microfilariae into the bloodstream. The mosquito takes the blood meal and gets infected. Up to several thousand microfilariae (first-stage larvae or L1) are shed daily. An infected dog may circulate several hundred microfilariae per ml of blood. Ingested L1 larvae reach malpighian tubules and undergo two subsequent temperature-dependent moultings to develop into third-stage larvae (L3). L3 larvae migrate to the labial sheath lumen in the vector mouthpart to initiate the cycle again.

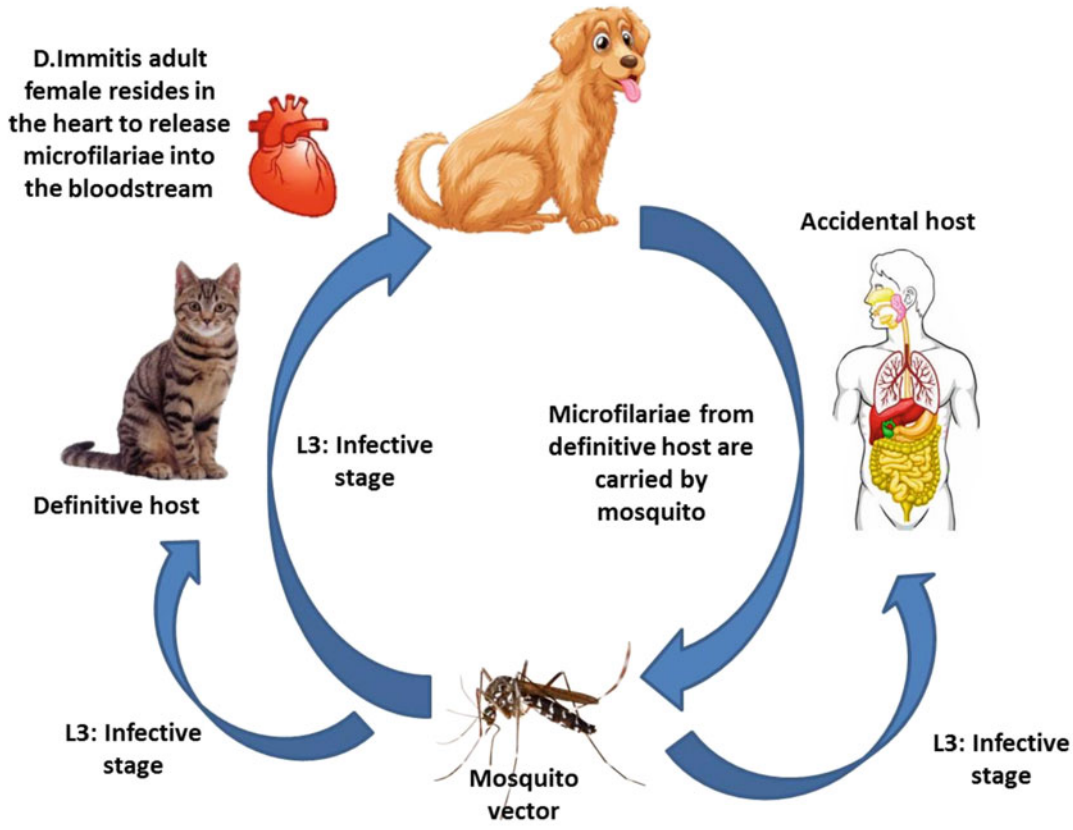

Fig. 3 The life cycle of *Dirofilaria immitis*

Humans act as an incidental host. The larvae embark on their journey but fail to mature. Their premature death results in granuloma formation in different internal organs and subcutaneous tissue.

Pathogenesis and Pathology

Lesions in animals predominantly occur in the pulmonary vessels and lung by *D. immitis*. They cause parasite load-dependent pulmonary hypertension ultimately leading to congestive heart failure. Generally, the larger right caudal lobar artery accumulates more worms than the left. The earliest lesions are characterized by endothelial cell junction disruption/dislodgement and denudation of the intimal surface. Intimal thickening narrows down the vascular lumen to cause pulmonary hypertension. The condition is associated with physical trauma, and metabolic and immune-mediated cytotoxicity by the parasite. In the cross-section, these ridges have a villous appearance, which is considered pathognomonic. Pulmonary blood flow is impeded primarily by a reduction in the cross-sectional area of the arterial vascular bed due to obliterative endarteritis of small peripheral branches. Eventually, with an increase in infection, the pulmonary vascular resistance becomes fixed and congestive cardiac failure gets manifested. Microfilariae play a minor pathogenic role in constituting pneumonia and glomerulonephritis.

Pathologically, the presence of a nodule is the characteristic. The nodule represents a pre-adult worm trapped in the defensive immune reactions causing ultimate death and disintegration of the

parasite. Histopathologically, four types of patterns are seen: (a) abscess type, the majority, characterized by necrotic matter containing neutrophils and eosinophils surrounding the nematode; (b) central zone containing nematode surrounded by epithelioid cells, histiocytes and foreign body giant cells; (c) decomposed nematode surrounded by occasional inflammatory cells inside fibrous tissue, mostly seen in lung; and (d) mixed pattern where nematode is surrounded by necrotic leucocytic infiltration with demarcating fibroblastic elements, seen in breast, epididymis, spermatic cord and mesentery.

Wolbachia spp. plays a key role in the pathogenesis of dirofilariasis. *Wolbachia* is the endosymbiont alpha-2 proteobacteria belonging to the *Rickettsiales* order inhabiting filarial worms, hexapods, crustaceans, etc. Studies suggest their role in moulting and embryogenesis of filariae. They are also found in hypodermal cords of adults of both genders and the female genital organs, suggesting their role in the long-term survival of adult worms. Experimental data suggest that *Wolbachia* provides the haem group to filariae, which is essential for cytochrome P450. Tetracycline antibiotics can block the intrauterine development of *D. immitis* microfilariae by depleting *Wolbachia*. *Wolbachia* surface proteins take part in the immunopathogenesis of dirofilariasis.

Immunology

The host–parasite relationship in dirofilariasis is immunologically complex due to (a) the wide range of hosts involved; (b) the presence of endosymbiont *Wolbachia* sp. contributing additional sets of antigens; and (c) immune evasion mechanisms.

Cell-mediated immunity plays a minor role as it gets obtunded by *D. immitis* proteins with detoxification and antioxidant properties.

Different antibodies of class IgM, IgG and IgE have been observed against every developmental stage with the highest levels corresponding to microfilaremia. While antibody-mediated complement activation and antibody-dependent cellular cytotoxicity provide a defence against microfilaria, they are ineffective against adult worms. Dead microfilaria and adult worms upon disintegration release *Wolbachia* spp. into the bloodstream. *Wolbachia* surface protein (WSP) is a potent immunogen and has been implicated in granuloma formation. Polyclonal antibodies against WSP have been detected in multiple tissues and immune cells of heartworm-infected dogs. Compared to dogs, a stronger immune response is seen in cats and possibly in humans, making them relatively unfavourable hosts.

Different subclasses of antibodies are noted in human pulmonary dirofilariasis cases with IgE, IgM and IgG antibodies formed against the excretory/secretory (E/S) antigens. Antibodies of IgG in nature against WSP are seen significantly associated with pulmonary dirofilariasis but not in *D. repens*-associated subcutaneous dirofilariasis.

The pulmonary nodule in human dirofilariasis cases is characterized by anIgG1-based pro-inflammatory response to WSP in comparison with IgE-based Th2 response against the parasitic proteins (aldolase and galectin) in those without pulmonary affection. Experimental data suggest that WSP contributes to the extension of the inflammation by the promotion of neutrophil chemotaxis and inhibition of apoptosis. Also, *Wolbachia* spp. possibly interacts with macrophages via lipopolysaccharide receptors.

Important immune evasion mechanisms employed by *Dirofilaria* spp. include (a) a short-term mechanism in L3 larvae by releasing large amounts of 6 kDa and 35 kDa surface antigens and (b) a long-term mechanism by pre-adult and adult worms by masking their body surface with glycolipids and heat shock proteins. E/S antigens also stimulate prostaglandin E2 and plasmin activation and retard monocyte transmigration.

Infection in Humans

Humans are considered to be accidental and dead-end host in dirofilariasis, although mature female *D. repens* carrying microfilariae have been reported in the literature. Pulmonary nodules are

commonly seen with *D. immitis* infection. These are frequently misdiagnosed as malignancy owing to their typical asymptomatic nature and incidental radiological discovery. A single peripherally located pulmonary nodule of diameter 1–3 cm is commonly seen; even five nodules have been described in a single person mimicking metastasis, histoplasmosis and Wegener's granulomatosis. Right lung and subpleural regions are frequent sites. Non-specific symptoms like chest pain, cough and haemoptysis are usually seen. Rarely, pleural effusion may be seen.

Subcutaneous dirofilariasis cases including ocular/periorbital cases have been reported extensively. These present as insidiously growing subcutaneous firm nodules. Mostly female individuals above the age of 40 years are affected, except in Sri Lanka where children also acquire the disease. Mostly the nodule is located at subcutaneous tissue, deep dermis, or submucosa, and rarely in muscle, lymph node or deep viscera. The upper half of the body (including periorbital) and upper limbs are more frequent sites.

D. repens has been the commonest pathogen in this scenario along with *D. tenuis*, *D. ursi*, *D. subdermata*, *D. striata* and *D. immitis* causing a minority of cases. A parasite nodule is always present except localization in subconjunctiva where it could be migratory and not trapped by the host's reaction. Data suggest the speed of migration in subcutaneous tissue could be 30 cm in 2 days, which is facilitated further by hot compress and ultrasound therapy. This could very well present as delusional parasitosis cases. Orbit, eyelid, subconjunctiva and intravitreous tissues are commonly affected, causing mild visual dimness and floaters to grave complications like cataract and retinal detachment. Subcutaneous *D. immitis* has been reported to involve liver, mesentery, conjunctiva, ocular chambers and even testicular arteries. The male external genitalia along with the spermatic cord and female breast have been affected by *D. repens*. Rarely, *D. repens* has been isolated from the lungs.

Infection in Animals

Canine cardiopulmonary dirofilariasis is a potentially lethal disease in dogs caused by adult *D. immitis*. The disease has a chronic course starting from the pulmonary arteries to lung parenchyma and right side of the heart. Worms cause proliferative endarteritis of the pulmonary arteries. Tunica intimal hypertrophy coupled with mechanical trauma leads to perivascular seepage of plasma proteins and blood cells into the lung parenchyma. Severity is proportional to the duration of infection, parasite load and host immune response. Affected arterial walls become rough and velvety and get ruptured resulting in haemoptysis and severe lung haemorrhage. Thromboembolism develops following death and disintegration of the worm resulting in severe inflammation. Inflammation along with arterial narrowing gives rise to pulmonary hypertension causing circulatory overload and tricuspid valve dysfunction. All these lead to congestive cardiac failure.

Immune-mediated glomerulonephritis is seen. The presence of IgG antibodies against *Wolbachia* in urine corresponded to microfilariae in renal capillaries. Heartworm-associated respiratory disease with primary pulmonary involvement is the characteristic clinical presentation in infected acts. Subcutaneous/ocular dirofilariasis is most commonly seen in dogs and is caused by *D. repens*, and very rarely by *D. immitis*.

Epidemiology and Public Health

Human dirofilariasis has been reported sporadically from different countries. Compared to *D. repens*, which is exclusive to the Old World, *D. immitis* has wider distribution globally (Table 1, Fig. 4). Cases have been reported sporadically from Costa Rica, Argentina, Venezuela and Colombia. Subcutaneous/ocular dirofilariasis due to rare species like *D. tenuis* and *D. ursi*-like species has been reported from North America. In the last decade, a greater rise of subcutaneous/ocular dirofilariasis cases has been attributed to

Table 1 Distribution of some *Dirofilaria* species of importance in humans

Species	Major distribution	Recognized vectors	Usual definitive host
Dirofilaria immitis	Temperate and tropical areas	Mosquito (*Aedes, Anopheles, Culex, Culiseta*)	Dogs, carnivores, cats
Dirofilaria repens	Old World	Mosquito (*Aedes, Anopheles, Culex, Mansonia*)	Dogs, carnivores, cats
Dirofilaria tenuis	North America	Mosquito (*Aedes taeniorhynchus*, *Anopheles quadrimaculatus*, *Psorophora* sp.)	Racoons
Dirofilaria striata	USA (Florida)	Mosquito (*Aedes taeniorhynchus*, *Anopheles quadrimaculatus*, *Culex quinquefasciatus*)	Panthers, bobcats
Dirofilaria ursi	North America	Black fly (*Simulium* sp.)	Bears
Dirofilaria subdermata	North America	Black fly (*Simulium* sp.)	Porcupines

the expansion from Southern Europe to the central and northern parts. Human dirofilariasis cases in India have been reported from coastal Karnataka, Kerala and Maharashtra. A few *D. tenuis* cases have also been reported from India.

Both *D. immitis* and *D. repens* are endemically widespread in European countries. Northern and Central European countries including France reported a higher prevalence of *D. repens*. The relative preponderance of *D. repens* over *D. immitis* has also been reported from Iran and Sri Lanka. Southern European countries and Italy are highly endemic for *D. immitis*. Central Asia has a high prevalence of *D. immitis*. Elevated prevalence rates have been reported from Malaysia, South Korea, Taiwan and Australia. *D. immitis* infection in canines has been well documented in the north-eastern states of India. Both *D. immitis* and *D. repens* infections have

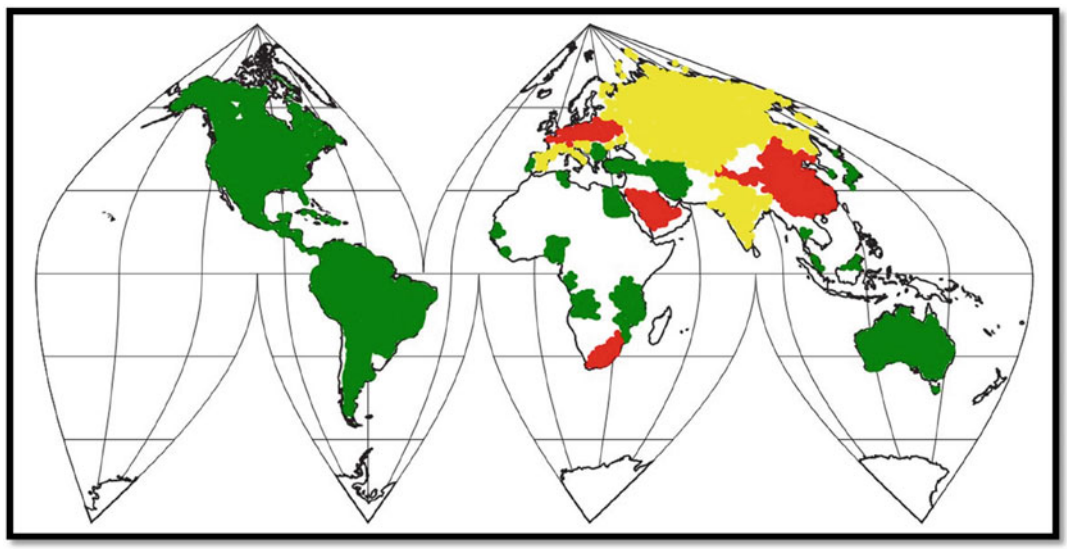

Fig. 4 Global endemicity of dirofilariasis. Green: *Dirofilaria immitis*; *red*: *Dirofilaria repens*; *yellow*: both *Dirofilaria immitis* and *Dirofilaria repens*

been reported from India. Feline dirofilariasis cases correspond to the highest level of endemicity in dogs and have been reported from Canada, Brazil, Venezuela, northern Italy, Japan and Australia. Based upon epidemiological studies, focal regions have been identified for coyotes, red wolves and foxes in Texas, California, Sierra Nevada and San Francisco, with *D. immitis* being the most prevalent species.

Culicid mosquitoes are efficient vectors due to their adaptability extending from the coastal areas to the mountain ranges. Multiple species of *Aedes*, *Culex* and *Anopheles* are involved. *Culex pipiens* is considered to be the potential primary vector.

Apart from the common species, human subcutaneous nodular granuloma has been reported with *D. tenuis*, *D. ursi*-like species, *D. subdermata* and *D. striata* from the USA. Black flies (*Simulium* spp.) have been implicated as a vector for some of these. Human infections caused by *D. ursi* have been reported along the US–Canadian border. *Simulium* sp. acts as the vector.

The actual public health importance of the non-overt human pulmonary dirofilariasis lies in the seriousness of the differential diagnoses including malignancy, tuberculosis and fungal infections.

Diagnosis

Diagnosis in Humans

Laboratory diagnosis of pulmonary dirofilariasis in humans (Table 2) is based on the following tests.

Microscopy

Fine-needle aspiration is done without any risk of parasitic embolization since the parasite has died already.

In the majority of subcutaneous/ocular dirofilariasis cases, the diagnosis is based on histological examination of the nodule (Figs. 5 and 6). The presence of external longitudinal cuticular ridges is characteristic for *Nochtiella* sp./*D. repens*, distinguishing it from *D. immitis*. Specific differentiating features include 95 to 105 longitudinal ridges placed at intervals measuring slightly more than the width of an individual ridge. Periodic acid–Schiff, Masson's trichrome and haematoxylin/eosin are the commonly employed stains. Nodules with dead and disintegrated parasite pose a challenge for identification. Careful histological examination may reveal decomposed parasite surrounded by occasional inflammatory cells. Special attention needs to be exercised for searching trilamellar cuticle, thick somatic muscle bundle and reproductive tubules. Eosinophilia in peripheral blood can be seen in only 20% of patients.

Serodiagnosis

ELISA and indirect haemagglutination assay for demonstration of specific antibodies in the serum are of limited value in the diagnosis of Dirofilaria infections in humans, due to their poor sensitivity and specificity.

Molecular Diagnosis

Polymerase chain reaction (PCR)-based techniques are helpful in diagnosis. However, the common practice of sending tissue samples in 10% formalin instead of methyl alcohol diminishes PCR positivity.

Other Tests

Chest radiogram shows homogenous spherical/ovoid opacity with well-defined borders. Calcified nodules might present as angiocentric lesions and may disappear.

Table 2 Diagnostic methods for dirofilariasis

Diagnostic approaches	Methods	Targets	Humans	Animal
Direct microscopy	Biopsy, larval extraction, necropsy	Larval/parasitic anatomical details (trilamellar cuticle, reproductive tubule, cuticular ridges)	Confirmatory Often difficult to identify the disintegrated worm anatomy particularly from the pulmonary cases	The adult worm can be visualized; morphological identification and speciation can be done
	Concentration of venous blood (modified Knott test, filter test)	Microfilaria	Not applicable	Good sensitivity and specificity; morphological identification and speciation can be done Poor performance in cats
Immunodiagnostics	Antigen detection (ELISA)	Adult female *Dirofilaria immitis* antigen (*Dirofilaria immitis* somatic antigen/DiSA, excretory antigen/DiE/S)	Variable sensitivity and poor specificity	Highly sensitive and nearly 100% specific Occult infection can be detected Poor performance in cats
	Antigen detection (immunochromatography)	Adult female *Dirofilaria immitis* antigen (*Dirofilaria immitis* somatic antigen/DiSA, excretory antigen/DiE/S)	Not available	Highly sensitive and specific Occult infection can be detected. Poor performance in cats
	Antibody (indirect haemagglutination assay)	Anti-*Dirofilaria immitis* antibody	Variable sensitivity and poor specificity	Useful in cats only
Molecular assays	PCR, sequencing, high-resolution melting analysis (HRMA)	Cytochrome oxidase subunit 1 (cox1), 18S-ITS1-5.8S, ITS1-5.8S-ITS2	High sensitivity and specificity; poor performance in formalin-preserved tissue samples	High sensitivity and specificity; HRMA can help in rapid diagnosis
		Wolbachia16S rRNA	Supportive diagnostic role	Supportive diagnostic role

Diagnosis in Animals

Laboratory diagnosis of pulmonary dirofilariasis in animals (Table 2) is based on the following tests.

Microscopy

Adult worms extracted from animal samples are examined microscopically for diagnosis. Poor sensitivity of fresh venous blood smear in demonstrating microfilaria mandates concentration methods like modified Knott test or filter test.

Modified Knott test is considered a sensitive and specific test for diagnosis of dirofilariasis in the canine population. In this method, venous

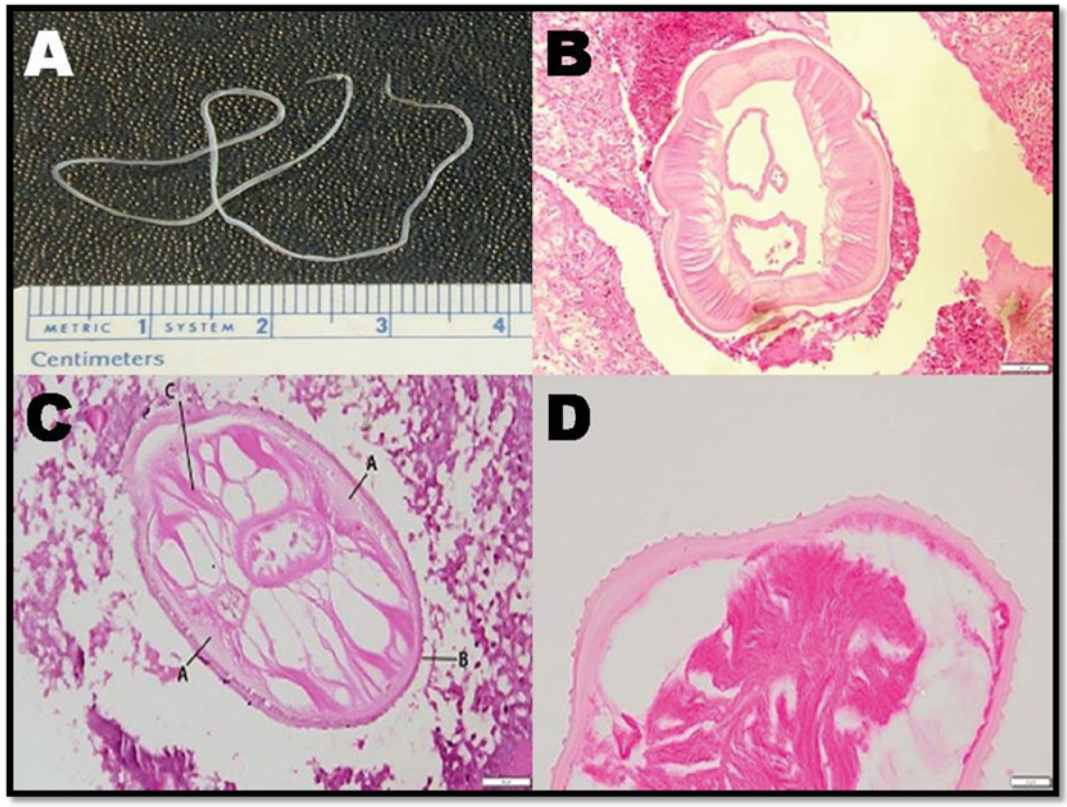

Fig. 5 Human dirofilariasis cases (**a**–**d**). (**a**) *Dirofilaria* sp. removed from the eye; (**b**) section of a *Dirofilaria immitis* worm showing the typical smooth cuticle (without ridges), musculature, paired uteri and small intestine; (**c**) section of *Dirofilaria tenuis*: '**a**' denotes the internal ridge, '**b**' denotes the cuticle (ridges) and '**c**' refers to the tall musculature; (**d**) biopsy specimen from breast nodule showing high-crested cuticular ridges. Note the distally spaced arrangement. These features distinguish *Dirofilaria subdermata* and *Dirofilaria ursi* distinctly among the subgenus *Nochtiella* (image courtesy: DPDx, CDC; https://www.cdc.gov/dpdx)

blood is mixed with 2% buffered formalin (1:10) followed by centrifugation (1500 rpm for 3–5 min). This is followed by staining of the sediment with methylene blue (1:1000) and microscopic examination. Filter test using millipore filter is another test used in the diagnosis. The test excludes the need for the centrifuge but is costly and shrinks microfilariae, thereby altering the measurements.

For differentiation of *Dirofilaria* species, morphological characteristics are compared.

Serodiagnosis

Antigen-based ELISA and immunochromatographic tests are used for diagnosis. These tests detect adult female *D. immitis* antigens with good sensitivity and nearly 100% specificity when two or more worms are present in the infected animals. These tests are not helpful if male worms only or immature female worms are present. When a negative antigen test result does not correlate with the presence of microfilaria or suspected active disease, heat treatment of serum (104 °C for 10 min in a water bath) is recommended to release blocked antigens. Dogs develop detectable antigenaemia 5–6.5 months

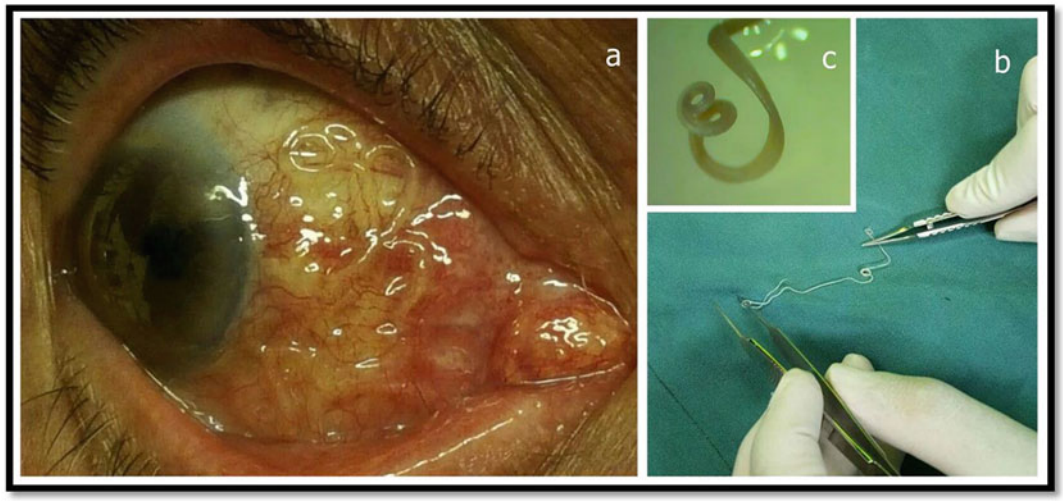

Fig. 6 Human dirofilariasis case (**a**) *Dirofilaria* visualized in subconjunctival space; (**b**) the extracted worm; inset (**c**) the coiled tail. Image reproduced from *BMC Infect Dis* **20,** 520 (2020) (http://creativecommons.org/licenses/by/4.0/)

after infection. Antigen tests are also useful to confirm the success of adulticide treatment when tested 5 and 9 months later. Both microfilariae and antigen tests are less useful in cats. Antibody-based tests hold promise in diagnosis of infection in cats.

Molecular Diagnosis

PCR-based methods can reliably differentiate *Dirofilaria* sp. from other filarial worms from animal samples. Molecular methods are best suited to diagnose in case of morphological abnormalities of microfilaria, particularly in dogs treated with medications or with co-infections. A recent method combining PCR and high-resolution melting analysis (HRMA) provides a rapid differentiation between *D. immitis* and *D. repens* from canine samples.

Treatment

In humans, the main modality is surgical extraction of the nodule or the worm for treatment of subcutaneous dirofilariasis. Pulmonary dirofilariasis needs no treatment as the parasite is already dead. Antinematodal medications like levamisole and thiabendazole have been tried in the past but are not used currently. The American Heartworm Society (AHS) in their latest guidelines (2020) advocated a 3-dose regimen of melarsomine (2.5 mg/kg; one injection followed at least 1 month later by two injections 24 h apart) for treatment in both symptomatic and asymptomatic dirofilariasis in dogs.

In cats, AHS recommends waiting for a spontaneous cure in the absence of overt clinical signs even if radiological evidence suggests dirofilariasis. Surgical extraction of the worm is always preferred.

Prevention and Control

Prevention of mosquito bite and breeding control are important steps in the prevention of human dirofilariasis. The use of mosquito nets and mosquito repellents is effective. Travel to endemic locations needs to be carefully planned with precautions.

For the canine population, regular *Dirofilaria* antigen along with microfilaria testing is

recommended over 7 months of age. Year-round administration of FDA-approved preventive drugs and EPA-registered mosquito repellents and ectoparasiticide application is endorsed by AHS. AHS recommends preventive medications for all cats in endemic areas during transmission season (warmer months) including kittens above 8 weeks of age. Monthly ivermectin (24 μg/kg) and milbemycinoxime (2 mg/kg) are oral formulations. Topical moxidectin (1 mg/kg) or selamectin (6 mg/kg) is also recommended.

Case Study

A 42-year-old male patient presented with dry cough and chest pain. The patient is a smoker (4 pack-days over the last 20 years) and has no contact with pets. In the last 1 year, he had a business meeting in Greece. Chest skiagram revealed a coin-shaped solitary pulmonary lesion in the right upper lobe. Clinical examination and laboratory parameters (leucocyte count, C-reactive protein, erythrocyte sedimentation rate, liver function test and serum electrolytes) were unremarkable. Tuberculin test and sputum testing were non-contributory. Follow-up after 2 months showed the absence of symptoms but no radiological change. CT scan of the chest showed a 1.6 cm non-calcified nodule in the right upper lobe abutting the parietal pleura without lymphadenopathy. Bronchoscopy and immunological/vasculitis profile were found to be negative. Surgically, a 1.6 cm greyish-yellow nodule was resected. Histopathological examination showed no malignancy but the presence of necrotic elements with fragments of a parasite characterized by a smooth surface and internal longitudinal ridges. The patient had an uneventful recovery without any further medical treatment.

Questions

1. How did the patient in the case study acquire the infection?
2. What could be the species involved? How would you proceed to differentiate?
3. What are the differences between *D. immitis* and *D. repens*?
4. What are the agents causing subcutaneous dirofilariasis?

Research Questions

1. What is the mechanism of high adaptability of *Dirofilaria* spp. in a vast animal population?
2. What may be the actual pathogenesis and pathology in dirofilariasis?
3. What is the effect of antibiotics on endosymbiont *Wolbachia* spp. in the killing of *Dirofilaria*?
4. Which anti-parasitic agent can be useful in eliminating the infection in animals?

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Thelaziasis

D. Ramya Priyadarshini

Learning Objectives

1. To understand the distinctive nature of transmission of the parasite through flies.
2. To study the involvement of the eye in thelaziasis and the laboratory diagnosis of the condition.

Introduction

The genus *Thelazia* consists of many species that infect both humans and animals. The definitive hosts are cats, foxes and dogs, and humans are the accidental hosts. Most of the infections, though rare, are caused by *Thelazia callipaeda* and *Thelazia californiensis*. *T. callipaeda* is transmitted by the face fly called *Musca autumnalis*, vector of the parasite. The embryonated eggs or primary-stage larvae are ingested by the flies, while feeding on lacrimal secretions from the conjunctival sac, lacrimal duct and gland in animals that host *Thelazia* spp. In humans, these eye worms parasitize the tear ducts and conjunctival sacs, when infected by the parasite.

History

The word *Thelazia* means *Oriental eye worm*. *T. callipaeda and T. californiensis* were the first two species isolated from animals. They were first described by A. Railliet and E.W. Price, among dogs and cats in the year 1910 and 1930, respectively. Human thelaziasis was first reported by O.L. Williams and C.A. Kofoid in the USA in 1935. In India, the first case was reported from Yercaud, a hill station in Salem District (Tamil Nadu), in 1948.

Taxonomy

The genus *Thelazia* belongs to phylum Nematoda; order Spirurida; suborder Spirurata; and superfamily Spiruroidea.

The genus *Thelazia* has many species that cause infection in animals and humans. *Thelazia lacrymalis* and less often *Thelazia rhodesii* cause infection in horses; *Thelazia gulosa*, *Thelazia skrjabini* and *T. rhodesii* in bovines; and *Thelazia leesei* in camels. *T. callipaeda* and *T. californiensis* are the two species that cause most accidental infections in the human eye.

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Genomics and Proteomics

The A + T content is more than 70% in the genome sequence of *T. callipaeda*. It is consistent with the genomes of nematodes from the order Spirurida. There are 12 protein-coding genes, which include nad4L and Nad6. *T. callipaeda* has one non-coding region. It also contains 2 transfer RNA genes and two ribosomal RNA genes. It is similar to most of the other spirurid nematodes.

The ribosomal ITS1 arrangements of *T. callipaeda*, *T. gulosa, T. skrjabini, T. rhodesii* and *T. lacrymalis* have been studied to differentiate the species. ITS1 sequence has been shown to be useful and promising for species-level identification of *Thelazia* species.

The Parasite Morphology

Adult Worm

Thelazia adult worms are creamy chalky white. Males measure 0.85 × 17.00 mm in size and are longer than the females (0.75 × 13.00 mm). At the posterior end, the female has a mid-ventral vulval opening and the male has a ventral curvature.

Morphologically, the numbers of pre- and post-cloacal papillae in the male and the vulval location in the female differentiate *T. callipaeda* from *T. californiensis* (Table 1; Fig. 1).

The *T. lacrymalis* female worm has a reproductive system with pairs of ovaries, oviducts and uteri. It has a single vagina and vulva. The ovaries have round-shaped oocytes, and the oviducts have spindle-shaped oocytes.

Scanning Electron Microscopy: Scanning electron microscopy helps to appreciate the surface ultrastructure of these worms. The density of the cuticular striations differs in males and females. In males, the transverse cuticular striations are arranged characteristically on the anterior surface. Their density in the anterior, middle and posterior regions is about 375, 220 and 240 rows per 1 mm length, respectively. In females, the transverse cuticular density in the anterior, middle and posterior regions is about 250, 170 and 375 rows per 1 mm length. The posterior end has encircled striations in both males and females.

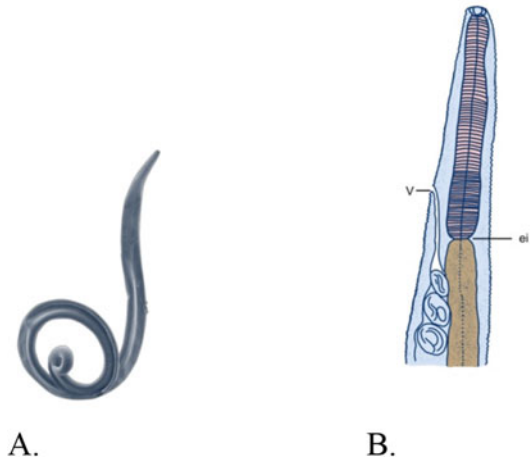

Fig. 1 (**a**) Adult worm, (**b**) female worm showing cup-shaped buccal cavity at the anterior end of *Thelazia* spp.

Eggs

Thelazia spp. eggs are oval in shape and measures 34–60 μm in size. The eggs are fully embryonated when they are laid, and they appear transparent.

Cultivation of Parasites

There are no data available on the cultivation of this parasite.

Table 1 Differences in the morphology of *Thelazia callipaeda* and *Thelazia californiensis*

Morphology	*Thelazia callipaeda*	*Thelazia californiensis*
Male: Pre-cloacal papillae	8–10 pairs	6–7 pairs
Female: Vulval opening	Present anterior to the oesophago-intestinal junction	Present posterior to the oesophago-intestinal junction

Laboratory Animals

Thelazia worms, isolated from the infected animal's eyes, are stored in normal saline in a container. The adult female is then dissected to release the first-stage larva. The face flies are maintained in the laboratory (*M. autumnalis*), and the vectors of the parasite are then allowed to feed on the first-stage larva present in the normal saline. Approximately 9–14 days after feeding of the first-stage larvae, the face flies are dissected to study the third-stage larva (L3) that has developed in the gut of the fly.

Life Cycle of *Thelazia* spp.

Hosts

The definitive hosts are cattle, horses, camels and dogs. Humans are the accidental hosts.

Intermediate Hosts

Thelazia species are transmitted by the drosophilid fly (*M. autumnalis*). The face fly are the intermediate hosts of the parasite.

Infective Stages

The third-stage larva (L3) is infective for humans.

Transmission of Infections

Third-stage larvae (L3) are accidentally transmitted by the face fly to susceptible hosts and accidentally to humans when they feed on their lacrimal secretions (Fig. 2). The vectors, while feeding on the host, deposit the larvae on the conjunctiva of horses, cattle, camels, dogs and other hosts. During a period of 3–6 weeks, the larva develops and matures in to the adult worm. *T. lacrymalis, T. rhodesii, T. gulosa* and *T. skrjabini* larvae and adult worms inhabit the lacrimal gland and its ducts. *T. skrjabini* is found in the nictitating membrane of the lacrimal ducts. *T. lacrymalis, T. gulosa* and *T. rhodesii* are found on cornea, conjunctival sac and under the eyelids in addition to the nictitating membrane. Following this, the adult female worm lays eggs in the tears of the definitive hosts (cattle, camel, horses, etc.). The face flies, which feed on the ocular secretions, ingest the embryonated eggs unintentionally. The embryonated egg develops in the body cavity of the fly into the first-stage larvae (L1) within a period of 15–30 days. The L1 larvae in the body cavity of the flies take about 3 weeks to develop into infective third-stage larvae (L3).

Pathogenesis and Pathology

Moderate to severe conjunctivitis and blepharitis are common in *T. rhodesii* infection in cattle. Invasion by the worm causes inflammation and necrotic exudation of the lacrimal gland and excretory ducts. In severe cases, the parasite causes keratitis, opacity, ulceration, perforation and even permanent fibrosis. *T. rhodesii* infection causes inflammation of the lacrimal ducts and sacs in horses.

Thelazia infections in humans involve varying degrees of inflammation of the eye including the conjunctiva. They may present with photophobia, oedema, corneal ulceration, epiphora, follicular hypertrophy of the conjunctiva and even conjunctivitis.

Immunology

Thelazia escapes the host immune system and multiplies within the host cells. Following the entry, the nematode sheds its protein coat, so the immune response is modulated to this different surface antigen. These nematodes secrete microbicidal agents, which help to escape phagocytosis. Because of their large size, these parasites escape phagocytosis by macrophages, which secrete microbicidal products extracellularly instead to facilitate their killing.

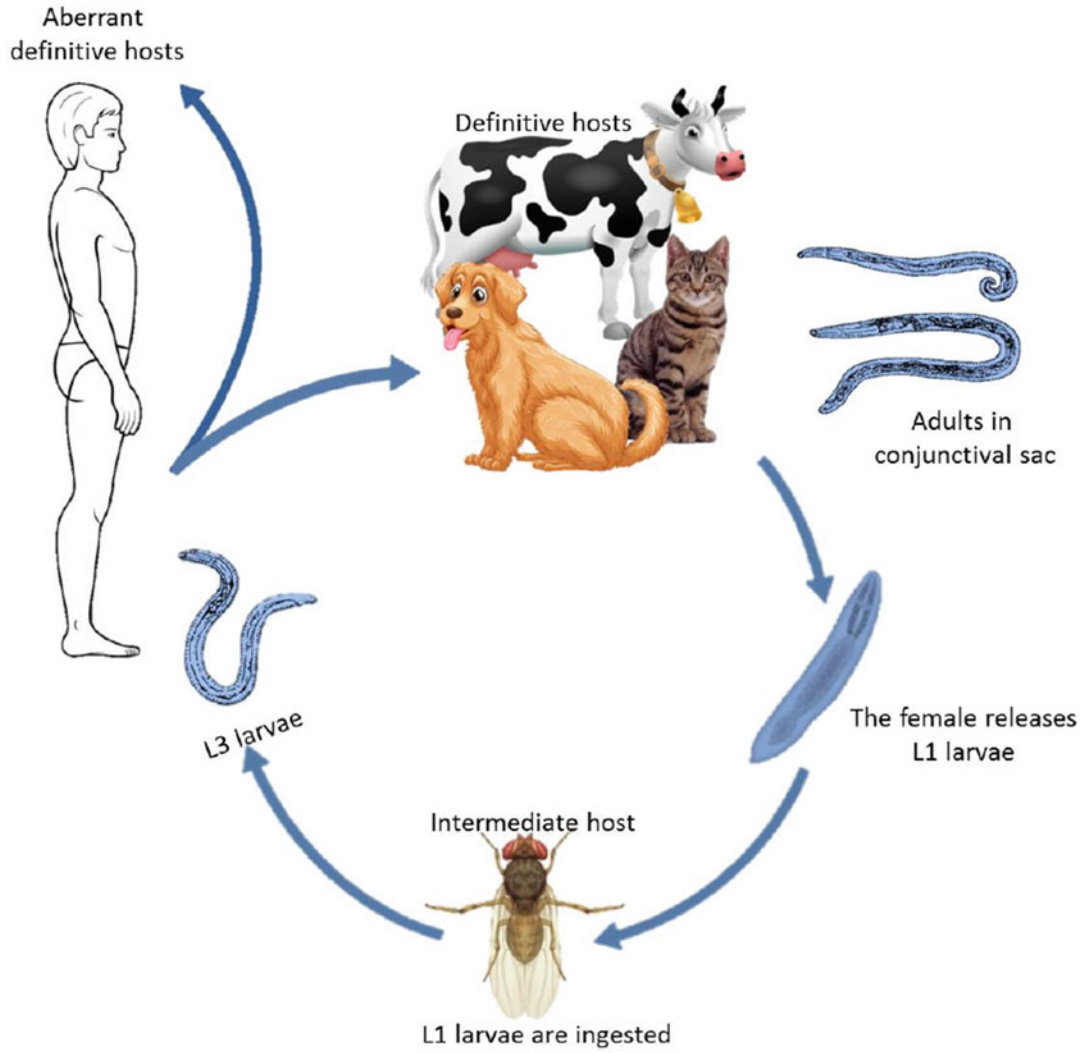

Fig. 2 Life cycle of *Thelazia callipaeda*

Infection in Humans

T. callipaeda and *T. californiensis* are the two species that cause accidental infection in the eyes. Most cases of human ocular thelaziasis (HOT) in Asia are caused by *T. callipaeda*.

These eye worms parasitize the tear ducts and conjunctival sacs. If they invade the posterior segment of the eye, they may cause serious damage to the infected eye. Symptoms become more severe following secondary bacterial infection. Pre-existing corneal and conjunctival trauma and conjunctivitis facilitate the entry of the larvae into the sub-conjunctival space and the vitreous cavity, thereby causing severe complications in the infected eye.

A case of intraocular inflammation with vitreous visual disturbances and intraocular thelaziasis with retinal detachment has been documented, with the recovery of the *Thelazia* adult worm from the vitreous fluid.

Infection in Animals

T. lacrymalis causes infection of horses worldwide, while *T. rhodesii* causes infection of horses in Africa, Asia and Europe. *T. gulosa* is the primary species that causes infection in the bovine species, in Asia, Europe and North America, while *T. skrjabini* causes bovine infection in Europe and North America. *T. Leesei* causes infection of camels in Russia and India.

Clinical manifestations of *Thelazia* infections in domestic and wild animals include conjunctivitis, cloudiness and corneal opacity. Usually, these worms live in the conjunctival sac, and since the worms have rough cuticle/skin, it irritates the cornea resulting in inflammation. The cornea may get ulcerated and perforated. If not treated, it may even lead to fibrosis of the eye.

Epidemiology and Public Health

The two most common species known to cause human thelaziasis are *T. callipaeda* and *T californiensis*. Approximately 250 cases of *T. callipaeda* human infestations have been reported worldwide including in India, China, Japan, Korea, Thailand, Russia and Indonesia (Table 2, Fig. 3). The Western USA has recorded a few cases of *T. californiensis* infestations in humans.

The prevalence of thelaziasis is higher in areas where humans live in close contact with animals, and it is also seen in areas with overcrowding and poor hygiene or sanitation. People with poor socio-economic standards, elderly farmers who are active in cattle rearing, people in more interaction with stray dogs and children who play in the farmlands are more susceptible to ocular thelaziasis.

Thelaziasis in animals shows seasonal distribution and depends on the normal cycle and vector activity of the face fly. The majority of cases are recorded during the rainy season in the months of July and August. The highest rate of *Thelazia* infection has been documented in cattle with poor living conditions and poor diet, and thus with low immunity. A slaughterhouse survey over a period of 8 months in Canada showed that nearly one-third (32 per cent) of cattle were infested with eye worms. A survey of different sites in Italy showed that nearly 23–60% of dogs, 5% of foxes and 100% cats were infested with *T. callipaeda.* In a survey conducted in Kentucky, 42% infestation of *T. lacrymalis* was found among horses.

Diagnosis

Thelazia infection of the eye needs to be clinically differentiated from other entities like foreign body sensation, increased lacrimation or conjunctival follicular hypertrophy.

Microscopy

Diagnosis is made by directly visualizing the worms or by ophthalmoscopy in the conjunctiva. After fixing with 10% formalin, they can be mounted in glycerine jelly and examined under a light microscope. Macroscopically, the worm appears as a thin, creamy white thread. The eggs and the larva, which are present in the tears and other ocular secretion, are the characteristic features of thelaziasis. Scanning electron microscopy helps to appreciate the ultrastructure of the worm.

Table 2 Distribution of *Thelazia* species of human importance

Species	Distribution	Intermediate host	Definitive host
Thelazia callipaeda	Europe, Asia	Fruit fly	Dog, cat, wolf, raccoon dog, red fox, European rabbit, human
Thelazia californiensis	Western North America	Lesser house fly	Dog, cat, human, domestic sheep, mule deer, American black beer

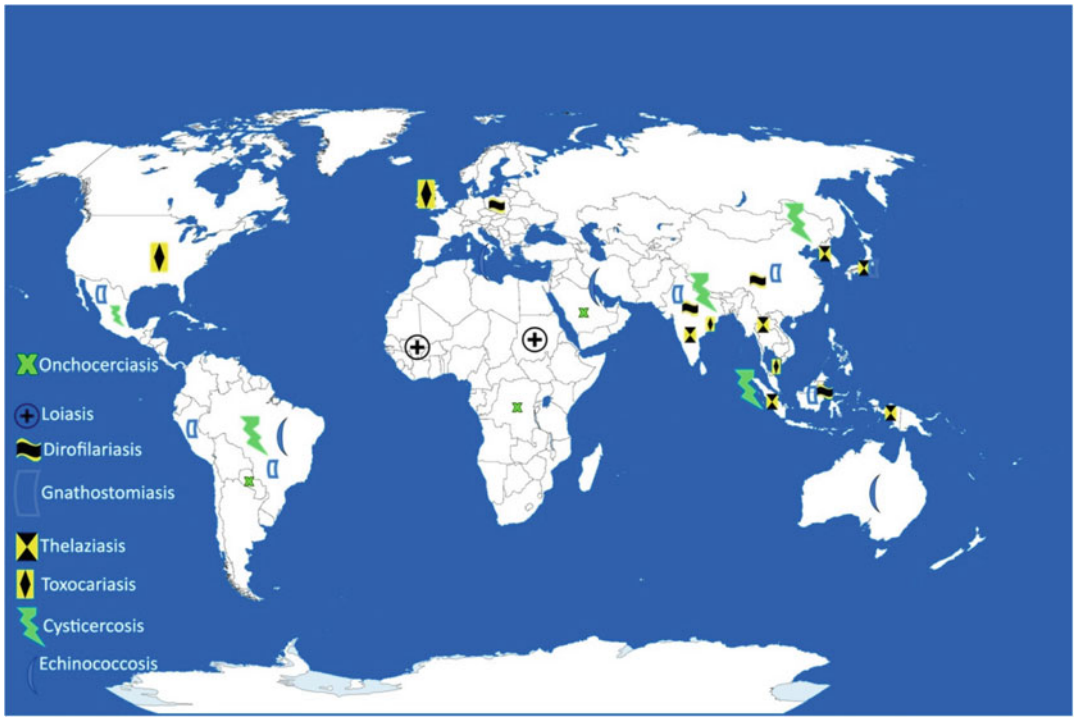

Fig. 3 World map showing endemicity of ocular parasitic infection

Serodiagnosis

Serological tests are yet to be developed for diagnostic purposes.

Molecular Diagnosis

A molecular diagnosis of thelaziasis helps in differentiation of species, thereby helping in treatment as different species of eye worms have different susceptibilities to antihelmintic drugs. The molecular identification also helps in studying their epidemiology and biology. Molecular diagnosis of both the larvae and adult worms can be performed using the first transcribed spacer of ribosomal DNA (ITS1) as a target sequence.

PCR, targeting the amplification of mitochondrial cytochrome c oxidase subunit 1 gene (cox 1), is useful for detection and identification of zoonotic *T. callipaeda* (Table 3).

Treatment

Treatment for Animal Infections

Drugs like organophosphates, 1% moxidectin, and combination of 10% imidacloprid and 2.5% moxidectin are effective in treating this infection in canines. The use of these drugs will eliminate the need for surgical removal of the worms. Removal can be performed using fine forceps under local anaesthesia followed by irrigation with Lugol's iodine or 2–3% boric acid. The symptoms resolve immediately once the worms are removed.

Treatment for Human Infections

Timely treatment of humans, especially children and the elderly, is essential to prevent a delay in recovery. The mainstay of treatment is mechanical removal of the parasite. Removal of the adult and the larval forms can be achieved by rinsing

Table 3 Diagnostic methods in thelaziasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Direct observation	*Thelazia* adult worms in conjunctiva	Gold standard test
	Examination of tear	*Thelazia* eggs	
Serology	Yet to be developed		
Molecular assay	PCR	ITS-1	High sensitivity and specificity *Limitations:* Skilled personnel are required to perform the tests

the conjunctival sac with saline. The parasite can also be immobilized using local anaesthesia followed by its removal using forceps and cotton swabs. Levamisole can be prescribed either orally or parenterally, at the dose of 5 mg/kg. For those parasites that cannot be removed manually, 2 ml of levamisole can be injected into the conjunctival sac. The drug shed in secretions of lacrimal glands immobilizes and kills the worm. Ivermectin in a dose of 2 mg/kg, injected subcutaneously, and echothiophate (0.03%) has also been found to be effective.

Prevention and Control

Using bed nets while sleeping, or shielding the eyes, face and nose while sleeping to prevent contact with vectors, maintaining personal hygiene, keeping the environment clean and raising public awareness of the disease are some of the preventive measures suggested to control thelaziasis.

Public health awareness, especially for farmers and other populations coming in close contact with animals such as cattle, horses, camels and dogs, and adaptation of coordinated disease prevention and control strategies in a community play an important role in control of infections in a community.

Case Study

A 50-year-old male farmer presented to the ophthalmology OPD with a history of foreign body sensation and irritation in his right eye for 2 months. The patient did not remember any history of injury. On examination, he had a dense cataract with no conjunctival congestion. The cornea and pupils were normal. The patient was advised to get cataract surgery done. His routine blood examination and blood sugar were found to be normal. A week later, cataract surgery was carried out in the right eye. During the operation, creamy white, motile, thread-like worms were observed. Those worms were removed with sterile forceps, preserved in 10% formalin and sent to the microbiology department for further identification. Following the removal of the worms, the surgery was carried out with cataract removal. Antiseptics were applied, and the patient was discharged with a vision of 6/6. Morphological identification was done, and *T. callipaeda* was reported.

1. What is the differential diagnosis in this case?
2. Are there any serological tests available to detect this nematode?
3. How does one differentiate similar types of nematodes?
4. How does animal-to-human transmission occur?

Research Questions

1. What are the transmission dynamics and seasonal dynamics of *Thelazia* in humans?
2. Are the studies on species of the parasite adequate or do they still need additional attention?

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Gongylonemiasis

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Learning Objectives

1. To understand parasitology of Gongylonemiasis in the infected lesions of humans.
2. To study various diagnostic modalities available to identify the parasite.

Introduction

Gongylonema infection is a zoonotic disease present worldwide, caused primarily by the ingestion of polluted water and raw food. *Gongylonema* infection has been recorded in sheep, goats, horses, cats, cattle, swine, poultry and many other wild and domestic mammals**.** Adult *Gongylonema* lives in humans as parasites for up to 10 years, affecting the oral cavity, oesophagus and pharynx. *Gongylonema pulchrum* infections in humans may often be misdiagnosed as delusional parasitosis.

History

Dr. Joseph Leidy found a worm in a child's mouth in the Philadelphia Academy in 1850. In 1857, Molin was the first to identify the parasite and named it *G. pulchrum*. The nematode was originally described as *Filaria hominisoris* and initially considered to be a guinea worm, *Dracunculus medinensis*. But the unusual position of the worm in the buccal cavity and the relatively small size failed to identify it as a guinea worm. The adult worms are variable in size, and hence they are difficult to identify morphologically. The length of the worm differs based on which host the worm is retrieved from.

Taxonomy

The genus *Gongylonema* is classified under kingdom Animalia; phylum Nematoda; class Secernentea; order Spirurida; and family Gongylonematidae.

The genus *Gongylonema* consists of 40 species, which are present worldwide. *G. pulchrum* and *Gongylonema verrucosum* are the two most common species that infect mammals. *Gongylonema ingluvicola* is an avian species that infects poultry.

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Genomics and Proteomics

Gongylonema contains 12 protein-coding genes, two ribosomal RNA genes, 22 transfer RNA genes and one non-coding region. The gene arrangement is the same as that of *Thelazia callipaeda*. The complete mitochondrial genome of *G. pulchrum* was derived by employing a long-range PCR.

The Parasite Morphology

Adult Worm

Gongylonema are long thread-like nematodes. The males measure approximately 29 mm in length and are shorter than the females. The female worms are relatively longer and measure nearly 59 mm in length. The length of both males and females varies depending on the host in which they are present. The worm is highly motile.

Head: Numerous cuticular platelets are arranged in the anterior end of both male and female worms. The anterior end of the worm possesses the raised cuticular bosses or plaques, which are the most salient feature of the nematode. These plaques are arranged in longitudinal rows. The entire length of the body has a striated cuticle. A pair of lateral cervical papillae is present. The buccal opening is small and extends along the dorsoventral direction. A cuticular elevation that encloses the labia is present around the mouth. Eight papillae are located both laterodorsally and lateroventrally, and two large lateral amphids are also present in the worm.

Caudal end: Phasmidal apertures are observed on the lateral side of the female tail. The male tail possesses 10 pairs of papillae and two phasmidal apertures. The caudal end has asymmetrical wing-like projections. The reproductive system of males possesses asymmetrical caudal alae and unequal spicules. Eggs are present in the uteri of females.

The worm has a digestive system with two openings, the mouth and the anus. There are no excretory organs and no circulatory system.

Eggs

Gongylonema eggs are ellipsoidal in shape and measure about 60 × 30 μm in size. They have thick, transparent shells, which contain a first-stage larva (Fig. 1). The eggs are unembryonated while laid by the adult worm. These eggs are not infective to humans.

Infective Larvae

L1 larvae take two moults to become L3 larvae, which is infective, and L3 larvae, however, do not complete their cycle unless they reach their definite host. The anterior end of the L3 larvae is blunt, and it possesses a cephalic hook and rows of tiny spines.

Cultivation of Parasite

Cultivation of *Gongylonema* is an intricate procedure, as it may require a specific environment,

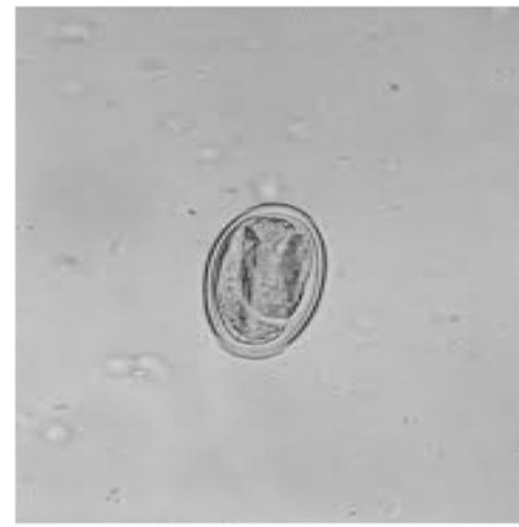

Fig. 1 Embryonated *Gongylonema* spp. egg

nutrition and various hosts for its growth. It may help in studying the morphology and producing the antigens, but culture is not used as a diagnostic test.

Laboratory Animals

Rabbit, cattle, etc., have been used to study the pathogenesis and immunology of *Gongylonema* infections. Experimental infection is carried out by inoculation of the third-stage larva obtained from infected beetles. Most of the worms have been recovered at necropsy from the oesophagus and cardiac end of the stomach. It was observed that the worm requires at least 50 days for its complete development.

Life Cycle of *Gongylonema pulchrum*

Hosts

Ruminants and pigs are the definitive hosts. Most often, in a zoonotic infection, humans are the accidental hosts.

Insects such as dung beetles and cockroaches are the intermediate hosts.

Infective Stage

The third-stage larva (L3) is the infective stage of the parasite.

Transmission of Infection

Consumption of contaminated food and water remains the main source of infection. Ingestion of coprophagous insects, mostly cockroaches and dung beetles infested by *Gongylonema*, results in gongylonemiasis (Fig. 2). The buccal mucosa inhabits the third-stage larva. The larva resides in the buccal cavity, especially in the cheek, below the tongue or in the floor of the mouth.

Human are infected by ingestion of contaminated food, water or an infected dung beetle. Following ingestion, the dung beetle lays its eggs near the upper oesophagus in the buccal cavity. The eggs hatch to the first-stage larvae (L1), which undergo two moults to become infective third-stage larvae, subsequently maturing into adult worms. The adult worm migrates back into the buccal cavity. Hence, humans do not excrete eggs in the faeces. This suggests that humans are the dead-end or accidental host for *Gongylonema*.

Animals are infected by the third-stage larva, through an intermediate host like dung beetles. The larva migrates into the upper gastrointestinal tract of the animals and undergoes three moults within 2 weeks. The final moult occurs after 5 weeks. Sexual maturation of the worm takes place at around 8 weeks from primary infection, and it migrates back to the oesophagus. After 10 weeks, the infected animal excretes the embryonated egg in the faeces.

Pathogenesis and Pathology

In most of the animals, there was no evidence of inflammatory response. *Gongylonema* species migrates into the mucosal layer by physically moving the cells without producing lytic enzymes. Because of this property, the basement membrane is not disturbed. And hence, there is minimal inflammatory response. This potentially reduces or evades the specific host immunity.

Heavy *Gongylonema* infections in animals cause gastrointestinal manifestations that lead to emaciation of animals. The nematodes are commonly present in the lumen of the gastric glands. They cause mild chronic oesophagitis, especially in cattle. Certain animals such as cattle show destruction, regeneration, hypertrophy and hyperplasia of oesophageal epithelium. At autopsy, serpentine tracks are noted in the mucosal layer and the vessels, surrounded by eosinophils and lymphocytes.

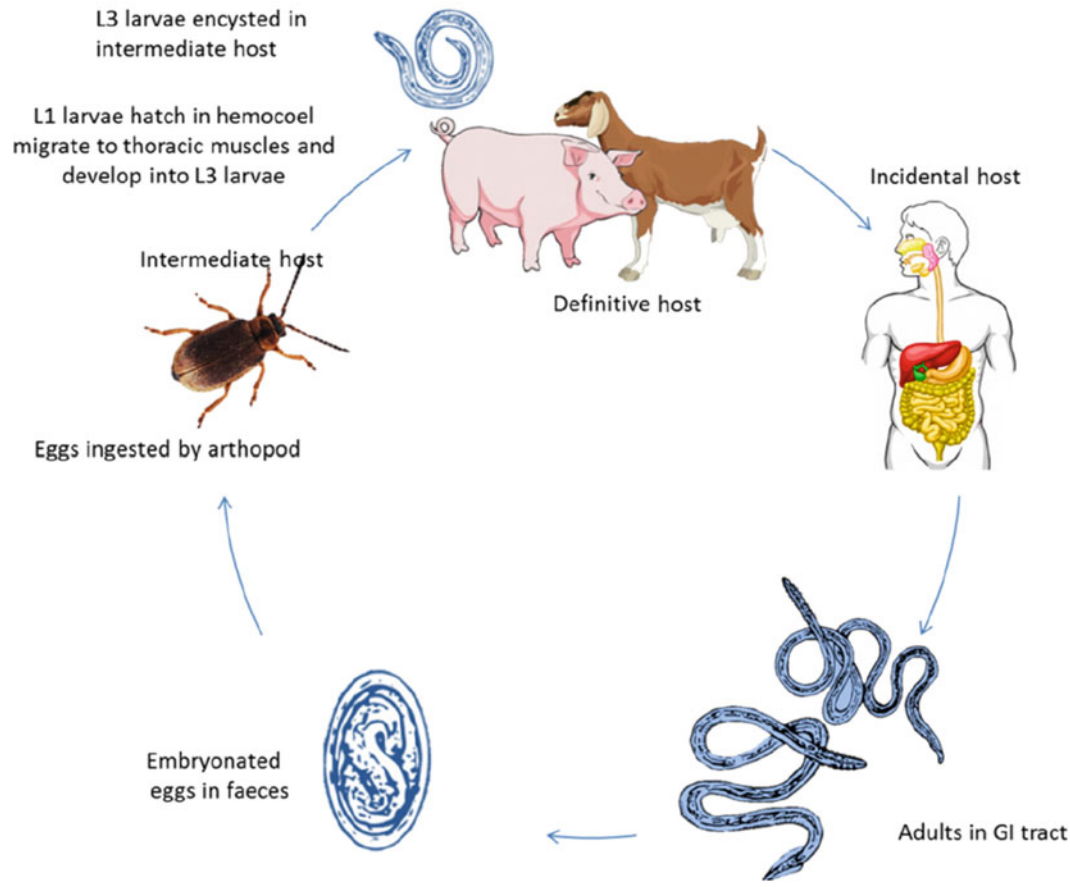

Fig. 2 Life cycle of *Gongylonema* spp.

G. pulchrum in infected humans gets lodged in the tunnels of stratum spinosum without producing any inflammatory reactions around the worm.

Immunology

Eosinophilia is noted in certain patients, but the immune response to *Gongylonema* infection is yet to be elucidated.

Infection in Humans

A moving sensation of the worm around the mouth, near the lips and in the soft palate, associated with foreign body sensation, is the most important symptom of *G. pulchrum* infection in humans. Typically, this movement is caused by immature adult worms. Excessive pain, expectoration of blood, numbness of tongue, vomiting, pharyngitis and stomatitis are also present in some patients.

Rarely, humans swallow the larva. It remains in the buccal cavity. After days and even weeks of discomfort, it is extracted by the infected individual when the creeping sensation is felt in the oral cavity. Worms are removed by the patients themselves from their tongue, lips, inner cheeks and gums. If the worm is not surgically removed, symptoms, once noted, can persist from 1 month to 1 year. Patients with an initial exposure to the worm experience moderate fever and flu-like symptoms.

Table 1 Distribution of some *Gongylonema* spp. of importance in humans

Species	Distribution	Intermediate host	Definitive host
Gongylonema pulchrum	Worldwide	Beetles, cockroaches	Domestic swine, sheep, goats, cattle and humans

Infection in Animals

Most *Gongylonema* infection in animals is benign and is usually asymptomatic. A slight inflammation of the oesophagus or stomach wall has been observed. The infected organs are identified incidentally during slaughter. But eggs are detected in the faeces of the infected animals.

Epidemiology and Public Health

Human *G. pulchrum* infections are not considered a major public health issue. Since the first documented case in 1850, there have only been 50 confirmed infections worldwide (Table 1). *G. pulchrum* infections have been reported from the USA, Laos, Morocco, China, Sri Lanka, Italy, New Zealand, Germany, Iran, Japan and Egypt. Ingestion of arthropod intermediate hosts or drinking water infected with infectious third-stage larvae (L3) causes human infection. Ingestion of infected bovine tissue, however, does not cause any human infection.

The prevalence of *Gongylonema* infection in goats and sheep is 39.6–55% in African countries; in bovines, infection ranges from 1% to 10% (Fig. 3).

Diagnosis

G. pulchrum infections in humans are often misdiagnosed as delusional parasitosis. Diagnosis of the infection is based on a high degree of clinical suspicion, supplemented with visual demonstration of the motile larvae crawling across the tissue of the oral cavity (Table 2).

Microscopy

Worms extracted either by the patient or by surgery (Fig. 4) are examined by microscopy for the identification of the worm. Identification to the species level is frequently difficult; hence, most cases are recorded as *Gongylonema* species. Since single worm is involved in most human infections, eggs are not normally found in stools. Occasionally, passage of egg is observed due to the ingestion of adult worms present in infected meat. The presence of *Gongylonema* eggs in the stool needs additional reconfirmation.

Immunodiagnosis

No serological or molecular methods are available at present due to the paucity of the number of infections. The polysaccharide antigen from the *Gongylonema* species was evaluated in intradermal skin tests in rabbits but was found to be unsuccessful.

Treatment

Surgical extraction and removal of the worm, followed by chemotherapy with albendazole, 400 mg twice daily for 21 days, are effective for the treatment of *G. pulchrum* infections in humans. Follow-up measures include periodic checks to ensure complete clearance, and elimination of the worms from the buccal cavity and oesophagus is important.

Ivermectin once a week for 4 weeks or mebendazole for three consecutive days, followed by monthly deworming, is recommended for the treatment of *Gongylonema*

Fig. 3 World map showing cattle endemicity of *Gongylonema* infection in parts of mid-regions of Africa, South America and Mexico

infection in animals. The infected animal and other animals need to be treated simultaneously to prevent egg shedding.

Prevention and Control

Avoidance of consumption of raw vegetables or other foods, possibly contaminated with the larvae, prevents transmission of *Gongylonema* infection to humans. Avoidance of drinking tap water and eating parboiled meat prevents further transmission. Keeping livestock away from feed contaminated with infected cockroaches and beetles is the best way to prevent *Gongylonema* infection in animals. Deworming livestock with antihelmintics is not indicated, because it is not cost-effective.

Case Study

A 40-year-old female writer presented to the OPD for the evaluation of an irregular area in her cheek, which she could feel with her tongue for 6 months. Recently, however, she had felt that the

Table 2 Diagnostic modalities in gongylonemiasis

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Direct observation	Adult worms in oral cavity	Gold standard test
	Stool examination	Presence of *Gongylonema* eggs	
Serology	Yet to be developed		
Molecular assay	PCR	*Cox*-1	High sensitivity and specificity *Limitations*: Requires skilled personnel

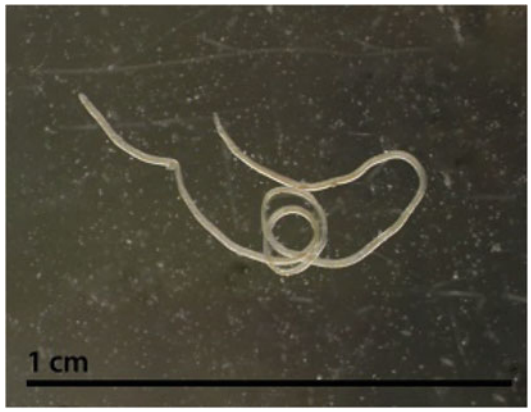

Fig. 4 *Gongylonema* spp. worm collected from a patient (courtesy: CDC)

patch was swollen and migrating. She was able to see a serpiginous thread-like structure in her buccal cavity. She also estimated that the thread-like structure moved approximately 2–3 cms per day. She had no other symptoms like fever, chills, nausea, vomiting, sore throat or other skin lesions. The patient had undergone appendectomy and tonsillectomy in the past. Her complete blood parameters were normal with no eosinophilia. Examination of the patient's mouth showed a superficial, filamentous and submucosal mass measuring 0.2 mm. The mass was sinusoidal in shape. The total size of the mass was 1 cm. Over a period of a few days, it migrated from one side of the buccal mucosa to the lower lip. With the use of topical anaesthesia, the buccal mucosa was anaesthetized, and the worm was gently teased from the mucosa. The entire worm was removed intact. It was placed in a 95% alcohol solution. There was no local bleeding or discomfort at the site. The patient was prescribed albendazole, 200 mg twice daily. The worm was identified as the *Gongylonema* species.

Questions

1. What are the differential diagnoses for this case scenario?
2. How does the transmission to humans occur?
3. What are the available diagnostic methods?
4. How do we prevent this infection?

Research Questions

1. Can molecular methodology overcome the morphology-based diagnosis of gongylonemiasis?
2. What research studies should be undertaken on the susceptibility of the *Gongylonema* spp. to antihelmintic agents?
3. What advanced studies are necessary to develop a more specific and sensitive diagnostic test for gongylonemiasis in animals and humans?

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Ternidens Infection

S. Pramodhini and Subhash Chandra Parija

Learning Objectives

1. To understand the importance of Ternidens infection and the need to differentiate it from Oesophagostomiasis which can present in similar manner.
2. To understand the importance of egg hatching techniques like the Harada–Mori method in definitive diagnosis.

Introduction

Ternidens deminutus, a nematode of zoonotic importance, affects both humans and non-human primates. Since the egg of the parasite resembles that of hookworm egg, *T. deminutus* has often been referred as false hookworm. The nematode is found most commonly in southern Africa, where it infects the large intestines of primates like baboons and vervet monkeys, whereas it has been documented only in monkeys in parts of Asia. A prevalence rate of up to 87% in humans has been reported in some surveyed populations in Zimbabwe. The similarity of *T. deminutus* eggs of parasite to that of hookworm poses a major challenge for both diagnosis and accurate prevalence surveys of soil-transmitted helminths (STH).

History

In 1865, these parasites were found in a vial collected during autopsy of a native of Mayotte, in the Comoro Islands of Mozambique. The autopsy was conducted by Monestier, who was a physician in the French navy. These parasites were initially identified as *Ancylostoma duodenale* and suggested as the aetiological agents of anaemia. In 1905, Railliet and Henry, while studying the collection of parasitic nematodes in the National Museum of Natural History in Paris, described this helminth as *Tropidophorus deminutus*. Subsequently, these authors established it as a new genus, *Ternidens,* in 1909. Smith, Fox and White in 1908 isolated a new worm called *Globocephalus macaci*, from a pig-tailed monkey, which died at the Philadelphia Zoo; later this worm was identified by Sandground as *T. deminutus*.

Taxonomy

The genus *Ternidens* belongs to phylum Nemathelminthes; order Strongylida; superfamily

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Strongyloidea and family Strongylidae. *T. deminutus and Ternidens simiae* are two pathogenic species that cause infections in humans and animals.

Genomics and Proteomics

The length and G + C content of the sequences of the second internal transcribed spacer (ITS-2) of rDNA of *T. deminutus* is 216 bp and ~43%, correspondingly. Studies have stated minimal (2.8%) difference in the nucleotide sequencing of parasites isolated from baboon and Mona monkey, but there was no sequence variation among *T. deminutus* parasites recovered from the baboon. These findings suggest a significant population variation or the existence of cryptic species within the *T. deminutus* species. Reports of ITS-2 sequence differences (27–48.3%) among the two taxonomic units of *T. deminutus* and hookworms (superfamily Ancylostomatoidea) formed the basis for the identification and delineation by PCR-based mutation scanning.

The Parasite Morphology

Adult Worm

T. deminutus adult males and females from humans measure 6–13 mm and 9–17 mm in length, respectively, and appear darker in colour than those adults worms isolated from baboons. Adult worms of *T. deminutus* are straight compared to the curved appearance of adult hookworms. Just below the buccal capsule lies the transverse cuticular fold. The cuticle appears opaque and has transverse striations. The sub-globose buccal capsule is large and swollen. It has three deep sets of teeth, an anteriorly facing mouth surrounded by a mouth collar and 22–24 bristles of the corona radii. The anterior end has four sub-median papillae and two lateral amphids. The oesophagus measures 525–840 mm in length. The males have a cup-shaped copulatory bursa, two spicules and a gubernaculum. The spicules measure 1116–1441 mm. Females have a protuberant vulva, located slightly anterior to the anus (Fig. 1).

Fig. 1 Adult worm of *Ternidens deminutus* (courtesy: Bradbury R. S. 2019. *Ternidens deminutus* Revisited: A Review of Human Infections with the False Hookworm. *Tropical medicine and infectious disease*, *4*(3), 106. Under Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/))

Egg

T. deminutus eggs are larger in size. They measure 70–94 μm in width and 40–60 μm in length. The greater ratio of width to length distinguishes *T. deminutus* eggs from those of hookworm eggs. Eggs have 4–32 morulae, which undergo further development within the egg and later hatch into larvae (Fig. 2).

Larva

Rhabditiform Larvae

The first-stage (L1) rhabditiform larvae of *T. deminutus* measure around 3.60 μm in length and 20 μm in width. The buccal cavity measures 10.5 × 1.5 μm in size. The oesophagus is 95 μm long. A refractile and spindle-shaped genital primordium measures 11.2 μm in length. A long flagella-like tail present in the distal end measures 70 μm in length. The second-stage (L2) larvae measure 620 μm in length and 32 μm in width, with an oesophagus of 140 μm in length.

The rhabditiform larvae *T. deminutus*, *Strongyloides* and hookworms appear to be morphologically similar to each other. Nevertheless, *T. deminutus* rhabditiform larvae can be differentiated from others on the basis of their long buccal cavity, longer tail and prominent genital primordium.

Filariform Larva

The filariform (L3) larvae measure 630–730 μm in length and 29–35 μm in width. The larva at its anterior end bears the head, followed by an indentation and a spear-shaped buccal cavity. The larva has the gut of characteristic "zigzag" appearance. The oesophagus measures 150–165 μm in length, nearly one third of the length of the intestine length, and shows a slight bulge distally. The oesophagus is separated from the intestine by two elongated sphincter cells. The intestine is palisade-shaped due to the presence of the ten pairs of large triangular cells. A genital primordium of 15 μm in length is found near the middle of the larva. The anus opening is present, 120–145 μm away from the tail end. It has a pointed tail. The filamentous end of the sheath extends a little, making it appear thread-like at the posterior end of the worm.

T. deminutus filariform larvae are readily differentiated from those of other "hookworm-like" such as *Necator* spp. and *Oesophagostomum* spp., by their greater length (702–950 μm), the "Y" form of the remnant buccal cavity, the rhabditoid oesophageal bulb, which appears wider and prominent, and the absence of sphincter cells between the oesophagus and the intestine. *Oesophagostomum* spp. filariform larvae have a round tail, and the anus opening is of much shorter distance from the tip of the tail (45–88 μm) (Fig. 3).

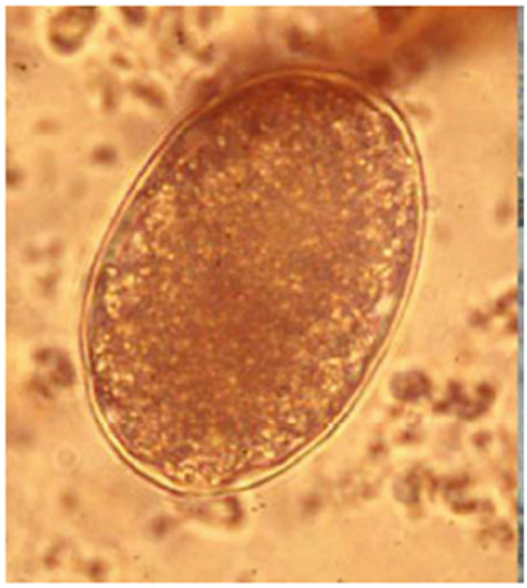

Fig. 2 Egg of *Ternidens deminutus* (courtesy: Bradbury R. S. 2019. *Ternidens deminutus* Revisited: A Review of Human Infections with the False Hookworm. *Tropical medicine and infectious disease*, *4*(3), 106. Under Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/))

Cultivation of Parasites

The Harada–Mori technique is a test tube filter paper method of stool culture. In this method, stool smeared on a moist filter paper is kept in a

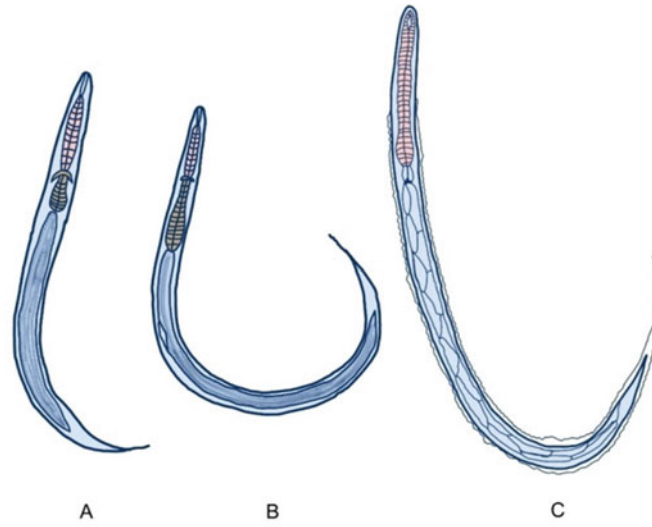

Fig. 3 Larval forms of *Ternidens deminutus:* (**a**) (L1) rhabditiform larva, (**b**) (L2) rhabditiform larva, (**c**) filariform (L3) larva

tube containing sterile water on incubation at room temperature for 8–10 days. The eggs of *T. deminutus*, if present in the stool of the host, hatch and develop into filariform larvae (L3). The method is used to differentiate the L3 larvae of *Ternidens* spp. from those of *Oesophagostomum* spp. and *Necator* spp. since eggs of all these species are morphologically similar.

Laboratory Animals

T. deminutus were identified from the autopsy of baboons, which were killed by poisoning to protect crops surrounding African settlements. In 1920 and 1930, experimental studies were carried out in human volunteers and in baboons either by ingestion of filariform larvae or cutaneous inoculation; however, this turned out to be unsuccessful.

Life Cycle of *Ternidens deminutus*

Hosts

Definite Hosts

Humans and other primates such as chimpanzees, gorillas, macaques and Cercopithecus monkeys.

Infective Stage

Filariform larvae (L3) are infective for humans and other primates.

Transmission of Infection

Humans and other primates acquire *T. deminutus* infection by ingestion of food contaminated with third-stage filariform larvae (L3). The larvae inhabit the large intestine, particularly the colon, and caecum in some individuals, unlike hookworms, which are primarily small intestine parasites. At these sites, L3 larvae attach and invade the intestinal mucosa and form nodules at the sites of attachments. The L3 larvae subsequently develop into L4 larvae. The L4 larvae are detached from the wall of the large intestine and re-enter the lumen, where they moult into adult worms. Finally, adult worms attach to the intestinal mucosa by their buccal cavity and start producing eggs that are released into lumen of the large intestine. The eggs start appearing in faeces of the infected hosts, 30–40 days after ingestion of the L3 larvae.

In the soil, the eggs become fully mature within 24–30 h of passage in the stool. The rhabditiform larvae (L1) hatch out of the eggs

after 48–72 h of presence in the soil. Further L1 larvae develop into L2 larvae after 2–3 days and finally into filariform larvae (L3), the infective stage of the parasite, after 8–10 days in the soil (Fig. 4).

Pathogenesis and Pathology

L3 larvae initiate *T. deminutus* infection by invading mucosa of the large intestine where they moult to L4 larvae and produce nodules or ulcers in the wall of the large intestine. L4 larvae detached from the wall of the large intestine moult into the adult worms in the lumen. The adult worms also produce ulcers or cystic nodules at the sites of their attachment in the intestinal wall. Heavy infections by adult worms may cause anaemia**.**

Immunology

Immune responses in chronic *T. deminutus* infection is characterized by an elevated serum IgG and IgA antibodies, specific to filariform larval antigens. However, the protective role of these serum antibodies against the nematode in infected humans is still unclear.

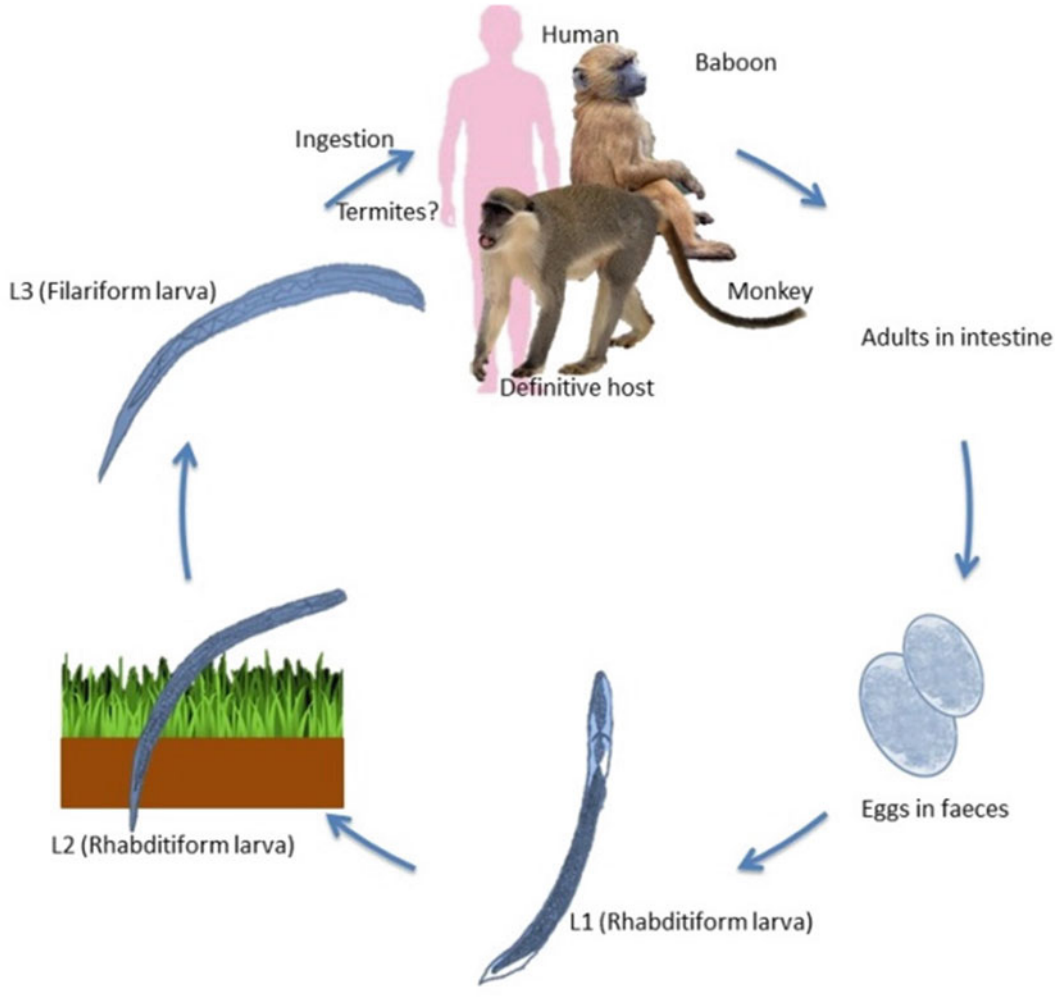

Fig. 4 Life cycle of *Ternidens deminutus*

Infections in Humans

The majority of human *T. deminutus* infections are asymptomatic.

The characteristic presentation of symptomatic chronic cases of human *T. deminutus* infection includes multiple intestinal abscesses, nodules or helminthomas of the large intestine. Adult worms may be located free in the intestinal lumen, or attached to the intestinal mucosa. Heavy infections caused by a large number of adult worms are frequently associated with malaise, obstipation and microcytic hypochromic anaemia. Co-infections of *Ternidens* with other intestinal helminthic infections have been documented in patients with poor nutritional status.

Infections in Animals

The first case of *T. deminutus* infection in primates was reported by Leiper, in a western lowland gorilla, which died at the London Zoological Gardens. Following this report, several reports of the infection in monkeys, baboons and chimpanzees were documented between 1906 and 1937, from countries in Africa and Asia. African non-human primates such as baboons and vervet monkeys are found to be more susceptible to *Ternidens* infections. The parasites are commonly found in the large intestine of primates and cause anaemia and nodules in the intestinal wall.

Epidemiology and Public Health

T. deminutus usually inhabits the large intestine of primates such as chimpanzees, gorillas, macaques and Cercopithecus monkeys in Africa, India and Indonesia. The infection has also been reported in nearly 21% of 100 Macaca mulattos from China.

Human *T. deminutus* infection has been recorded from sub-Saharan Africa (Rhodesia, Tanzania), with only one case from Thailand and two from Suriname (Table 1). No human infection has been documented from Asia, although the infection has been recorded in monkeys.

Diagnosis

The diagnosis of *Ternidens* infection is based on various laboratory methods (Table 2).

Microscopy

Stool microscopy is frequently helpful in the detection of *Ternidens* egg in the stool. These eggs, however, need to be differentiated from other hookworms based on their size and other features. Recovery of eggs in the stool is increased after concentration of stool either by the saturated salt flotation method or by the formalin-ethyl acetate sedimentation method.

Adult worms can be recovered and identified in (a) stool specimens following purgation or (b) histopathology specimen of the large intestine obtained during autopsy. It is considered to be the gold standard in diagnosis of *Ternidens* infection.

In Vitro Culture

In vitro culture of larvae to L3 stage is helpful to detect and identify *Ternidens* infection. Harada–Mori stool culture is used to detect and identify L3 stage of *Ternidens*.

Table 1 Distribution of *Ternidens* spp. of importance to humans

Species	Distribution	Definitive host
Ternidens deminutus	*Humans*—Sub-Saharan Africa, for example Rhodesia and Tanzania *Primates*—Africa, India and Indonesia	Humans, primates such as chimpanzees, gorillas, macaques and *Cercopithecus* monkeys

Table 2 Diagnostic methods for *Ternidens* infection

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Stool microscopy	Direct demonstration of ova and parasite	Stool concentration techniques are needed to increase the sensitivity
	Purgation or autopsy	Direct demonstration of adult worm	Gold standard method for diagnosis
Immunodiagnostics	Antibody (IFAT)	IgG and IgE antibodies	Important role in epidemiological studies *Limitation:* Cross-reaction with patient infected with related helminths
Molecular assays	PCR	ITS2	Utilized to study the prevalence and distribution of the species *Limitations:* Require skilled personnel

Serodiagnosis

Indirect immunofluorescent test (IFAT), surface precipitation test, etc., are used for the detection of specific antibodies against *T. deminutus*, in the sera for the diagnosis of *T. deminutus* infection in humans. The IFAT, which used adult worm antigen, in the test, however, showed cross-reaction with sera from a patient infected with related helminths such as *Necator americanus.* The surface precipitation test employed exsheathed larvae that were incubated with immune serum at 4 °C, following which they were sectioned and examined by electron microscopy. Serological assays also play an important role in epidemiological studies.

Molecular Diagnosis

Genetic characterization of *T. deminutus* has been performed by sequencing the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA (rDNA). This molecular characterization of *T. deminutus* was carried out in the adult worm isolated from the olive baboon and the Mona monkey. The molecular methods are extremely useful not only for identification but also for understanding the prevalence of the *Ternidens* spp. in the community (Table 2).

Treatment

Thiabendazole and pyrantel pamoate are highly effective for the treatment of *Ternidens* infections in humans, with high cure rates. Pyrantel pamoate was observed to be effective for *T. deminutus* infection with high rates of cure but with a few side effects. Albendazole, mebendazole and ivermectin have also been evaluated for the treatment of the condition with good efficacy.

Treatment of helminthic pseudo-tumours and helminthic abscess is primarily by surgical excision of the involved bowel, or removal of worms from nodules.

Prevention and Control

Prompt disposal of human and animal faeces prevents hatching and contaminating soil with the *Ternidens* eggs, which make it important for control of the parasitic infection. Routine veterinary care of pet animals and animals in zoos with regular deworming reduces environmental contamination with the zoonotic hookworm eggs and larvae. Personal hygiene and safety measures to avoid skin contact with sand or soil prevent infection with these nematodes.

Case Study

A 35-year-old man presented with fever, abdominal pain, tenderness and a right lower quadrant mass. Exploratory laparotomy showed a live worm, which was extracted from the mass found at the ileum. The worm was identified as *T. deminutus*.

1. Name the clinical condition of this patient.
2. What is the gold standard method for diagnosis of this condition?
3. How does human *Ternidens* infection occur?
4. Mention the differentiating features with other hookworms.
5. What are the various modalities of treatment of *Ternidens* infections?

Research Questions

1. How do we carry forward the research available on the knowledge about biology, transmission or the extent of its effects on primate hosts regarding *Ternidens* spp.?
2. What role is played by *Ternidens* when there is co-infection with other helminths and the contribution of *Ternidens* in causation of anaemia?

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Oesophagostomum Infection

Rahul Negi, Rahul Kunwar Singh, V. Samuel Raj, and Tribhuvan Mohan Mohaptara

Learning Objectives

1. To learn that the presentation of the cases may resemble those of acute appendicitis or hernia.
2. To be aware that *Oesophagostomum* infection need to be considered in the differential diagnosis of acute abdominal pain.

Introduction

Oesophagostomiasis is a zoonotic parasitic disease of the gastrointestinal system caused by the nematodes parasite *Oesophagostomum* spp. belonging to family Strongylidae. It is also known as nodular worms and infects both animals and humans. Eight species of *Oesophagostomum* are known to infect the primates including the commonly reported *Oesophagostomum bifurcum*, *Oesophagostomum aculeatum* and *Oesophagostomum stephanostomum*. The oesophagostomiasis in humans is caused by *O. bifurcum* characterized by the formation of nodule in the intestine. The disease affects about 0.25 million people globally, and another 1 million are at risk of infection.

History

Oesophagostomum was first reported from cattle and pigs as early as the nineteenth century. However, the first case of oesophagostomiasis in human beings was observed by Railliet and Henry during the early twentieth century, while performing autopsy of an old African man living near Omro River in southern Ethiopia. The parasites were observed in the tumours of the caecum and colon. Second case of the disease was reported by H.W. Thomas in 1910, during viscera examination of a native Brazilian patient, who died due to severe dysentery. Since then, several cases of human oesophagostomiasis in endemic areas have been observed**.** In 1911, Leiper described the first adult worm causing oesophagostomiasis.

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Taxonomy

The genus *Oesophagostomum* belongs to subfamily Oesophagostominae; family Strongylidae; superfamily Strongyloidae; order Strongylida; class Secernentea; and phylum Nematoda.

Genomics and Proteomics

Sequencing of complete mitochondrial genomes of *Oesophagostomum asperum* and *Oesophagostomum columbianum* from small ruminants has identified 36 genes, which include 12 protein-coding genes, 2 rRNA genes and 22 tRNA genes. Proteomic analysis of *Oesophagostomum dentatum* during larval transition revealed three proteins namely intermediate filament protein B, tropomyosin and peptidyl prolyl cis–trans isomerase, which were suggested to be involved in the moulting process of the worm.

The Parasite Morphology

Adult Worm

O. bifurcum adult female measures 6.5–24 mm in length (Fig. 1) and is longer than the male, which measures 6–16.6 mm in length. Like other nematodes, it has developed multinucleated digestive tract and reproductive system. It also has the characteristics buccal capsule and club-shaped oesophagus that differentiate it from hookworms. The nematode shows cephalic groove and observable secretory pore (*stomum*) at the oesophagus level. The worms have an outer covering with a flexible and tough cuticle without any segmentation like other roundworms. Both male and female adult worms have a cephalic inflation and oral opening consisting of internal and external leaf crowns (Fig. 2). Males are differentiated from females by the presence of bell-like copulatory bursa and paired rod-like spicules in their tail end.

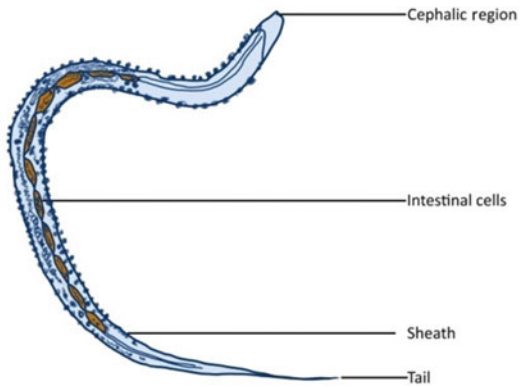

Fig. 1 An *Oesophagostomum* spp. adult worm

Eggs

Eggs of *O. bifurcum* are morphologically similar to the eggs of *Necator* or *Ancylostoma*. They measure around 60–75 μm in length and 35–40 μm in width. The eggs when shed in faeces are in the later stage of cleavage containing several cells (Fig. 2).

Larva

The L3 filariform larva is the infective stage, and it measures about 800 μm in length and 30 μm in width. It has got a sheath, which has transverse striations. The posterior end of the larva tapers to a slender thread-like tip. The characteristic feature is the presence of 16–30 triangular intestinal cells in the body. The L3 larva is exceptionally resistant to adverse environmental conditions and may survive total desiccation for almost 6 months or a sub-zero temperature for a few days.

Cultivation of Parasites

The sheathed L3 larvae of *Oesophagostomum* spp. can be isolated from stool of infected host by a method of small-scale agar gel migration in vitro. Alternatively, phosphate-buffered saline containing 15% serum and 10% liver extract may be used for hatching the eggs into L3 larva. For best results, culture is incubated for 5–7 days at

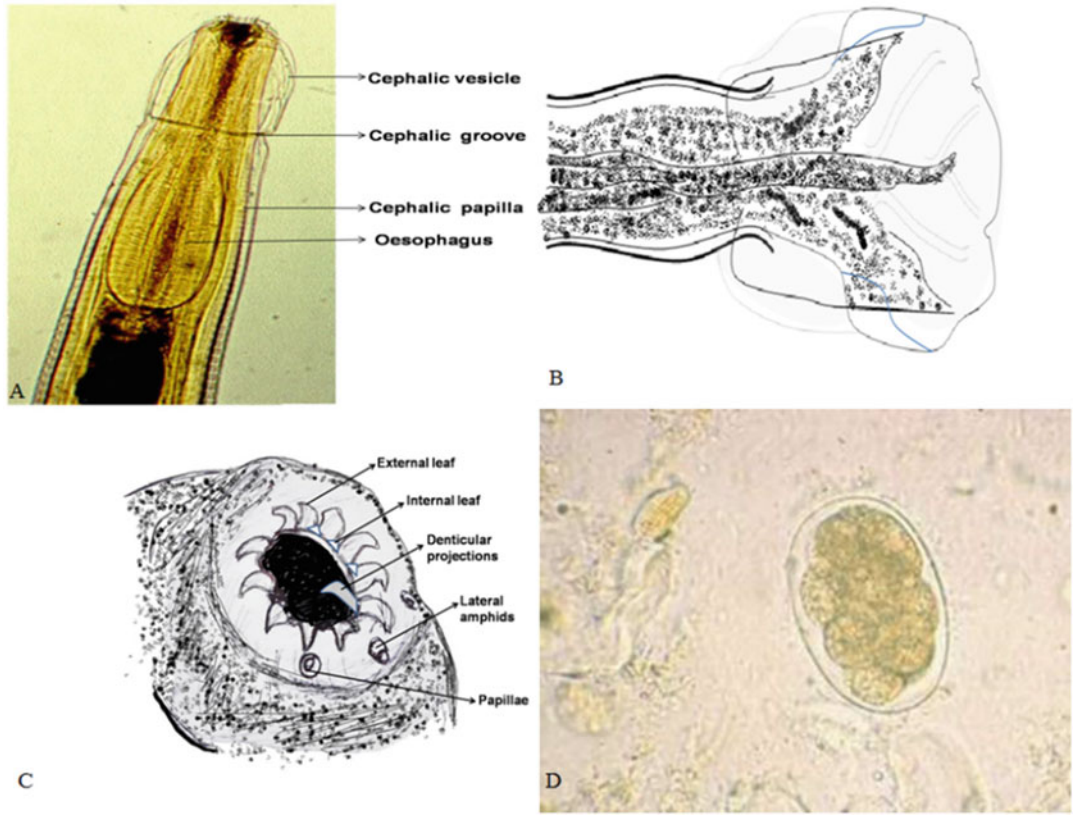

Fig. 2 An *Oesophagostomum* spp. adult worm description (**a**), bursa copula of a male (**b**), anterior end with external leaf crown (**c**), egg with multiple segments (**d**). (part label **a** and **d** are adapted from Juventus, 2006)

26 °C, pH 6.5 and relative humidity ≥80–90%. The L3 larva in the culture is grown to L4 stage using cell-free API-1 medium with or without glutathione (reduced) or a bovine haem. The bovine haem is provided using "unbound" haemin or the bound haemin component of Fildes' reagent. L4 larva is separated from L3 through sedimentation and may be kept in 0.9% sodium chloride at 37 °C until further use. It has been observed that in medium containing bovine haem, development continued in young adult males and females within 25–35 days.

Laboratory Animals

Crossbred piglets, Bengal goats (*Capra hircus*) and Holstein calves are used for experimental infections to study the mechanism of infection, host–parasite interaction and comparative analysis of the development of *Oesophagostomum* spp. These animals before experimental infection are maintained helminth-free under intensive rearing conditions until use.

Life Cycle of *Oesophagostomum* Spp.

Hosts

Sheep, goat, swine, non-human primates and humans are the definitive hosts for the parasite.

Infective Stage

The infective stage of *Oesophagostomum* is filariform (L3) larva.

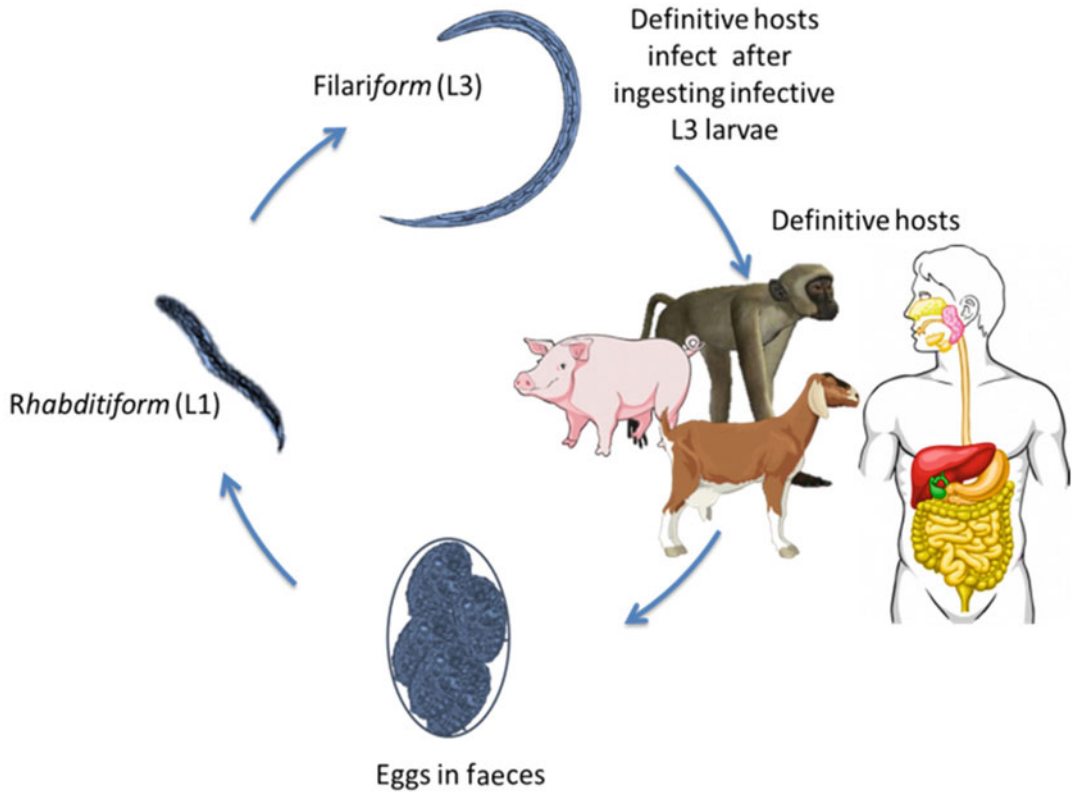

Fig. 3 Life cycle of *Oesophagostomum* spp.

Transmission of Infection

Humans and other mammals such as sheep, goat, primates and swine acquire the infection by ingestion of food and water contaminated with infective filariform (L3) larva (Fig. 3). In stomach and small intestine, the larva loses its cuticular sheath, and after penetrating the intestinal mucosa, it transforms into L4 stage at the submucosal site. This results in the formation of 1–3 mm size nodules in the intestine, particularly in large intestine, and hence the name *nodular worms*. The L4 larva matures into the adult forms, and after fertilization, the female starts laying eggs at about 30–40 days post-infection. In humans, full development may not happen and the worms remain immature or without egg production.

The eggs are shed with faeces and embryonated to produce the L1 larvae, which are released. In about a week's time, and under favourable temperature and humidity, L1 larva moults into L3 larva, which remains encysted within the L2 larva. This is the infective stage of the parasite.

Pathogenesis and Pathology

The development of the disease process starts with the ingestion of *Oesophagostomum* third-stage larvae (L3). Some of the larvae begin to infiltrate the mucosal layer of colon and ceacum leading to intradermal bleeding. Majority of larvae encyst in mucosa and initiate the destruction of proximate muscularis mucosae. The larvae either persist there or penetrate further even into the serosa at the site of attachment.

A marked inflammatory reaction is triggered by larvae in the wall of the gut. The repeated invasion of mucosa by *Oesophagostomum* larvae leads to hypersensitivity reactions. It also results in inflammatory oedema with mucosal thickening and lymphatic thrombosis. Some larvae are killed, but many of the parasites moult and reattach to the surface of the intestinal mucosa. The fibrotic nodules may develop around the larvae, persist and are associated with secondary bacterial infections.

Immunology

The immune response of the host to *O. dentatum* has been studied in pigs. A suppressed Th1 and Treg-type immune reactions were observed corresponding to a predominant Th2-type immune response. In the BALB/c mice model, extract from adult *O. dentatum* induced Th2 and regulatory responses. Stimulation of bone marrow-derived dendritic cells induced the production of regulatory cytokines IL-10 and TGF-β.

Infection in Humans

The acute abdominal pain, mimicking appendicitis, is the most common manifestation of *O. bifurcum* infection in humans. The condition is associated with moderate fever and discomfort in the lower-right area of the abdomen. Vomiting, diarrhoea and anorexia are the uncommon manifestations.

Other manifestations include intestinal obstruction, mimicking a hernia, and development of large, painless cutaneous masses in the lower abdominal area. *Dapaong tumour* is a common manifestation and presents as an abdominal inflammatory mass with fever. The well-defined tumour develops near the abdominal wall. These tumours are 3–6 cm in size, soft, bulbous, periumbilical and painful. In rare cases, the worm invades and perforates the intestinal wall, causing purulent peritonitis, or may migrate to the skin causing cutaneous nodules.

Infection in Animals

Oesophagostomiasis in animals manifests as either acute or chronic infections.

The acute infection is characterized by loss of weight and appetite, and aqueous or mucous diarrhoea. The chronic infections manifest as anaemia, oedema and persistent diarrhoea leading to profound weakness of the infected animals.

Epidemiology and Public Health

Oesophagostomiasis is endemic in as many as 35 countries of the world (Fig. 4). Most of the cases have been reported from Africa specifically in Ghana, Togo and Uganda (Table 1). A total of 156 cases of oesophagostomiasis caused by *O. bifurcum* were reported from villages of northern Togo and Ghana, in West Africa. Cases have also been documented from countries of Asia like Malaysia, Brazil and Indonesia and parts of America. The disease is estimated to affect about 0.25 million persons worldwide, and another 1 million individuals are at risk of acquiring the infection.

Human infection is most commonly found in children between 5 and 9 years of age. Females older than 5 years have higher risk of infection than the males. Although the factors contributing to discrepancies in age, demography and gender are not exactly known, nevertheless, it is believed that exposure to contaminated water and strength of immune responses may be important. A strong correlation between infections caused by *O. bifurcum* and *Necator americanus* in the individuals living in endemic villages has been described. Inadequate sanitation, few farming

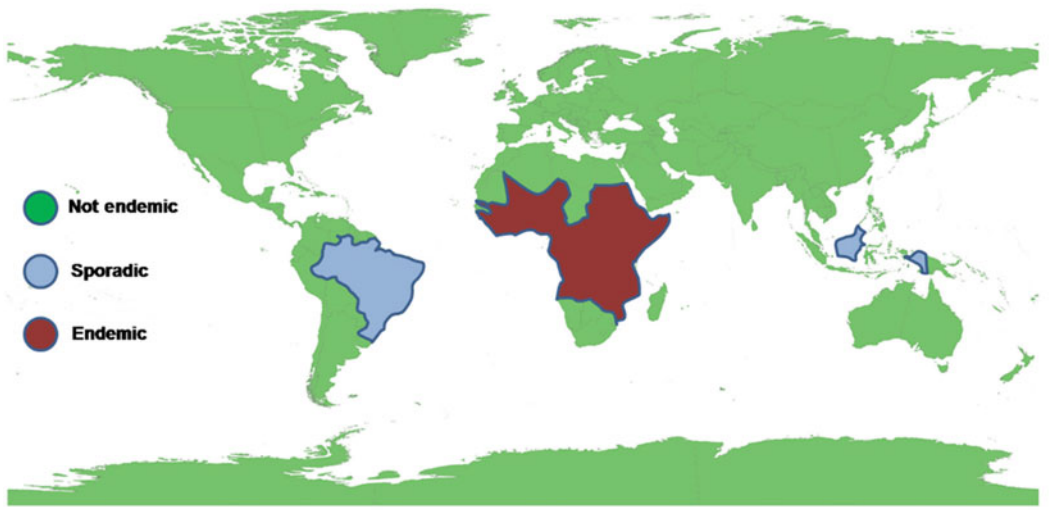

Fig. 4 Global distribution of *Oesophagostomum* spp. infection

practices and lack of good quality potable water possibly may be some of the contributing factors.

Diagnosis

Definitive diagnosis of oesophagostomiasis is made by demonstrating the *Oesophagostomum* larva or young adult forms in the nodules of the intestine, or in the excised tissue following surgery. Microscopic analysis of the intestinal tissue for multinucleated cells indicates the presence of larvae and young adult (Table 2).

Microscopy

Demonstration of *O. bifurcum* eggs in stool by microscopy suggests the tentative diagnosis of oesophagostomiasis. The eggs that are round and measure 60–75 μm by 35–40 in size are found in larger numbers in stool during acute manifestation of oesophagostomiasis. However, *Oesophagostomum* eggs and other hookworm eggs are morphologically similar to each other; hence, coproculture is carried out for specific diagnosis based on the morphology of the larvae hatched out of the eggs.

In Vitro Culture

Coproculture can be performed, which enables the eggs to hatch to L1 larvae that subsequently develop into L3 larvae. The specific diagnosis is made by identification of the L3 larva. Phosphate-buffered saline containing 15% serum and 10% liver extract may be used for hatching of the eggs into L3 larva. For best results, culture should be incubated at 26 °C,

Table 1 Distribution of *Oesophagostomum* spp. of importance to humans

Species	Distribution	Intermediate host	Definitive host
Oesophagostomum bifurcum	Ghana, Togo, Uganda, Brazil, Southeast Asia, including Indonesia and Malaysia	No intermediate host	Humans, monkeys

Table 2 Methods for diagnosis of *Oesophagostomum* infections in humans

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Biopsy/stool sampling/ coproculture	Larval sections in tissues/ fluids and eggs in stool	*Drawback*: Invasive and insensitive, not easy to distinguish and time-consuming
Immunodiagnostics	Antibody (ELISA)	IgA, IgE, IgG4 antibodies	Good sensitivity and specificity
Molecular assays	PCR, multiplex PCR	16SrDNA, ITS-2-21	High sensitivity and specificity *Limitations:* Require skilled personnel
Imaging technique	Ultrasound/ sonography	Intestinal and abdominal wall	Reduce invasiveness, increase reliability of chemotherapy

pH 6.5 and relative humidity ≥80–90% for 5–7 days. This technique, nevertheless, is time-consuming.

Serodiagnosis

A sensitive and specific ELISA test for diagnosis of human oesophagostomiasis has been described in the literature using *O. bifurcum* crude-soluble antigens. The IgG ELISA showed cross-reactivity with other helminth infections. However, detection of IgG4 fraction by ELISA has been described to be highly specific although sensitivity could not be ascertained because of lack of definitive parasitological diagnosis. Apart from it, a number of workers have described immunoassays to detect antibodies in sheep and goats using various antigens derived from *O. columbianum.*

Molecular Diagnosis

PCR-based assays are being increasingly used for the diagnosis of oesophagostomiasis. PCR with specific *O. bifurcum* genetic markers is highly sensitive and specific for the detection of the parasite genome in the serum. The multiplex PCR with stool samples is also a promising method for the simultaneous detection and identification of *O. bifurcum* along with *Ancylostoma dudodenale* and *Necator americanus* with 100% specificity and sensitivity.

Other Tests

Sonographic imaging is being increasingly used for the diagnosis of oesophagostomiasis. The ultrasound method and sonographic images have the advantages of detecting nodules in the abdominal wall prior to the chronic phase of the infection. These non-invasive procedures minimize the invasive surgical procedures to minimum and also allow sufficient time for chemotherapy of the infection.

Treatment

Both narrow and broad-spectrum anthelmintics are used for the treatment of *Oesophagostomum* infections in humans. Narrow spectrum anthelmintics such as pyrantel and morantel are effective against adult worms, but not against the larvae.

The broad-spectrum anthelmintics such as albendazole are highly effective against oesophagostomiasis. The drug acts by inhibiting the tubulin polymerization, which in turn inhibits the glucose uptake by the parasite. A single dose of albendazole (400 mg) given orally is effective to eradicate the parasite from infected case. The recurring administration of albendazole to people in an endemic area of northern Ghana has shown a dramatic decrease (~90%) in prevalence of *O. bifurcum* infection within a year and up to ~98% within 2 years. Albendazole (200–400 mg) in combination with amoxicillin (250 mg) is recommended for up to 5 days

depending on the severity of the disease. In case of fistulae or abscess, incision and drainage of the lesion are recommended followed by chemotherapy.

Recently, trans-cinnamaldehyde (CA) has been evaluated and was found to be effective against *O. dentatum* larvae in vitro. The compound, when administered in vivo, the infection was not significantly reduced, possibly due to the rapid absorption or metabolism of trans-cinnamaldehyde.

Prevention and Control

Oesophagostomum infections are primarily transmitted through oral–faecal route by the L3 stage, the infective stage of the parasite. Hence, adequate cleaning and cooking of meat and meat products and vegetables, boiling of drinking water, and maintenance of proper sanitation and hygiene are the suggestive measures to reduce the infection in the area endemic for the disease. Since the development of eggs to infective L3 larvae takes about 1 week, removing all manure in shorter intervals can break the life cycle and reduce the infectivity of the environment. As the infection is primarily limited to a particular region of Africa, resource mobilization within and around the endemic area targeting this parasite may be undertaken to prevent or reduce the disease burden**.**

Case Study

An eight-year-old Malaysian boy showing the symptoms like abdominal pain and weight loss was admitted to a hospital. Blood examination was performed but found normal. Ultrasonography of colon showed the presence of "target" or "bulls eye" and "pseudokidney" appearances. The laparotomy analysis also showed that the abdominal cavity was filled with a fluid. The intestinal wall was covered with hundreds of pale, pea-sized, nodules. When the bowel was dissected, the nodules were ruptured and the thick yellow pus leaked out, which included an 11 mm long worm moving aggressively. Further examination confirmed that infection was caused due to *Oesophagostomum* worms.

1. Mention the definitive diagnostic methods of *Oesophagostomum* infection?
2. What is the disadvantage encountered during stool microscopy in this condition?
3. How do you treat this infection?

Research Questions

1. What is the mechanism of maintenance of infectivity and survival capacity of the *Oesophagostomum* larva in extreme drought and low temperatures?
2. Even though humans are considered as unsuitable host for *Oesophagostomum*, yet why some larva complete their development in humans?
3. Is there any possibility of human-to-human transmission of oesophagostomiasis?

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Mammomonogamiasis

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Learning Objectives

1. To understand the importance of this parasite in causing acute upper respiratory tract infection.
2. To understand the value of bronchoscopy for removal of the worm.

Introduction

Mammomonogamiasis is an infection of the respiratory system caused by the nematode belonging to genus *Mammomonogamus*. The condition is well documented in cats, cattle, deer, elephants, goats, orangutans, sheep, wild yaks, but rarely, in humans in subtropical and tropical regions of the world. The genus *Mammomonogamus* included only four known species: *Mammomonogamus laryngeus, Mammomonogamus nasicola, Mammomonogamus gangguiensis* and *Mammomonogamus auris.* Out of these four species, only one, namely *M. laryngeus,* is recognized to cause accidental infestation in human beings and resides in the trachea, bronchus or larynx.

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History

Earlier, *Mammomonogamus* was considered as kind of gapeworms belonging to the genus *Syngamus,* due to its close resemblance with the later causing infection in birds. The worm has been first isolated from cattle in Vietnam and named as *Syngamus laryngeus* by Railliet in 1899. Later, based on the phylogenetic relationship it has been described as a member of different genus, *Mammomonogamous*. The word *Mammomonogamus* is comprised of Latin word *mamma* (breast), as well as Greek words, *mono* (single) and *gamos* (marriage). The foremost cases of mammomonogamiasis in humans were reported from St. Lucia Island by Dr. A. King and from Caribbean region by Leiper in 1913. *Mammomonogamus* species exhibit weirdly low host specificity compared with the other members of order Strongylida.

Taxonomy

On the basis of morphological features, such as the state of permanent copulation and the site of

S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
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infection, genus *Mammomonogamus* is classified under the family Syngamidae of phylum Nematoda (Ryzhikov 1948). Further, family Syngamidae has been classified into two subfamilies: Syngaminae and Stephanurinae. The subfamily Stephanurinae is represented by the species, *Stephanurus dentatus*, causing infection in pigs. The subfamily Syngaminae has five genera, such as *Syngamus, Cyathostoma* and *Boydinema,* causing infection in birds, as well as *Rodentogamus* and *Mammomonogamus,* causing infection in mammalians.

The taxon *Mammomonogamus* belongs to the order Strongylida due to its close relation with hookworms. However, unlike hookworms, the oral cavity of *Mammomonogamus* is devoid of cutting plates and teeth.

Genomics and Proteomics

Very limited studies have been done at the molecular level for *Mammomonogamus*. The profiling of *M. laryngeus* ES (excretion–secretion) proteins was evaluated using SDS-PAGE electrophoresis, and its enzyme activity was determined in a Columbian study. The four bands separated from these proteins including the most dominant band of 94.4 kDa and diffuse bands of 72, 108 and 122 kDa, among which 108 kDa had shown protease activity. It is suggested that the proteins present in these bands have significant role in penetration of skin by the parasite and its movement through the host's connective tissues.

The Parasite Morphology

Adult Worm

M. laryngeus is a haematophagous roundworm with some unusual characteristics. The parasite appears Y-shaped, and the worms are found *in copula*. The female worm has elongated red-brown coloured arm, whereas male worm has shorter yellowish coloured arm (posterior bursa) attached to vulva of female located near the centre. Thus, a "Y"-shaped structure is formed by the association of male and female worms, which remains permanently united in the adult stage. Adult worms of *M. laryngeus* appear blood red to red-brown in colour owing to absorption of the host's blood (Fig. 1).

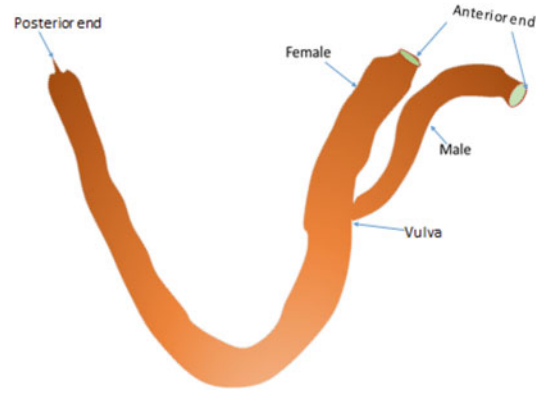

Fig. 1 *Mammomonogamus laryngeus* adult worms

The adult female worm is 8.7–23.5 mm long and 0.55–0.57 mm wide. It possesses a lengthy or short tail with pointed posterior end and possesses a genital opening in the middle of the body for copulation. The uterus contains many eggs. The male worm is 3–6.3 mm long, 0.36–0.38 mm wide and possesses spicules for copulating with female worm. Spicules are 23–30 μm long.

The worms have an oral opening or a mouth present at their anterior end and a posterior opening. The mouth is thick-walled and has a cup-shaped buccal capsule and short or long ribs. It consists of eight to ten small teeth located deep in the buccal cavity at its base without leaf crowns. These teeth are not used for attachment. The oral aperture adjoins the circular rim and is bounded by lips.

Eggs

The female *M. laryngeus* lays many eggs that are ovoid to ellipsoid, non-operculated and 40 x

80 μm in size. The eggs have spicules measuring 23–30 μm in length and have outer wall, which is thicker than the hookworm eggs. Eggs are expectorated out in the sputum or excreted in faeces of infected humans.

Cultivation of Parasites

Cultivation of a pathogen is important for understanding the life cycle, detection of drug resistance, production of vaccines and therapeutic agent screening. There are only a few in vitro techniques such as Harada–Mori culture technique available for cultivation of *Mammomonogamus* spp.

Laboratory Animal

Attempts to establish mammomonogamiasis in grown-up kittens and cats using hatched larvae have not been successful. This has led to the hypothesis that an intermediate or paratenic host is required to complete the life cycle of the parasite.

Life Cycle of *Mammomonogamus* spp.

The life cycle of *Mammomonogamus* spp. has not been elucidated completely.

Hosts

Definitive Hosts

Ruminants like cattle are considered as definitive hosts for *Mammomonogamous* spp. Most common species *M. laryngeus* causes rare infection in humans.

Intermediate Hosts

Not confirmed. It may be arthropods, snails or earthworms. No biological or mechanical vectors are known. It is also not clear whether they require intermediate hosts or not to complete the life cycle.

Fig. 2 Egg of *Mammomonogamus* sp. isolated from faeces of an African forest elephant. The surface of egg shell shows typical division into irregular rectangular fields by striation (adapted from Červená, 2017)

Infective Stage

It may be eggs or larvae or may be the adult worm itself.

Transmission of Infection

Transmission of parasite is suggested to be through oral–faecal route, where parasite enters the host's body by ingestion of food or water containing larvae or embryonated eggs (Fig. 2). Currently, there are two hypotheses that explain the possible life cycle of *Mammomonogamous* spp., especially in endemic areas.

Hypothesis 1: Mammomonogamus infection may be caused by intake of the mature worm through contaminated food or water. The infective agents reach the trachea via larynx and cling towards mucosal walls of the respiratory tract. After that, the worms perform sexual

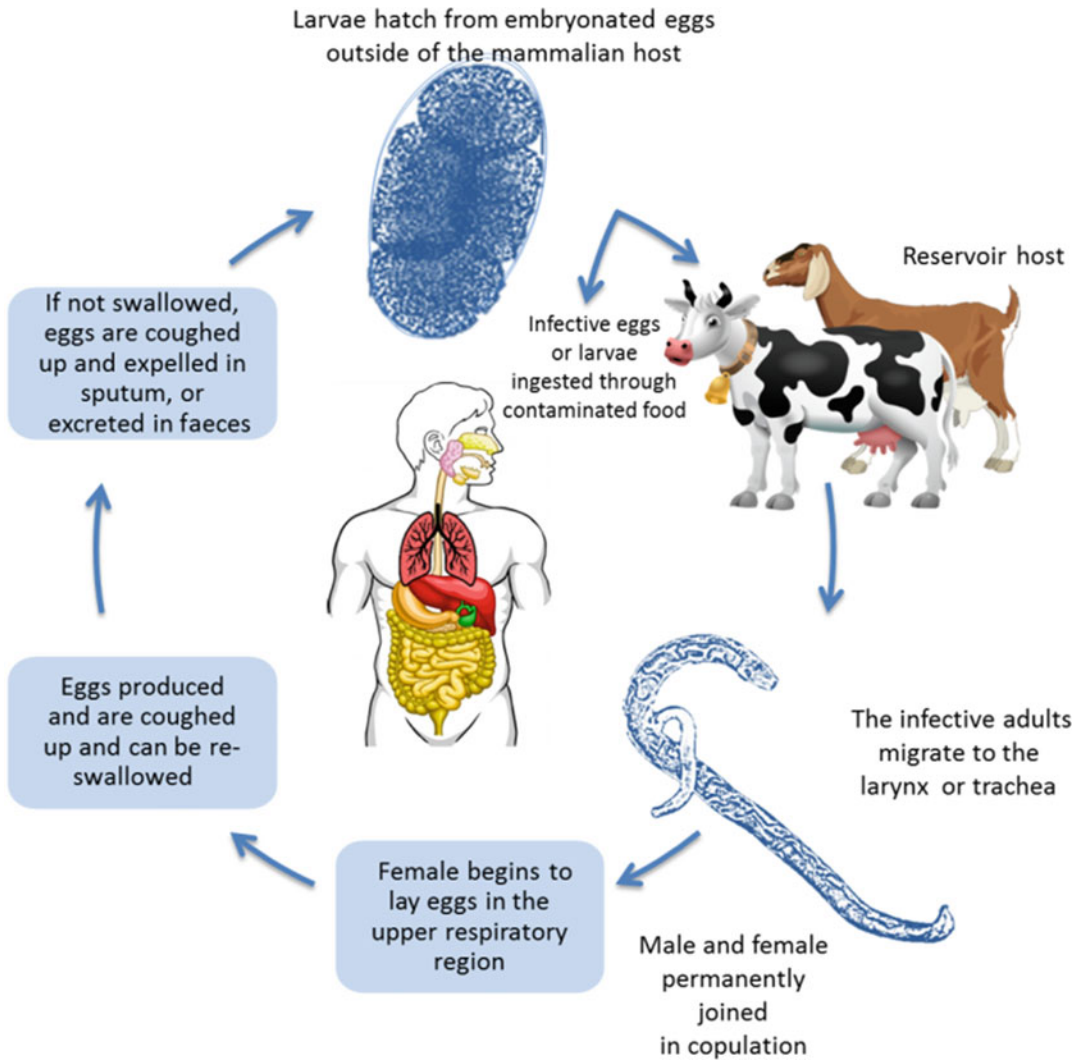

Fig. 3 Life cycle of *Mammomonogamus* spp.

reproduction, and female worm starts to lay eggs in the respiratory tract of host. The eggs are exiled in sputum or re-swallowed and exit in faeces of the infected host. Thus, the development of egg to larvae does not occur within the human body.

Hypothesis 2: The infective agents (embryonated eggs or infective larvae), after ingestion, move to the intestinal region, migrate along the intestinal walls and, through mesenteric veins, reach to the alveoli area, where the eggs/larvae grow into an adult worm within 7 days. Further, the adult worms move upwards to the larynx and reproduce. The female worm lays eggs, which come out from host body through faeces or sputum. The eggs hatch after 3 weeks to produce larvae.

Figure 3 depicts a tentative life cycle of *Mammomonogamus*. Further research is required to elucidate the complete life cycle; nevertheless, both eggs and larvae appear to be infectious.

Pathogenesis and Pathology

So far, little is known regarding pathogenesis of *Mammomonogamus* infection. After infection,

the worms show an incubation period of 6–11 days. The development of symptoms does not occur until the worms develop into mature form and cause cough and asthma-like symptoms because of hampering the bronchial air passage. These symptoms may appear in ruminants, domestic ungulates and the human hosts. Other symptoms, such as inflammation in bronchi or haemoptysis, might appear due to attachment of worms to cells of the mucosal region and phagocytosis of host RBC. Pathological changes in tissue appear to be minimal.

Immunology

The immune responses in host body evolve through innate and acquired immunity. Host and parasite both try to protect themselves with immune responses. *Mammomonogamus* secretes the immunomodulatory molecules, which may halt or change the host immune response to support their survival in the host body.

Infection in Humans

Incubation period varies from 6 to 11 days.

M. laryngeus infection in humans is associated with severe chronic non-productive dry cough and haemoptysis. In a few cases, the infection shows asthma-like symptoms and transient pneumonitis. Generally, the villagers like ranchers and farmers are at risk of infection (Sossai et al. 2007). Most of the patients harbour only single pair of worms; however, some may harbour multiple pairs of the parasite. So far, no re-infection of *M. laryngeus* has been reported.

Infection in Animals

Clinical manifestations in infected animals begin as febrile illness with conditions such as cough and asthma-like symptoms. Most cases progress to a persistent cough, and at times haemoptysis. These conditions persist for several months, along with a low-grade fever, if they remain untreated. Weight loss and pneumonitis, but not anaemia, have also been reported.

Epidemiology and Public Health

Mammomonogamus spp. causes infection in various animal hosts, particularly in the ruminant population. The infection in human beings is quite uncommon, with only around 100 cases documented till date. Most cases have been documented from the endemic areas of Caribbean Islands, Brazil, Africa, India, Korea, China, Malaysia, Philippines, Thailand and Vietnam (Table 1). Countries like the United Kingdom, the USA, Canada, Australia and France, though not endemic, also have documented cases because of the high number of travellers contracting with this disease parasite.

Diagnosis

The definitive diagnosis of mammomonogamiasis is based on demonstration of adult *Mammomonogamus* by bronchoscopy or endoscopy, or by recovering the adult worm from cough samples (Table 2).

Table 1 Distribution of *Mammomonogamus laryngeus*

Species	Distribution (Endemic countries)	Distribution (Non-endemic countries)	Intermediate host (possibly)	Definitive host
Mammomonogamus laryngeus	Caribbean Islands, Brazil, Africa, India, Malaysia, Philippines, Vietnam, China, Korea and Thailand	Australia, Canada, USA, UK and France	Earthworms, snails and arthropods	Humans, cattle

Table 2 Diagnostic methods for mammomonogamiasis in humans

Diagnostic method	Sample	Target	Notes
Bright-field and fluorescence microscopy	Biopsy or sputum or stool	Y-shaped adult copula/ eggs	Easy, rapid, reliable
Scanning electron microscopy (SEM)	Biopsy or sputum or stool	Y-shaped adult worm *in copula*/ eggs	Very reliable study ultrastructural details of parasite can be studied *Drawback*: Requires skilled personnel
Bronchoscopy or endoscopy	–	Adult worm	Less time taking, safe procedure *Limitations:* invasive procedure
DNA amplification and sequencing	Biopsy or sputum or stool	Mitochondrial cytochrome c oxidase subunit I (cox1) gene	High sensitivity and specificity *Limitations:* require skilled personnel

Microscopy

M. laryngeus infection is diagnosed by observing the eggs of *M. laryngeus* in the faeces or sputum. However, the eggs closely resemble the eggs of hookworm, but differ from them by having much thicker shell divided into irregular rectangular fields by striation (Fig. 4).

In vitro Culture

Harada–Mori culture technique has been used for the demonstration of nematodes at its larval stage. The technique employs a filter paper to which faecal material is added and is then inserted into a test tube, and incubated at 30 °C. Further, moisture is provided by addition of water in test tube to provide suitable conditions for hatching of egg and development of larvae. The water sediment is screened daily to look for living larvae. After recovery of the worms, minute details of the genital structures and anterior end may be examined using bright-field microscopy, fluorescence microscopy or scanning electron microscopy.

Serodiagnosis

No antigen or antibody detection methods are available for diagnosis of *Mammomonogamus* infection in either animals or humans.

Molecular Diagnosis

No standard molecular diagnostic method is available on date. Recently, cox1 gene sequence homology analysis has been used for the identification of *Mammomonogamous* spp.

Treatment

Removal of worms by bronchoscopy or manually from patients results in resolution of symptoms. Sometimes, there may be spontaneous expulsion of worms during violent coughing and resolution of infection. Though there are no conclusive studies on the efficacy of anthelmintics for the treatment of *Mammomonogamus* infection, combined therapy by anthelmintics such as albendazole 200 mg and mebendazole 100 mg, for 3 days with three times a day, is effective. Alternatively, one dose of ivermectin (200 μg/kg) with a 2 days' course of thiabendazole (1250 mg bid) followed by final 2 days' course of albendazole (400 mg bid) may also be prescribed.

Prevention and Control

Food hygiene, safe water and proper sanitation are effective preventive and control measures. However, to prevent the spread of zoonotic parasitic diseases like mammomonogamiasis, the

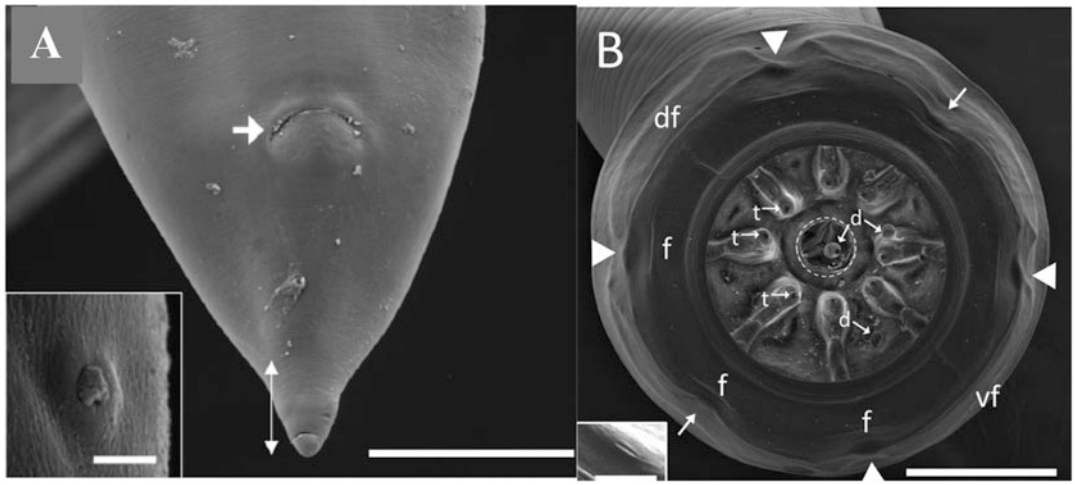

Fig. 4 Scanning electron microscopy of an adult female *Mammomonogamus laryngeus* worm. (**a**) Ventral view of posterior end showing anus (*arrow*) and cone-shaped tail with a pointed tip (*double arrow*). Inset: detail of phasmid (**b**), internal view of the anterior end showing the oesophagus valve (*dotted circle*), debris, which is probably the host's tissue (d), eight ribs and the same number of teeth with rounded tips (t). Externally, it is possible to identify four external papillae (*arrowhead*), two amphids (*arrow*) and eight festoons organized as follows: two pairs of six small festoons (f) and the two largest ones located at the ventral (vf) and dorsal (df) faces of the mouth. Inset: detail of one amphid. Scale bar 100 μm (adapted from Lopes-Torres et al., 2020)

slaughter houses of animals need to be regularly monitored for the presence of parasite, so that the quality control and safety of the meat product for consumers are ensured. Prophylactic measures need to be employed to prevent the potential risk of infection in workers of slaughter houses.

Case Study

A 28-year-old female student of animal sciences residing in Brazil presented with dry cough and throat pain. Following an examination, the physician prescribed cough medications. The biochemical profile, differential WBC count, urine and stool examinations were normal. Subsequently, after 60 days, the patient suffered from productive cough with fever. Antibiotic amoxicillin and paracetamol were given to her based on the findings of pneumological examinations. In the coming days, the patient continuously suffered from cough with or without mucus and has taken self-medication with cough and cold drugs. However, the patient did not get rid of frequent and violent coughing and was admitted to the intensive care unit for 10 days. There, she was administered with antibiotic levofloxacin, mucolytic syrup acetylcysteine and nebulized with sympathomimetic drug fenoterol hydrobromide and broncholdilator ipratropium bromide. After few months, the patient again appeared in hospital with severe illness due to frequent and violent productive coughing along with the sensation of formication in pharynx. Subsequently, a Y- shaped worm, red in colour, was observed in her sputum after few days (Fig. 1). After expectoration of parasites, the patient gets rid of coughing. Further examination by the parasitology laboratories confirmed that the worm was *M. laryngeus* couple. The worms recovered from this patient were 20 mm (female) and 5 mm (male), but in stool test there were no parasite eggs detected.

1. What are the parasites which cause pulmonary involvement?
2. What are treatment option for this condition?
3. How is the infection acquired?

Research Questions

(a) What are the reservoir host and the intermediate host?
(b) What is the mode of infection and the infective stage?
(c) How common is it among the putative animal hosts like cattle?
(d) How to develop a serological test for humans or animals?

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Part VI

Infections Caused by Pentastomids, Arthropods and Ectoparasites

Pentastomiasis

Sourav Maiti

Learning Objectives

1. To review the clinical presentation of pentastomiasis and misdiagnosis with tuberculosis or malignancy.
2. To understand the critical role played by microscopy in its diagnosis.

Introduction

Pentastomiasis is an often forgotten zoonotic infection caused by the pentastomes, a peculiar group of vermiform endoparasites. An annulated elongate body or tongue-like shape distinctly characterizes these unique '*tongue worms*'. A single mouth flanked by two pairs of hooks initially generated the misimpression of five mouths naming as *pentastome*. *Armillifer armillatus* and *Linguatula serrata* are the causative agents in more than 90% of infection in humans. Largely, being asymptomatic and self-limited, opportunities to diagnose and treat the condition are restricted.

History

Pentastomes possibly parasitized the carnivorous dinosaurs in the Mesozoic times. Chabert, a French veterinary surgeon, first observed the parasite in 1787 in the nasal cavity of dogs, possibly *Linguatula* sp., but thought of a tapeworm. In 1845, Wyman first reported *A. armillatus* from western Africa and identified the adult pentastome in the nasopharynx of an African rock python within the next 3 years. However, Pruner (1847) in the meantime reported the first human pentastomiasis case from Cairo, Egypt. Stiles (1891) prioritized the name '*Porocephalus*'.

Taxonomy

Hesitancy to place the pentastomes under Annelida or Arthropoda is persisting. The advent of molecular techniques points their relative closeness to the crustacean arthropods, but most authorities prefer to retain them under a unique minor phylum Pentastomida. Inclusion in a more comprehensive phylum Lobopodia is under consideration.

The present taxonomical status for the common pentastomes infecting humans belongs to families Porocephalidae and Linguatulidae in the order Porocephalida; class Pentastomata; and phylum Pentastomida.

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Linguatulidae family includes the genus *Linguatula* containing the medically important species *Linguatula serrata*. Porocephalidae family consists of numerous members with *Armillifer* genus being the commonest. Order, Cephalobaenida, contains primitive pentastomes affecting lizards and snakes. The species, among the pentastomes infecting humans, are *Linguatula serrata*, *Armillifer armillatus*, *Armillifer moniliformis*, *Armillifer grandis*, *Armillifer agkistrodontis*, *Porocephalus crotali*, *Porocephalus taiwana* and *Sebakia* sp. Reports of human infections by *Leiperia cincinnalis*, *Railietiella hemidactyli* and *Railietiella gehyrae* are also present.

Genomics and Proteomics

Mitochondrial genome sequencing of *A. armillatus* shows similarity to metazoan traits including 37 genes. Gene rearrangements suggest its inclusion within the phylum Arthropoda. However, studies suggest that the pentastomes might be closer to the nematodes than the arthropods. *A. agkistrodontis* has a complete mitochondrial transcript of length 16,521 bp containing 13 protein-coding genes (PCGs), 22 tRNA genes and 2 rRNA genes. The same number of PCGs, tRNA and rRNA prevails in *A. grandis* and *L. serrata* mitochondrial DNA with smaller genome lengths (16,073 bp and 15,328 bp, respectively). All pentastomes show an inherent A + T bias in their mitochondrial genomes. Genomic studies have been refining the much-debated taxonomical status of the pentastomes. It provides important genetic markers for the epidemiological studies too.

Proteomic studies are important for providing useful insights into the pathogenesis of pentastomiasis. However, studies are lacking in this aspect. Two proteins from *A. armillatus*, namely a serine endopeptidase and a G-protein-coupled receptor kinase, are under research. A 48 kDa metalloproteinase from the frontal glands is used for diagnostic purpose.

The Parasite Morphology

Adult Worm

Pentastomes have elongated cylindrical or flat tongue-like bodies divided into a short cephalothorax and a long abdomen (Fig. 1). Males are shorter than the females. Length varies from few mm to 15 cm. The cephalothorax contains a mouth with two pairs of chitin hooks. The non-segmented abdomen has encircling pseudoannulations in *A. armillatus* (18–22), *A. moniliformis* (around 30), *A. agkistrodontis* (7–9) and *A. grandis* (more than 25). A straight tube of primitive digestive system is flanked by paired frontal glands. An extensive reproductive system and the absence of respiratory and circulatory systems are the characteristic features of the worm. The genital pore in the female is located anteriorly in family Cephalobaenida and at the posterior end in family Porocephalida.

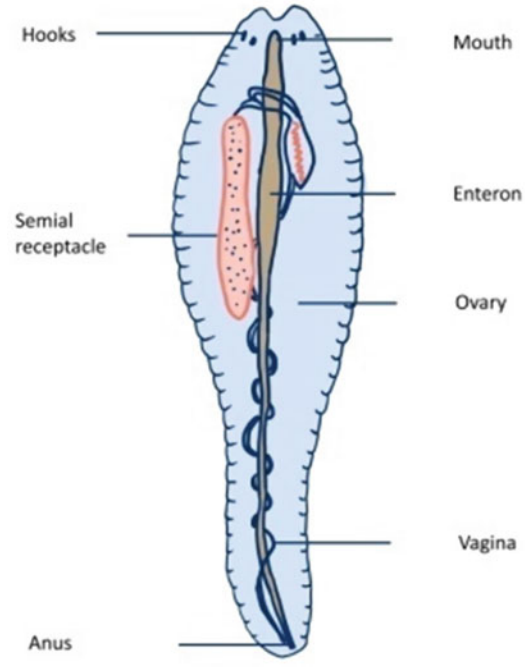

Fig. 1 Schematic diagram of adult *Armillifer armillatus* adult

Eggs

Eggs are ovoid (105 μm × 125 μm), double-shelled containing a mite-like embryo when deposited.

Larvae

The first-stage larva (L1) has rudimentary appendages that are lost subsequently upon moulting (Fig. 2). The third-stage larva (L3) or the nymph has hooks and morphology akin to the adult in a miniature form. Apart from *L. serrata*, which has spines, all pentastome larvae have smooth cuticle. The 5- to 10-μm thick cuticle contains numerous sclerotized openings. Sebaceous glands and muscle fibres, both circular and longitudinal, lie under the cuticle. *Armillifer* sp. (L3) larvae are 9–23 mm long, cylindrical with spiral rings. *L. serrata* (L3) larvae are 4–6 mm long, flat, annulated with a row of spines on each annulus.

Fig. 2 Schematic diagram of a pentastome larva

Cultivation of Parasites

In vitro culture has not been successful for pentastomes. Experimentally, *P. crotali* eggs withstood desiccation for 2 weeks and survived 6 months in water. Development requires an appropriate living host.

Laboratory Animals

Hamsters serve as experimental paratenic hosts. Recently, a multi-host model has been developed for *A. agkistrodontis*. This type of model helps in understanding the pathogenesis and the transmission. The model uses snakes as the definitive hosts and rodents as intermediate hosts.

Life Cycle of Pentastomes

Hosts

Definitive Hosts

Snakes are the definitive hosts for *Armillifer* sp. and *Porocephalus* sp., while dogs and wolves are the hosts for *Linguatula* sp.

Intermediate Hosts

Various rodents and monkeys act as the intermediate hosts for *Armillifer* sp. and *Porocephalus* sp. Ruminants like sheep and goat serve as intermediate hosts for *Linguatula* spp.

Humans act as an aberrant intermediate host, rarely aberrant/accidental definitive host for *L. serrata*.

Infective Stage

Third-stage larvae are the infective stage for the definitive hosts, while embryonated eggs are infective for the intermediate hosts.

Transmission of Infection

Definitive host acquires infection by ingestion of the third-stage larvae in infected rodent tissue (or herbivore tissue, for *Linguatula* sp.). Intermediate hosts, including humans, acquire infection by ingestion of eggs from food and water contaminated with snake droppings (Fig. 3).

The reptiles carry the adults in their respiratory passage. Mating takes place 3–4 months after infection. The fertilized female deposits embryonated eggs in the snake's oral cavity after 4–8 months, which are discharged outside or passed in the faeces. An intermediate host such as rodent ingests egg through water or vegetations. Inside the rodents, the first-stage larva (L1) hatches out in the intestine and penetrates the gut wall to enter the peritoneum or spread haematogenously. It loses appendages and encysts in the internal organs to transform into the infective third-stage larva (L3) after moultings. A definitive host eats the rodent tissue to release the larvae in the stomach. The L3 larvae then actively migrate to the lungs, mature and reinitiate the life cycle. In aberrant intermediate hosts such as humans, the larvae distribute in various organs and peritoneum but die within 2 years, disrupting the cycle.

In *L. serrata*, carnivore mammals carry the adult pentastomes in their nasal passages. After the embryonated eggs are deposited on grass and vegetations, the herbivores (sheep, goat) take up with similar events following. In place of carnivores, if a human being takes up the infected herbivore tissue the infective larvae migrate to the throat to provoke *halzoun* or *marrara syndrome*. Rarely, *L. serrata* larvae develop into adults in humans.

Pathogenesis and Pathology

In the definitive hosts, adult pentastomes do not provoke significant tissue reactions. In the intermediate hosts, varied tissue reactions occur but of mild degree, probably due to the immunomodulatory action of the excretory/secretory proteins coating the cuticle. Humans ingest the pentastome eggs through food and water contaminated with snake droppings, handling and consumption of contaminated snake products such as meat and bile. The eggs hatch to release the first-stage larvae, which penetrate the gut wall with stylet, aided by the frontal gland enzymes and enter abdomen to encyst in the peritoneum and other viscera. Typically, a thin-walled cyst encases a single larva. With moulting, the larva grows larger in size, nearly 1000-fold and the cuticle merges with the cyst wall preserving the sclerotized openings and spines of *L. serrata*. This increased volume creates pressure effects on the adjacent viscera. In a couple of years, the larvae die with granuloma formation in a few cases, particularly in humans acting as an aberrant definitive host. A hyalinized calcified nodule is the final stage of an encysted pentastome.

The exact cause of death of the parasite in the infected host is unknown. Depending upon the stage, three patterns emerge during biopsy/autopsy: (a) necrotic pentastomida granuloma occurs commonly. This reveals a disintegrated parasite into amorphous and calcified debris preserving a C-shape and the perioral hooks. (b) The presence of a viable larva occurs in recent infection; and c) least commonly, the occurrences of a cuticle granuloma. It consists of the cuticular remnants shed during a migrating larva appearing as refractile structures stained by periodic acid–Schiff.

In visceral linguatuliasis, pearly protuberant nodules of size smaller than 1 cm are seen over the peritoneum, pleura and under the capsules of liver and spleen. These '*Linguatula bodies*' are probably a larva migrans effect due to the migration of the larvae. Different mechanisms act in halzoun/marrara syndrome where the humans act as aberrant definitive hosts for *L. serrata*. The migrating larvae create mechanical and hypersensitive events resulting in violent cough, asphyxia and congestive oedema of the gum, tonsils and the Eustachian tubes.

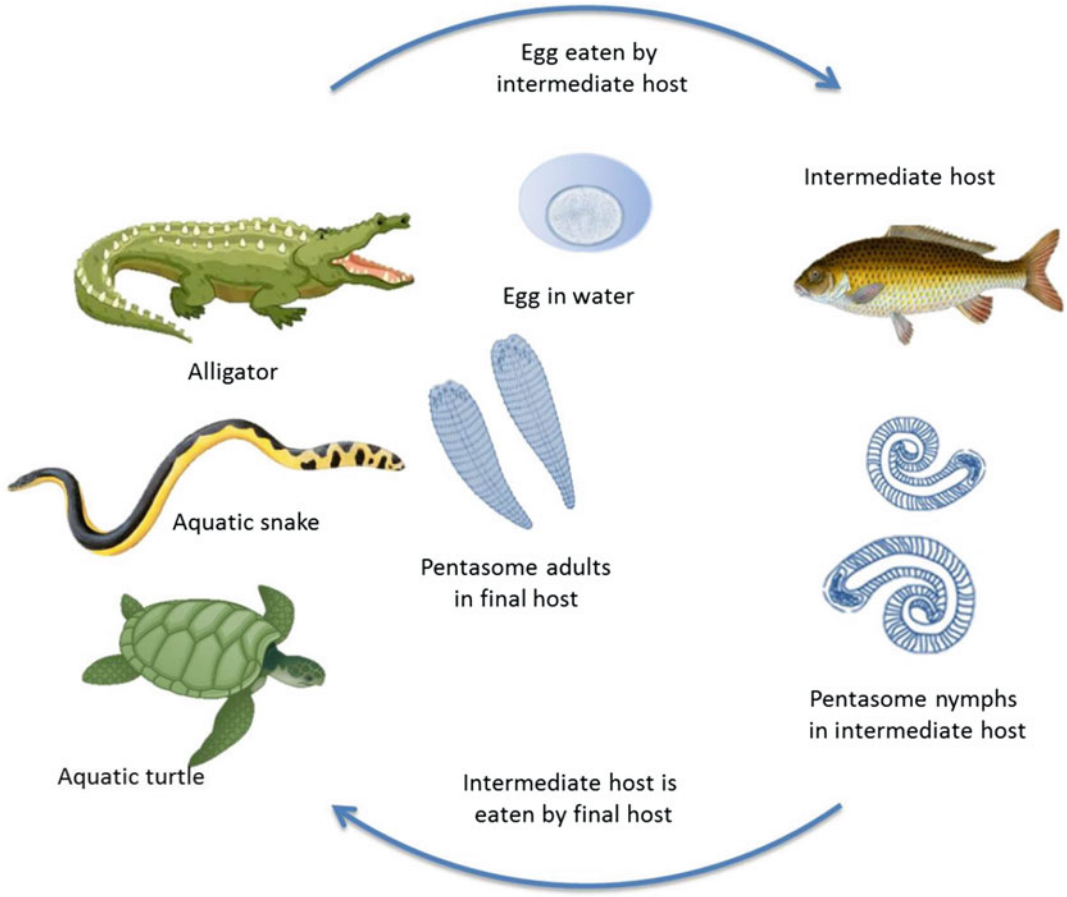

Fig. 3 Life cycle of a pentastome

Immunology

The pentastomes behave like a true parasite by escaping from or downregulating the host immune responses. The encystment of the larva and frontal gland secretions coating the cuticle possibly hinder exposure of the antigenic epitopes to the host immune system. The literature in immunological experiment in human infection is scarce. However, autopsy studies indicate granulomatous tissue response with foreign body giant cells can occur after the parasite disintegrates. A possibility of delayed hypersensitivity exists. The death of larvae in humans could be due to an immune response; nevertheless, a scientific explanation is lacking.

Infection in Humans

Visceral pentastomiasis and nasopharyngeal pentastomiasis are two forms of pentastomiasis in humans.

Visceral pentastomiasis, acquired by ingestion of pentastome eggs, is usually asymptomatic. In symptomatic infections, the condition has protean manifestations. Peritonitis, pneumonitis, lung collapse, meningitis, nephritis and pericarditis have been documented. The condition involving the eye causes iritis, subluxation of the lens, secondary glaucoma, conjunctivitis and lacrimal canaliculitis. Rarely, the condition may present with acute or chronic non-specific abdominal symptoms.

Table 1 Distribution of the major pentastomes causing human infections

Species	Major distribution	Definitive host	Intermediate host
Armillifer armillatus	West and Central Africa, the Arabian peninsula, Malaysia	Python	Rodent, monkey
Linguatula serrata	Cosmopolitan; predominantly the Middle East	Dog, wolf	Ruminants
Armillifer moniliformis	Southeast Asia	Cobra	Rodent, monkey
Armillifer grandis	Central Africa	Python	Rodent, monkey
Porocephalus crotali	North, Central and South America	Rattlesnake	Rodent, monkey

The nasopharyngeal pentastomiasis is caused by ingestion of the nymph of *L. serrata*. The larvae travel to the nasopharynx to induce paroxysmal cough, discomfort in nose and throat, sneezing, dysphagia and vomiting. Asphyxia, Eustachian tube congestion and aural pruritus may also occur.

Cases of creeping subcutaneous human infection with *R. hemidactyli* have been reported in the Southeast Asian tribes who eat live lizards as a folk remedy for respiratory disease. A pruritic serpiginous burrow over a patient's abdomen, caused by *Sebakia* sp., has been reported.

Infection in Animals

Visceral linguatulosis caused by *Linguatula* sp. occurs in sheep, cattle and rodents. Usually, animal infection is asymptomatic. Diarrhoea, reluctance to eat or stand and gradual emaciation, occurs in symptomatic infections. Adult *Linguatula* worms appear in the tongue, nasal passage, frontal sinus and tympanic cavities of dogs. True autoinfection also occurs in snakes.

Epidemiology and Public Health

The majority of human infections are caused primarily by *A. armillatus* and distributed mainly in the West and Central Africa (Table 1). *A. armillatus* infection in African immigrants in America and Europe has been documented. *A. moniliformis* is the commonest isolate from human cases in Malaysia. *A. grandis* caused a large series of human ocular infections from the Sankuru district of the Democratic Republic of Congo. *P. crotali* cases have been reported mainly from the Americas though they occur worldwide. Pentastomiasis due to *Sebakia* sp. has been reported from Costa Rica.

However, a precise estimate of prevalence is unattainable owing to the asymptomatic and self-limiting nature of the disease. Wide discordances among the prevalence rates obtained by serological, radiological and post-mortem surveys have been noted. Radiological prevalence rates in Nigeria and Congo are much lower than the seroprevalence statistics at Ivory Coast (less than 1.5% versus 4.2%). On the other hand, autopsy series pop up with much higher prevalence ranging between 22.5% (in Congo) and 33% (in Nigeria). A high prevalence rate of 45.5% was observed in West Malaysia from consecutive 30 autopsies on aborigines. Autopsy prevalence statistics from Cameroon also range between 7.8 and 12.6%. These differences reflect possibly the failure of imaging methods to detect non-calcified nymphs and low sensitivity of the serological tools compared to the large scope of detection by the autopsy.

Canine infection with *Linguatula* sp. has numerous public health implications. Prevalence among the canine population from Nigeria is more than 35%. A study of the slaughtered dogs from the dog markets in Nigeria demonstrated a high prevalence of 48.26% with the puppies being affected the most (55.45%). The people in impoverished rural and semi-urban areas commonly consume dog viscera propagating the disease.

Snakes, especially their meat and the bile, have been in use for animalistic rituals and medicinal purpose. Tropical snake farming, python totemism and a shift from the bushmeat to the reptile meat create an economy-driven transmission opportunity. China reports very few cases of pentastomiasis mostly caused by *Porocephalus taiwana* and *Armillifer agkistrodontis*. *A. moniliformis* has also been detected in Cynomolgus monkeys, cockroaches and wild rats. The house geckos and lizards have been shown to harbour *Railietiella* sp. The estimated prevalence rate varies from 1.8% to 20.7% in Malaysian wild animals.

Diagnosis

Various diagnostic methods are available for diagnosis of pentastomiasis (Table 2).

Microscopy

Histopathology is frequently helpful. Characteristic sclerotized cuticular openings are often demonstrated in the tissue sections stained by haematoxylin and eosin. Movat's pentachrome stain of the tissue section appears to be better. The presence of the cuticular spines distinguishes *L. serrata* larvae from *Armillifer* sp. The presence of the striated muscle fibres differentiates these from those of cysticercus, spargana and nematodes but not from the fly larva. The latter is distinct by the presence of the tracheal tracts and no cuticular sclerotized opening.

Microscopy of an intact larva, if available, is highly useful for species identification of the pentastome. A lower number (7–9) of annulations suggests *A. agkistrodontis,* whereas relatively large numbers of (18–22) annulations suggest *A. armillatus*. Positions of the genital pore and annulations are helpful features in diagnosing adult worms.

Serodiagnosis

Various antibody-based tests such as gel diffusion, immunoelectrophoresis, immunofluorescence, ELISA and Western blot are used in serodiagnosis of pentastomiasis both in humans and in animals. Most of these tests use a crude antigen from canine omentum containing pentastome larvae. An ELISA method using a 48 kDa frontal gland metalloproteinase has shown increased efficacy. The 97 kDa and 37 kDa bands on Western blot with *L. serrata* help in diagnosis.

In animals, a sandwich ELISA for detecting antibodies against excretory/secretory antigens of *L. serrata* showed excellent sensitivity compared to microscopy in animals.

Antigen-based methods are yet to be evaluated.

Molecular Diagnosis

Polymerase chain reaction (PCR) and DNA sequencing have been highly useful for molecular diagnosis. The tests differentiate *A. agkistrodontis*, *A. armillatus* and few other species confidently. BLAST analysis following the sequencing of a 424 bp amplicon is the usual procedure. However, high cost and limited access prohibit their routine use. Necrosis and formalin fixation of tissue hamper nucleic acid detection by PCR.

Other Methods

Crescentic or coiled opacities on lung radiology (Fig. 4), liver imaging (Fig. 5) or discovery during laparotomy or autopsy are frequently used methods in the diagnosis of the condition.

Table 2 Diagnostic methods for pentastomiasis

Diagnostic approaches	Sample	Methods	Targets	Comment
Microscopy	Biopsy/ autopsy/ parasite	Histology H&E stain Movat's pentachrome stain	Sclerotized cuticular openings, cuticular spines, striated muscle layer, annulations, calcified circumoral hooks	Diagnostic; morphologic speciation
Antibody detection	Serum	Immunofluorescence Gel diffusion ELISA Western blot	Antibody against crude antigen, frontal gland 48 kDa metalloproteinase	Poor sensitivity
		Sandwich ELISA	Antibody against *Linguatula serrata* excretory/secretory antigen	Good sensitivity in goats and dogs
Molecular	Biopsy/ autopsy/ worm extract	PCR DNA sequencing	mtDNA	Mainly research purpose; high cost and limited access; does not cover all species

Treatment

The asymptomatic nature and eventual death of the parasite limit the opportunity and requirement of treatment in human visceral pentastomiasis.

Acute abdominal cases in human visceral pentastomiasis are treated by exploratory laparotomy and peritoneal lavage to diagnose and remove the larvae. In a few cases, as many as 100 *A. armillatus* nymphs were removed by laparotomy. *Halzoun/marrara syndrome* requires

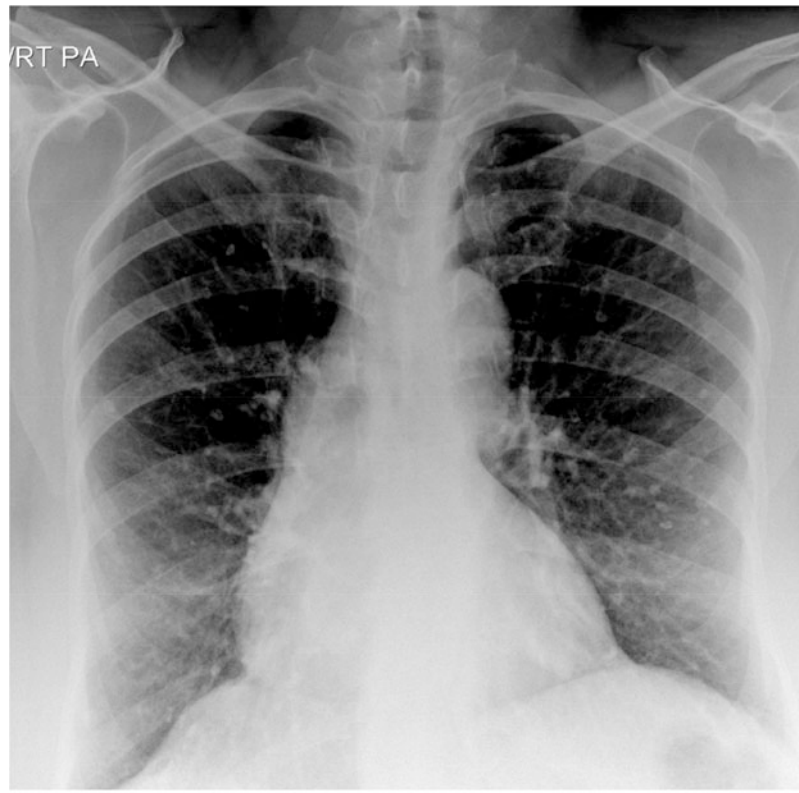

Fig. 4 Chest radiograph showing multiple tiny C-shaped opacities in the lungs in a visceral pentastomiasis case (image reproduced from BJR Case Rep 2019; 5: 20180058; CC by 4.0)

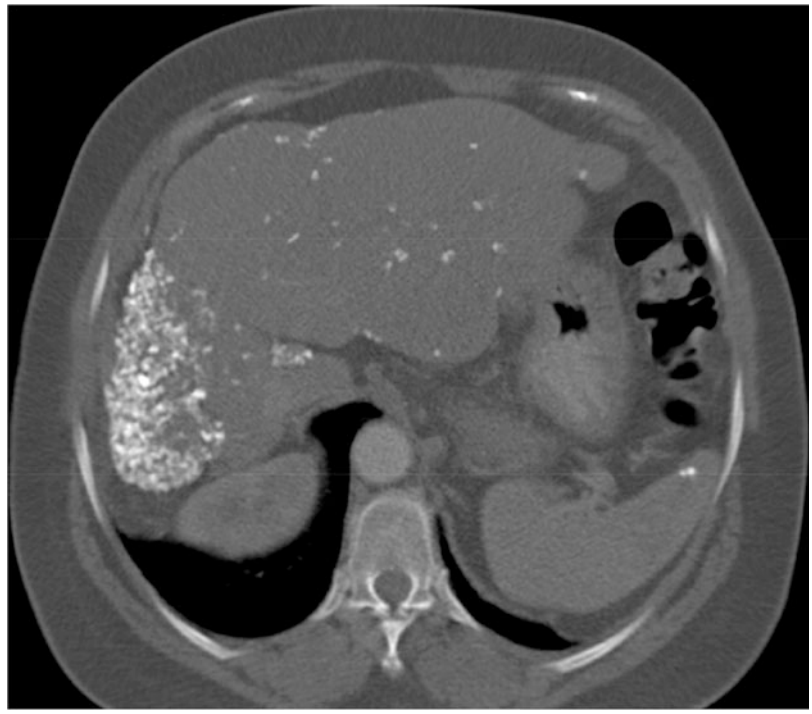

Fig. 5 Multifocal calcification in the liver and spleen in a visceral pentastomiasis case (image reproduced from BJR Case Rep 2019; 5: 20180058; CC by 4.0)

surgical removal of free or encysted parasites in eye, throat or nasal passage.

No validated medical treatment is available for visceral pentastomiasis. Medical therapy aims to kill and remove dead worm excreted out in the stool. Monotherapy and combination therapy with praziquantel, albendazole and mebendazole have been in practice along with Chinese traditional medicines. Diethylcarbamazine has been suggested for the treatment of infections by *Linguatula* sp. Associated allergic manifestations respond to therapy by antihistaminic and/or corticosteroids. Ivermectin has shown some curative effects in pentastome infections in animals such as snakes and captive lizards.

Prevention and Control

Since snake dropping and contaminated food and water are important sources of infection, food and water hygiene is necessary to prevent the infection. Cautious and hygienic handling of contaminated snake products such as their muscle and bile and avoiding their consumption are essential preventive measures. Avoidance of eating uncooked meat (dog, rodent and sheep) and avoidance of contact with infected reptiles needs are important personal preventive measures. Screening of canine population helps in controlling *Linguatula* sp. infection in these animals.

Case Study

A 50-year-old man comes to you with acute abdominal pain and vomiting. He was having constipation for the last 7 days. He is a professional snake hunter and avidly takes folk medicines. Clinical examination shows marked epigastric tenderness. You ordered a straight X-ray abdomen, which surprisingly showed numerous tiny C-shaped opacities in the abdomen with a collection of gas under the diaphragm. You decided for an emergency laparotomy.

1. What could be those opacities?
2. Are the opacities causing the problem in this patient?

3. What could be the possible findings during this laparotomy?
4. How would you proceed further?

Research Questions

1. What is the mechanism of the pentastome larval death in humans?
2. What are the immunological aspects of pentastomiasis particularly the immune evasion strategies and the indiscernible host response?
3. What should be the therapeutic approach need elucidation in relation to one health concept?

Further Readings

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Scabies

Sumeeta Khurana and Bhavana Yadav

Learning Objectives

1. To review the spectrum of various clinical forms of scabies.
2. To evaluate the diagnostic modalities for diagnosis.

Introduction

Scabies is a skin condition of humans and animals caused by the mite, *Sarcoptes scabiei,* which remains a significant public health concern worldwide regardless of socio-economic status. Scabies was declared as a neglected tropical disease by WHO in 2009 and associated with significant morbidity in humans and mammals. An intolerable pruritic rash characterizes typical scabies. The most severe form (crusted/Norwegian) of scabies has become a critical re-emerging ectoparasitosis in the developed nations, especially among vagrants, institutionalized older population, individuals with intellectual disability and immunocompromised individuals. Infested individuals require prompt management because a misdiagnosis can lead to outbreaks, morbidity and an increased economic burden.

History

The earliest recorded reference to scabies either in humans or in animals dates back to Biblical times (1200 BCE). Later, Aristotle reported that "*lice*" would "escape from little pimples if they are pricked"; researchers believed it to be about scabies mite instead of lice. Based on archaeological evidence and Egyptian hieroglyphics, scabies was present for the past 2500 years. From the time of the Romans and Greeks, it was known as gale or itch and well described in areas of filth and poverty. Celsus, a Roman physician, coined the term "*scabies*" to describe this disease. Although various civilizations over several eras have recognized scabies, cause of the infection remained a mystery.

S. scabiei mite derived its name from the Greek word "*sarx*" (flesh) and "*koptein*" (to cut or to smite) and the Latin word "*scabere*" (to scratch). Giovanni Cosimo Bonomo discovered scabies mite in 1687 as the cause of the "*itch*", and scabies became the first disease of humankind with a recognized cause. Bonomo documented the scabies aetiology in his famous letter entitled, "*Observations concerning the flesh worms of the human body*", written to his mentor Francesco Redi, the naturalist. Bonomo, with the help of Giacinto Cestoni, described the behaviour

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S. C. Parija, A. Chaudhury (eds.), *Textbook of Parasitic Zoonoses*, Microbial Zoonoses,
https://doi.org/10.1007/978-981-16-7204-0_59

of mite and its treatment principles. Despite such convincing evidence and a translation of the text into English 16 years later, the discovery remained forgotten for around the next 200 years. With the publication of the treatise by Hebra in 1868, it was universally accepted in the nineteenth century. In the 1940s, during World War II, Kenneth Mellanby, an entomologist, studied the transmission and treatment of scabies.

Taxonomy

The genus *Sarcoptes* belongs to family Sarcoptidae; order Astigmata; subclass Acari; class Arachnida; phylum Arthropoda; and kingdom Animalia.

S. scabiei is the species that causes infections in both humans and animals. Different biological forms of *S. scabiei,* which are morphologically indistinguishable but are host-specific and have differences at physiological level, are given in Table 1.

Genomics and Proteomics

Genomic data have provided some insights into the host preference and phylogenetic relationships of scabies mites. An annotated draft genome of *S. scabiei* var. *canis* is available in the Vector Base database and NCBI, while the var. *hominis* and var. *suis* genomic data are available in the GigaScience repositories and NCBI. The var. *canis* and var. *hominis* genome contains 10,644 and 13,226 putative coding sequences, respectively.

The proteins associated with essential biological processes of mite are studied from information obtained from genomic data. More than 150 proteins present in extracts of mite have been classified using MALDI-TOF/TOF mass spectrometry using the predicted proteome software packages. In the blood of patients with scabies, all of these proteins are detected by antibodies, either IgM or IgG. Other proteins do not bind to the circulating antibody and are believed to be involved in the immune system regulation of the host to protect the mite.

Morphology

Mite has four stages in its life cycle: adult, egg, larva and nymph.

Adult

S. scabiei is a tiny mite, barely visible to the naked eye. The females are 0.3–0.5 mm long and 0.25–0.4 mm in width. The male is smaller, about two third the size of female. The colour of mite varies from creamy white to yellowish with brown sclerotized legs and mouthparts. The mite possesses a thin cuticle without heavily sclerotized shields, lacks stigmata and tracheae and respires directly through the tegument. The body is broad, oval-shaped with fine striations on the flattened ventral surface and dorsally convex tortoise-like surface. The demarcation into cephalothorax and abdomen is indistinct (Fig. 1).

These mites lack the eyes and possess four pairs of short legs, two pairs on front and two at the back. The front two pairs of legs end in bell-shaped sucker-like structures called pulvilli, which is used for attachment to skin. The hind two pairs of legs are not visible in the dorsal view. The third pair in males and third and fourth pairs in female mites end in long bristle-like sensory structures called setae.

The average life span of the mites is around 30–60 days.

Egg

The eggs laid by the adult mite are oval and about 0.10–0.15 mm long. These hatch out into six-legged larvae and then moult progressively into eight-legged nymphs and then adult mites. It takes about 14 days for an egg to develop into adult mite.

Table 1 *Sarcoptes* species of humans and animals

Species	Host
Sarcoptes scabiei var. *hominis*	Humans
Sarcoptes scabiei var. *canis*	Dogs, cats, pigs, foxes, rabbits
Sarcoptes scabiei var. *suis*	Pigs, dogs, rabbits
Sarcoptes scabiei var. *bovis*	Cattle
Sarcoptes scabiei var. *equi*	Horses
Sarcoptes scabiei var. *ovis*	Sheep, goats, camels
Sarcoptes scabiei var. *caprae*	Goats, cattle, sheep, dogs

Larva

The larva is a six-legged structure emerging out of eggs that increases in size and metamorphosis into a eight-legged nymph.

Nymph

The nymph is essentially the smaller version of adult mite.

Cultivation of Parasites

In vitro culture of *S. scabiei* is not yet possible. Mites required for the immunological and morphological studies are obtained from the cutaneous scabies lesions of man and animals.

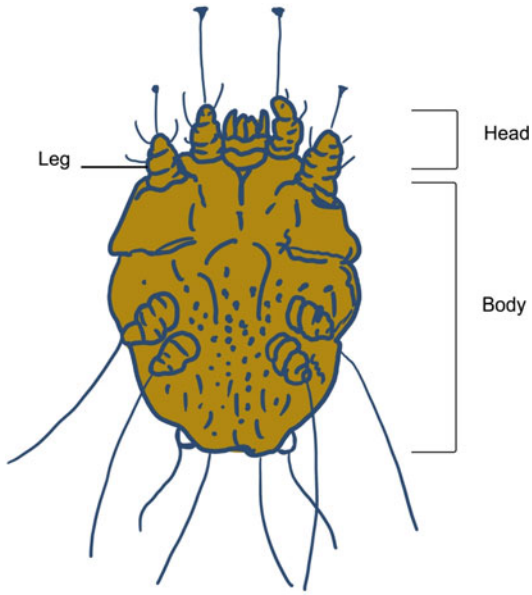

Fig. 1 Schematic diagram of ventral view of adult *Sarcoptes scabiei* mite

Laboratory Animals

S. scabiei is a highly host-specific parasite and produces only transient, a self-limiting lesion in its non-preferred host. No method has been established so far to maintain the viability of mite in the laboratory for more than 24–48 h. Moreover, it is tough to collect mites from humans in large numbers, as a typical form of human scabies involves 10–15 mites per person. Ample numbers of mites for research purposes are obtained from a crusted scabies patient but access to such hyper-infested individuals remains sporadic. Thus, various immunological, host–parasite interaction, biological, proteomic and genomic research depends on the animal model of scabies mites.

Laboratory animals such as rabbits or pigs are used to study various properties of mite populations, including host–parasite interactions and immune-modulating ability. In cross-infectivity studies (dog mites on rabbits), *S. scabiei* var. *canis* whole mite antigen extracts were used to study the immunopathogenesis of scabies on mice, rabbits and humans. A rabbit/canine model is extremely valuable to study the immunological perspective of scabies.

However, the majority of the trans-species studies remained unsuccessful due to transient and self-limiting infestations along with the logistical issues of access for international research and regulatory restrictions.

In various studies, experimental infections were induced by encrusted lesions obtained from the ears of chronically infested pigs. Pigs

have been used as an animal model to study scabies. They are the natural host of *S. scabiei* var. *suis* and develop epidermal, morphological, biochemical and immunological changes similar to humans. The clinical manifestations of scabies in pigs closely resemble human crusted (Norwegian) scabies. Moreover, complement system of a pig is comparable to humans. The porcine model consistently provides heavy mite burden (>6000 mites/g skin) and prolonged infestation period (6–12 months) on treatment with the immune-suppressive drugs.

Life Cycle of *Sarcoptes scabiei*

S. scabiei is an obligate parasite to humans.

Its life cycle consists of four developmental stages: egg, larva, nymph (protonymph and tritonymph) and adult—the adult mite gets transferred to a person's skin by dermal contact or via fomites.

Transmission of Infection

The most prevalent form of transmission of scabies is direct skin contact from one person to another. It is common among family members and seen in institutional environments. It can be spread through sexual contact as well. In the case of typical scabies, the fomites play a minor role in disease transmission. However, in crusted scabies, the inanimate environment of patients/residents has been shown to be heavily contaminated with mites and contribute to disease transmission. In patients with crusted scabies, mites can be found on bedsheets, curtains, beds and clothing. The risk of infection is related to the burden of the mite on the person infected and the length of contact.

The fertilized female mite burrows preferentially into superficial layers of the skin, depositing 2–3 eggs daily during her life period of 4–6 weeks. The burrows appear as tiny greyish or skin-coloured raised serpentine lines. It takes 3–4 days for the hatching of eggs into larva. The larvae migrate to the surface of the skin and create new burrows. Around 3 days later, they leave the burrows and moult to the next stage of development, i.e. nymphs. The nymphs either migrate to the skin surface or remain below the skin surface, where in the next 3–4 days, they moult into adults. The egg develops through larva and nymph and becomes fully adult mite in 10–15 days. The adults are small with a length of 0.3–0.4 mm in females and 0.25–0.35 mm in males. The life span of man is usually 1–2 days, and it spends this time in search of unmated females. Mating occurs only once, and the female mite lays eggs for the rest of her life. The entire cycle takes about 2 weeks (Fig. 2).

The eggs are laid by the female at a rate of about two to three eggs a day for about 2 months. While a female lays around 180 eggs in her lifetime, only 10% of eggs usually survive and give rise to mature mites. Bathing, scratching or rubbing the skin eliminates much of the eggs from the skin. It is estimated that 3–50 mites can reside in a single human host.

Mites survive for many days off the host. When the mite falls off from its host, it lives at room temperature for 24–36 h with average humidity (21 °C and 40–80% relative humidity) and much longer with high humidity at lower temperatures. The capacity of the mites to infest the host decreases with increased time away from the host.

Pathogenesis and Pathology

In the epidermis, the female mite burrows and deposits eggs for many days and causes different pathological changes in the skin of humans and mammals. Female mites, guided by the host odour and thermal stimulus, travel 2.5 cm/min. The burrow containing mite body parts appears as a cleft in the upper epidermis. Acanthosis, parakeratosis, spongiosis and thick eosinophilic dermal infiltrates are found in the epidermis. In the dermis, there is diffuse infiltration of lymphocytes and histiocytes, sometimes accompanied by neutrophils and eosinophils. Dermal infiltrate can be profusely dense in nodular skin lesions.

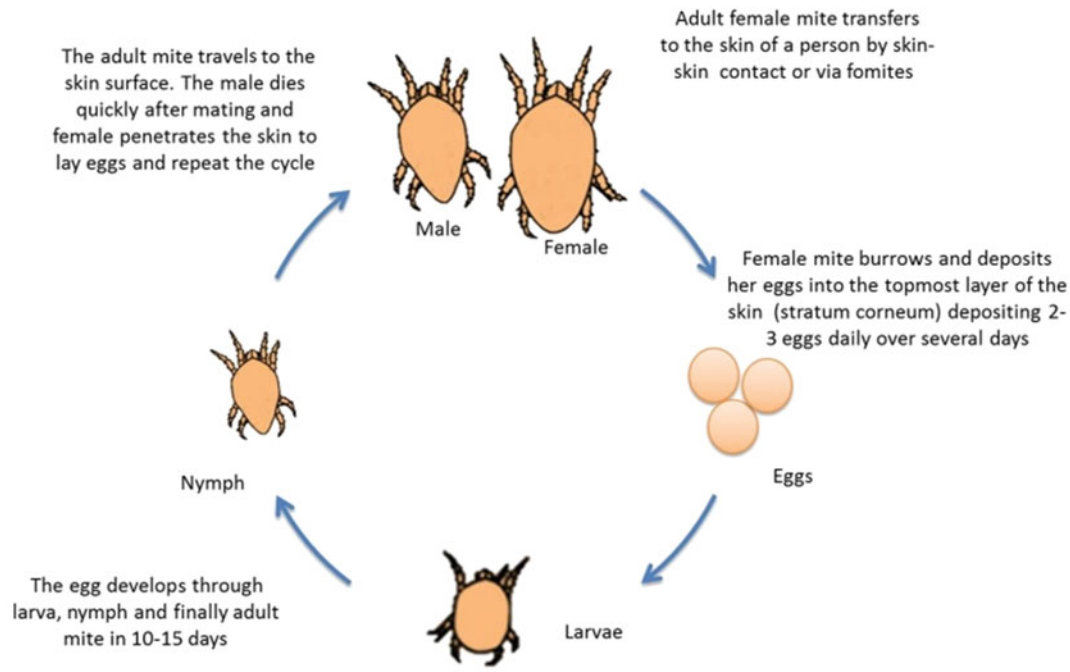

Fig. 2 Life cycle of *Sarcoptes scabiei* mite

Immunology

The immune responses to scabies are complex. Scabies has previously been described as the "*worst itch*" in patient's life, highlighting the significant scratching of this disorder. Scabies pruritus can occur either due to the scabies mite's overt action or because of the host's immune response against it.

Both the Th1 and Th2 immune responses are observed in classical scabies. A primarily Th2 (non-protective) reaction is, however, present in crusted scabies. The immune responses of Th1 are regulated by CD4+ and CD8+ T cells that secrete TNF-a, IFN-γ and IL-2 cytokines. To combat extracellular parasites, Th2 cells secrete various interleukins including IL-4, IL-5 and IL-13, and regulate humoral immunity by enhancing antibody production. The Th2 response stimulates eosinophil development and activation along with downregulation of cell-mediated immunity. Cytokine signalling, in particular IL-23, IL-6, IL-1 β, IL-18 and TGF-β, stimulates the Th17 cellular response and IL-17 secretion. Due to a reduction in CD4+ T cells, immunocompromised patients (HIV) are associated with severe disease. Ageing and immunosuppressive conditions like AIDS are associated with a reduction in the activity of Th1 cells and an increased frequency and severity of disease.

Scabies mite infestation initiates potential humoral immune responses, especially in crusted scabies, by upregulating the IgG, IgE and IgA specific antigens. Levels of IgG, IgE, IgA and IgM are reported to be elevated in typical scabies. The C3a and C4a complement fragments function on particular receptors and cause the release of mediators (histamine and TNF-alpha) by mast cells that contribute to the inflammatory response. In crusted scabies, low circulating serum levels of complement C3 and C4 were identified, indicating possible complement function defects. Scabies mite-induced protease paralogues (SMIPPs) and serpins (SMSs) have also been documented to inhibit complement activation and promote in vitro bacterial development, possibly shielding mites from complement-mediated

destruction. In animals, sarcoptic mange results in comparatively late inflammatory and adaptive immune responses during infection, typically 4–6 weeks after initial mite contact.

Infection in Humans

Classic Scabies

It is present in the form of pruritus, which is often stated to be more extreme at night. It is documented that the clinical appearance of a primary infestation of scabies occurs 4–6 weeks after diagnosis (Fig. 3). Usually, two types of skin rash are observed: (1) papular or vesicular lesions consistent with the position or proximity of burrows and (2) a more generalized itchy papular eruption unrelated to apparent mite behaviour that is believed to be an immunological reaction. A burrow is the classical feature of scabies. Within the burrow, egg cases and mite faecal balls, or scybala, are present. Unfortunately, for an unaided eye, these burrows are barely apparent and are frequently absent. Interdigital web spaces, wrists and limbs, anterior axillary folds, periumbilical muscle, pelvic girdle, including buttocks, knees, male penis and female nipples, are the places of predilection of the human mite.

Pyoderma is frequently linked with untreated scabies, mainly due to secondary Group A *Streptococcus* (GAS) and *Staphylococcus aureus* infections. Cellulitis, invasive bacterial infections and acute post-streptococcal glomerulonephritis (APSGN) are more severe sequelae.

In infants and very young children, the body areas that are classically involved are the head, neck, palms, feet and body folds—scabies in older adults manifests as severe pruritus with a barely noticeable inflammatory reaction. In bedridden patients, the back is usually involved.

Bullous Scabies

It is a rare subtype of classic scabies, with few reported cases till now. The highly pruritic bullous lesions most commonly cluster on the trunk and extremities but may involve the neck, genitals, feet, thighs and inguinal folds. Risk factors include male sex and adults older than 65 years. It can mimic bullous pemphigoid clinically and immuno-pathologically.

Nodular Scabies

The strongly pruritic, dark pink to brown, solid nodules are seen in nodular scabies. They present most commonly around the groins, on penis or scrotum, on the buttocks, on the axillary anterior fold and around the navel. Despite effective treatment of the disease, nodules survive for months after treatment.

Crusted (Norwegian Scabies)

Following the disease's description by Boeck and Danielssen in leprosy patients in Norway in 1848, crusted scabies was referred as Norwegian scabies. The disease is characterized by the formation of hyperkeratotic crusts on the face, head, ears, throat, feet, nail beds or nail plates with substantial involvement. This type of scabies is extremely infectious since vast amounts of mites are trapped in the thick crusts (up to two million) and are quickly shed from the infected skin in scales and flakes. People of crusted scabies were known as "*core-transmitters*". The aetiology of crusted scabies is commonly due to either iatrogenic or serious diseases such as advanced HIV and human T-cell type 1 leukaemia virus infection, lymphoma and immunosuppression. The formation of fissures and secondary bacterial infections by *S. aureus* and *Streptococcus pyogenes* is widespread and is associated with elevated mortality rates for this type of disease. Generalized lymphadenopathy can be present, and a prominent finding is peripheral blood eosinophilia.

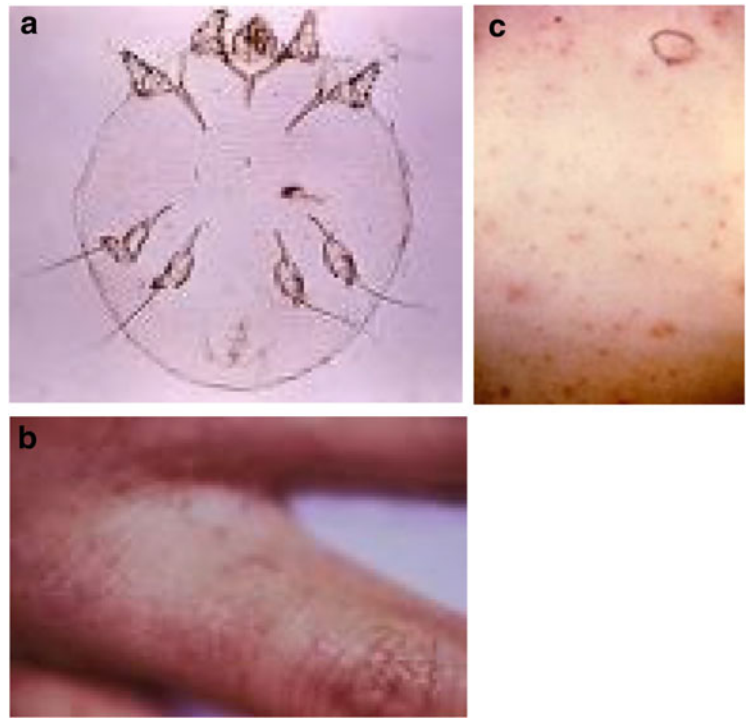

Fig. 3 Clinical manifestations of *Sarcoptes scabiei* mite. (**a**) Ventral view of *Sarcoptes scabiei* var. *hominis* Source: https://phil.cdc.gov/Details.aspx?pid=6301, (**b**) Pimple-like rash in the web between the fingers of hand, due to the human itch mite, *Sarcoptes scabiei* var. *hominis* *Source:* https://phil.cdc.gov/Details.aspx?pid=4801 (**c**) Skin rash in a human being, caused by the mites associated with a case of canine scabies, *Sarcoptes scabiei* var. *canis*, otherwise known as sarcoptic mange. Source: https://phil.cdc.gov/Details.aspx?pid=3972

Infection in Animals

S. scabiei mite produces *sarcoptic mange* in domestic and wild animals.

In mammals, the clinical symptoms of mange present as slightly elevated red papules seen on the body's sparsely haired areas. The sarcoptic mite burrows deep into the epidermis, causing skin inflammation, and intense pruritus with consequential scratching leading to excoriation. In advance cases, the death of animals occurs secondary to infection, dehydration and impaired thermoregulation. Alopecia, scaling and crusting of the skin with dry serum exudate are found when mange is left untreated.

Distribution of lesions varies with host species, but common sites are legs (dogs), inner thighs (cattle), neck (horses, cattle), muzzle, ears and face (cats, dogs, sheep, goats, pigs), trunk (pigs) and tail (dogs, cattle). The transfer of mites between a group of animals is most likely by direct contact or by infected bedding.

Epidemiology and Public Health

Scabies is one of the commonest dermatological conditions, which can affect individuals of any age and socio-economic status. The condition is included in the list of NTDs of the World Health Organization. The global prevalence is estimated to be 200 million people, according to WHO, while more effort is needed to determine the burden of the disease. Estimates of prevalence vary from 0.2% to 71% in the scabies-related literature. The highest prevalence is seen in East and Southeast Asia, Oceania and Latin America. This is confirmed by a high prevalence of scabies in humid, tropical regions where overcrowding encourages the rapid spread of mite scabies. In 2015, it was estimated that the scabies contributes to about 0.21% disability-adjusted life-years (DALYs) (Table 2).

Prevalence of scabies is found to be related to poverty, crowding and hygiene. Poverty and overcrowding are key influences, and it is suspected that overcrowding has a substantial effect on the dissemination of scabies.

Table 2 Epidemiology of *Sarcoptes scabiei* of importance in humans

Species	Distribution	Host
Sarcoptes scabiei var. *hominis*	Estimated global prevalence is approx. 200 million The highest prevalence is seen in eastern Asia, Southeast Asia, Oceania and tropical Latin America Higher prevalence is associated with poverty, crowding and poor hygiene	Humans

Malnutrition can predispose people to scabies. It is a significant health concern in many developing countries. The most vulnerable groups in resource-poor communities are young children and the elderly. In industrialized countries, scabies outbreaks may occur as sporadic cases or as institutional outbreaks in schools, nursing homes, hospitals, prisons, retirement homes and areas of overcrowding.

Increased personal hygiene only postpones the onset of symptoms and reduces their severity. Swimming may have an adverse effect on treatment effectiveness when drugs have been washed off rather than affecting scabies themselves.

Scabies is an important infection in populations of wild canids, ungulates, boars, wombats, koalas, cats, large apes and bovids. It is an epizootic disease. It is suggested to be a significant cause of mortality among red foxes (*Vulpes vulpes*), coyotes and common wombats (*Vombatus ursinus*). Fifty to 95% of pig populations are infested with *S. scabiei* mite worldwide. The scabies mite is considered a ubiquitous parasite among some economically important livestock with reports documented from numerous countries including Europe, Australia, Africa and Asia.

Extreme mange-related pruritus in animals interferes with milk production, leather quality and weight gain and can cause severe economic losses on primary industries. In addition, the mite has the potential to disperse quickly in highly vulnerable species, limit reproduction and cause mass mortality events.

Diagnosis

Scabies is suspected clinically based on a standard history of pruritus that worsens at night, and the spread of inflammatory rash in characteristic areas. Sometimes, more than one member of a family may be involved (Table 3).

There are several invasive and non-invasive approaches for parasitological diagnosis. KOH burrow scratching, skin biopsy, burrow ink test, adhesive tape test, serology and molecular techniques are invasive tests for scabies diagnosis. Dermoscopy, videodermoscopy, reflectance confocal microscopy and optical coherence tomography are non-invasive diagnostic methods.

Microscopy

Skin Scraping

The diagnosis is confirmed by the presence of mites, larvae, eggshell remains or faecal pellets at the end of the burrow. One or two drops of mineral oil, saline or potassium hydroxide may be added to the lesion, and a sterile brush or sterile needle is used to carefully remove the lesion. The specimens are examined under a low-power light microscope. Microscopy is highly specific but insensitive for ordinary scabies, due to the low mite burden. The number of places sampled and/or frequent scrapings, and the expertise of the microscopist are the other factors that may affect the sensitivity of scrapping.

Skin Biopsy

The burrow made by the mite is seen inside the horny layer during the histopathological inspection. The end of the burrow enters the Malpighian skin layer, where a rounded body can be seen as the female mite. Larvae, eggs and faecal deposits containing eggs within the stratum corneum are suggestive of scabies. Histopathological findings

Table 3 Diagnostic methods for *Sarcoptes scabiei* infections in humans

Diagnostic approaches	Methods	Targets	Remarks
Direct microscopy	Skin scraping/skin biopsy, burrow ink test, scotch tape test	Visualization of mites, larvae, eggshell remains or faecal pellets	*Drawback*: The number of scrapings, and the expertise of the microscopist may affect sensitivity
Dermoscopy/ videodermoscopy/ reflectance confocal microscopy (RCM)	Examination of skin containing mite in burrows	Mite head and trailing burrow can be visualized in the "*delta-wing jet*" sign	It is a rapid non-invasive technique that can be used to screen the asymptomatic contacts and family members and to monitor the response to therapy
Immunodiagnosis	ELISA or intradermal skin test	Mite antigens	Cross-reactions to house dust mite
Molecular assays	PCR	(cox1) mitochondrial cytochrome c oxidase enzyme	Still in research settings only

of scabies are spongiosis, hyper-granulosis, epidermal tunnels (burrows) and perivascular dermal infiltrate. Node biopsy reveals a thick chronic inflammatory infiltrate that may be pseudolymphomatous. Thickened horny layers are seen in crusted scabies with heavy mite burden.

Burrow Ink Test (BIT)

The suspicious area is scrubbed with ink and wiped with an alcohol swab. A characteristic "*zigzag or S pattern*" of the burrow may be seen in scabies by the naked eye.

Adhesive Tape Test

Transparent adhesive tape is sliced into smaller strips and then tightly applied to the lesion and moved directly to a slide for microscopic inspection. Although the technique is quick, its sensitivity is low.

Dermoscopy

It is considered a sensitive and useful method for diagnosing scabies with reasonable sensitivity and specificity. On 10x magnification, the mite head and trailing burrow can be visualized in the "*delta-wing jet*" sign. Another sign known as "*Wake sign*" is specific for scabies, and it points to the location of mite and its product. In crusted scabies, dermoscopy reveals a hyperkeratotic appearance with many burrows. However, it has a low sensitivity and specificity in mild infestation. Therefore, when videodermoscopy is not possible or for the screening of suspected lesions prior to scraping, dermoscopy can be used.

Videodermoscopy (VD)

It is a rapid non-invasive technique that allows the concurrent examination of dermoscopic features of scabies at higher magnification. At 400× magnification, it shows a clear evidence of burrows containing the mite. Moreover, the eggs and the faeces of the mite are also seen. At the end of a linear fragment (burrow containing eggs or faeces), VD shows a dark brown triangular structure (pigmented anterior part of the scabies mite) called "*jet with a contrail*". The precision of VD is equal to scraping, and it is not painful for children. It can be used to screen the asymptomatic contacts and family members and to monitor the response to therapy.

Reflectance Confocal Microscopy (RCM)

RCM is a non-invasive technique that visualizes the epidermis and papillary dermis in vivo at resolutions equivalent to histology using light reflectance of the cellular structure. It allows

visualization of burrows, mite, larvae, eggs and faecal material. It is also used as a tool to detect viability of mite after treatment. The drawback of this technique is non-availability, cost and time-intensiveness (approximately 10 min per lesion).

Optical Coherence Tomography

It is similar, but with greater precision, to ultrasonography. It is possible to classify mites, larvae, urine and burrow contents with this method.

Immunodiagnosis

An enzyme-linked immunosorbent assay (ELISA) to detect antibodies and an intradermal skin test are several other studies that are in progress. Cross-reactivity between scabies antigens and house dust mites has restricted the production of ELISA-based tests against scabies antigens.

Molecular Diagnosis

Direct skin-based evaluation for infection using traditional PCR targeted at the S subunit 1 (cox1) mitochondrial cytochrome c oxidase enzyme coding gene is being used but has yet to prove its usefulness for diagnosis.

Treatment

Patients and their household contacts should be treated even if they are asymptomatic. In addition, the bedding, clothing and surfaces should be decontaminated thoroughly. A hot cycle wash and a hot drying cycle can be used to wash bedsheets and clothes.

Most commonly, 5% permethrin, 2–10% precipitated sulphur, 10–25% benzyl benzoate, 10% crotamiton, 0.5% malathion and 1% lindane are topical scabicides used. Only permethrin or sulphur may be used in infants. The patients should be instructed to apply topical agents from the neck down to the whole body and wipe it clean after 8–14 h, to repeat in 1 week to kill any mites that have survived the first application or those hatched out of eggs. Face and scalp should also be treated in infants and the elderly. Supportive medication to relieve itching involves topical steroids, emollients and antihistamines. If secondary infection is present, antibiotics should be recommended.

Parenteral ivermectin may be given as a single dose in patients at 200 micrograms/kg and then repeated after 7–14 days in order to kill freshly hatched mites. It is effective in handling institutional or group outbreaks, with signs of scabies being quickly diminished. In the treatment of crusted scabies, ivermectin is especially very successful, in conjunction with keratinolytics and topical 5% permethrin. It is contraindicated for use in pregnant women or small children.

Prevention and Control

Avoiding close skin-to-skin contact with contaminated scabies is the only way to prevent scabies. In order to discourage potential re-exposure and re-infestation, all close contacts, including sexual contacts, should be treated even if asymptomatic. It is also necessary to treat people with crusted scabies and their near associates, as this type of scabies is easily spread due to high mite loads.

The recent inclusion of scabies as neglected tropical disease is a promising action and scabies should gain attention in relevant health policy in both developed and developing countries. In order to promote an increase in scabies research, funding would be needed. The focus areas of research in scabies include the development of comprehensive scabies diagnosis testing and better treatment and control methods, particularly in the light of the emerging threat of drug resistance.

Case Study

A 12-year-old boy from a middle-income family was taken to a clinic with history of intense scratching on his body and scrotum over the last

20–21 days. The other members of the family have also endured scratching over the last 4–5 days. The boy's parents had applied Betnovate ointment (0.1% betamethasone valerate) to the body of the patient. It briefly decreased itching for 2–3 days, but it recurred with severe itching. The papular lesions present on the groin of the child became nodular. With slight tenderness, his father developed crusted lesions.

1. What is the differential diagnosis of this case?
2. What investigations should be performed?
3. What treatment should be advocated?
4. What advice for prevention should be given?

Research Questions

1. What are the immunological events in skin and peripheral blood during scabies which may provide insights into immunological therapies?
2. What systematic, accurate and economical diagnostic approach should be developed for scabies that can be accessible in underdeveloped countries?
3. What is the optimal treatment modality?

Further Readings

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Myiasis

Aradhana Singh and Tuhina Banerjee

Learning Objectives

1. To have the knowledge about the occurrence of myiasis in different organ systems.
2. To understand the difficulty in identifying the various genera and species by microscopy since a thorough knowledge about the same is necessary.

Introduction

Myiasis is a skin infection caused by developing larvae of different fly species of the order Diptera. The most common flies causing human infestations include *Cordylobia anthropophaga* and *Dermatobia hominis*. In mammals, the larvae survive on host's tissue, body matter or ingested food material. The series of infestations by the larvae mainly vary depending on body location and relationship with the host. Typically, myiasis is a major problem in animals leading to severe economic loss for livestock industries globally. Frequent human cases have been reported from the rural tropical and sub-tropical regions. Myiasis is among the top five conditions representing 7.3–11% of the cases in tourist-associated skin diseases.

History

Myiasis (noun for Greek word *mya* or fly) was first described by Frederick William Hope in 1840. The term was used for defining the diseases caused by dipterous larvae in contrast to the diseases caused by insect larvae. Although the term myiasis was first used in 1840, the condition has been known since ancient times.

Zumpt in 1965 defined myiasis as 'infestation of the live vertebrate animals feeding on host's tissue, liquid body matters, or ingested food of host'. Bishopp first proposed the anatomical classification, which was later modified by James in 1947. However, Patton found it unsatisfactory and gave an organization system depending on the grading of parasitism shown by the disease-causing fly.

Taxonomy

The flies causing myiasis belongs to family Calliphoridae and Sarcophagidae; superfamilies Muscoidea and Oestroidea; sub-section Calyptratae and Acalyptratae (*Drosophila melanogaster, Piophila casei* species causing human myiasis); sections Schizophora and

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Aschiza (*Eristalis tenax, Megaselia scalaris* species causing human myiasis); infraorder: Muscomorpha (*Hermetia* sp., *Scenopinus* sp. causing human myiasis); sub-orders Brachycera and Nematosera (*Psychoda albipennis, Telmatoscopus albipunctatus* species causing human myiasis); and order Diptera.

Genomics and Proteomics

Despite variations in the morphology of the myiasis causing flies, the chromosome number in them is quite stable at 12, with 5 pairs of large metacentric chromosomes and a single pair of small, heteromorphic sex chromosome. Variations in mitochondrial DNA (mtDNA) and nuclear DNA by restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD), respectively, have been used to differentiate the genetic structure of these flies. Two distinct domains related to conserved and variable sequences in the control region (CR) of mtDNA have been identified. The CRs contain several conserved sequence blocks (CSBs). They are the potential regulatory elements in these flies. These CSBs indicate similarity in the regulatory mechanisms for replication and transcriptional processes in the flies. Duplication in genes has been recently found in their mitochondrial genomes. Much of the genome analysis for species differentiation and for better understanding of evolutionary aspects remains yet to be studied.

The composition of proteins for metabolism and antigenic properties in the larva of the myiasis causing flies depends on the different stages of the larva. Several larval proteins such as arylphorin, larval serum protein (LSP-2), paramyosin, tubulin and tropomyosin are mostly identified in the second stage of larva. Proteins like filamin, fumarase, enolase and heat shock protein (HSP-70) are expressed in advanced stages of the larva. Ribosomal proteins are important in all the biological processes. Upregulation of several genes encoding proteins like nucleotide (ATP/ADP) carriers, respiratory oxidases and ferritin has often been associated with various clinical presentations of myiasis.

Morphology

Different stages of development of myiasis causing flies are depicted in Fig. 1.

Adult

Adult flies are encountered much lesser as compared to the larvae. *Cochliomyia* sp. is distinguished from other myiasis causing flies by its unique blue to blue-green colour with dark longitudinal lines on the thorax. An adult screwworm is double the size of the common housefly.

Eggs

Egg hatching in *D. hominis* within the host is triggered by changes in the ambient temperature.

Infective Larvae

In most of the myiasis causing flies, the larvae (Fig. 2) undergo three stages of development commonly called as *instars*. The shape of the mature maggot of myiasis causing flies is from typical to oval, and the size ranges from 11–25 mm depending upon the species.

Cochliomyia hominivorax maggot has bands of spines encirculating anterior margin of each body segment. *C. anthropophaga* maggot has three curved slits in the spiracles and numerous black spines. *Cordylobia rodhaini* maggot has scattered spines and three sinuous slits in each posterior spiracle. *D. hominis* has spine in rows and pair of flower-like anterior spiracles. Second *instar* of the *C. hominivorax* shows dark coloration of the dorsal tracheal trunk over one-half of their length in the terminal section. Other species

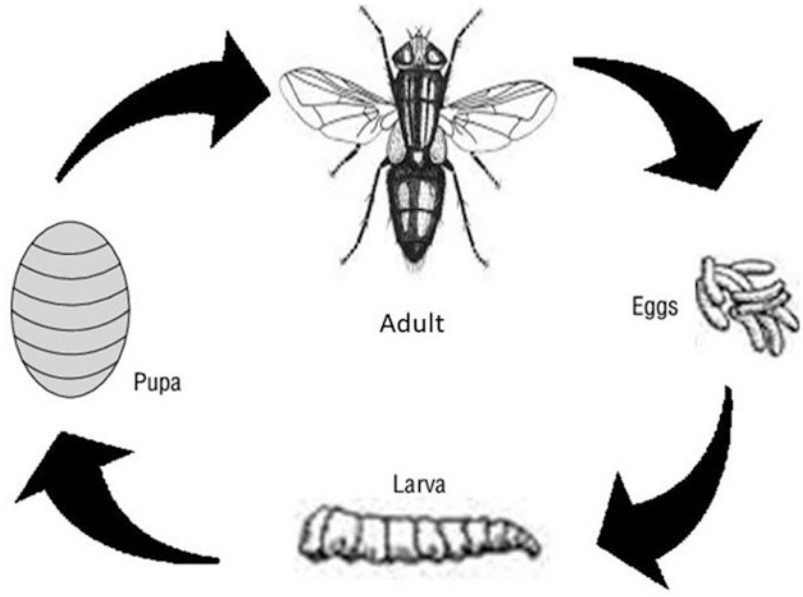

Fig. 1 Different stages of development of myiasis causing flies

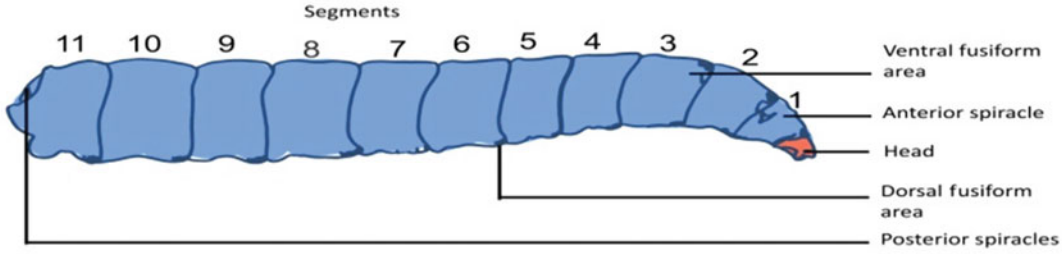

Fig. 2 Schematic diagram of the larva

have less marked pigmentation of dorsal tracheal trunk.

Cultivation of Parasite

In vitro cultivation of different stages of flies causing myiasis is carried out in 'fly box' containers with tightly fitting lids with single-exit points for collection of different stages of larva and pupa of the flies. Food waste and animal excreta are usually kept in the boxes as media for growth and development of the eggs at optimum temperature and humidity. Larva in huge numbers at different stages is harvested from the exit point. The large-scale production of the larva is carried out for pharmacological and industrial uses.

Laboratory Animals

Most of the warm-blooded vertebrates are infested or infected with myiasis causing flies. Therefore, a wide range of laboratory animals are susceptible to myiasis. However, for experimental studies, laboratory-reared mice models including the balb-c mice have been extensively used.

Life Cycle of Myiasis Causing Flies

Hosts

Sheep, goats, humans, horses, donkeys, cattle, reindeer, deer, wild animals and birds are the hosts for the family Oestridae (botflies). The

hosts range from sheep, cattle, humans, dogs to wild animals for the family Calliphoridae (blowflies). Camel, cattle, birds, humans and wild animals are the host of the family Sarcophagidae (flesh flies).

Infective Stage

Larva is the infective stage. The larvae feed for 5 to 10 weeks in the subdermal cavity getting oxygen through the hole in the host's skin.

Transmission of Infection

Female *D. hominis* deposit their mature egg on a vector, i.e. blood-sucking arthropod (usually mosquito or a tick) (Fig. 3). As the vector takes the blood meal, the eggs respond to temperature change and hatch to liberate the larva. The larvae then enter the host's skin through hair follicles or wounds and burrows in the skin. After a week of infestation, the larvae moult to second *instar* and then to the third *instar* in 2–3 weeks. The larvae feed on host tissue exudates and in a month's time the grown third *instar* larva crawls out of the host to pupate in the soil. The adult flies emerge from the pupal cases in 2–3 weeks. The extremely sensitive antennae of the adult flies allow the mature males and females to identify each other. Other genera of flies have more simple life cycle as they lay eggs directly in or in close vicinity of the wounds of host.

Pathogenesis and Pathology

The myiasis caused by flies usually commences with deposition of the eggs on the skin of the host. The eggs embryonate enters deep into the skin of the infected host, facilitated by the bite of mosquitoes or through pre-existing trauma or wounds. The larvae released from the embryonated eggs undergo further development under the skin. The larvae undergo complete development at a fixed site or may migrate to other sites. As they wander, they leave behind extremely pruritic, erythematous linear marks on the skin. The larva may develop completely into an adult fly or emerge as such from the wounds. The infection ends with the death of the larva without sequelae.

The entry of the larvae into the skin is characterized by an ulcerated epidermis. Inflammatory cells containing lymphocytes, neutrophils, eosinophils, fibroblasts, histiocytes, mast cells and Langerhans cells are found in the dermis along with the larva. In the dermis, the larva is protected by its cuticle and striated muscle.

Immunology

Immune responses to larva of 'myiasis causing flies' depend on various factors including larval stages, site of infection and others. Both local inflammatory reactions and specific immune responses are seen. On entry through the skin, the larval antigens induce both innate and adaptive immune responses in the host. The larval antigens stimulate the natural killer cells, eosinophils, mast cells, neutrophils causing polyclonal T-cell activation and activation of alternate complement pathway. Secondary immune responses are induced by interactions between these antigens and major histocompatibility complex (MHC) class II molecules. The release of interleukins (IL-12) from the antigen-presenting cells (APCs) stimulates both Th1 and Th2 responses and also releases several pro-inflammatory and immunoregulatory cytokines.

Infection in Humans

Various clinical manifestations of myiasis are observed in humans.

Cutaneous myiasis is the commonest manifestation. The condition presents as furunculi, wound or migratory myiasis. Furunculi forms typically present as nodules or papules on the skin with complaints of pain, pruritus and 'something crawling underneath' sensations. Lesions

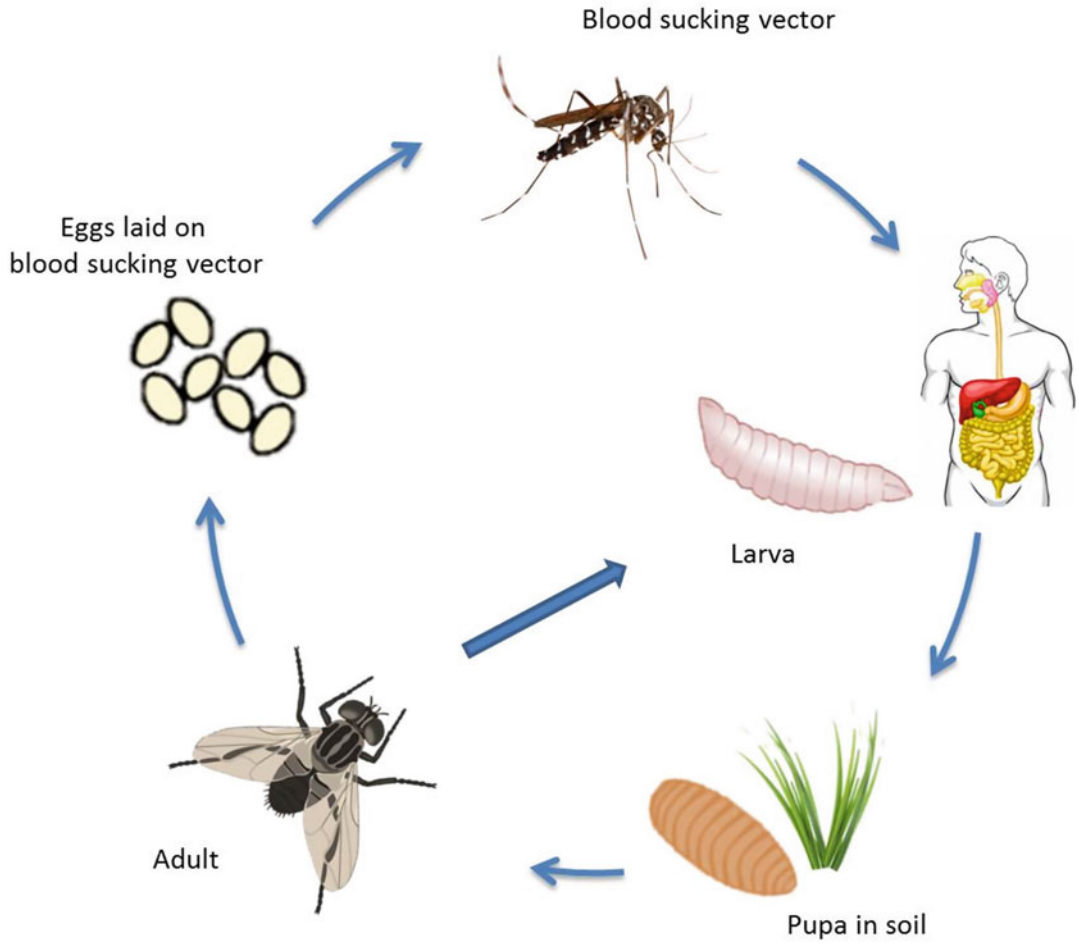

Fig. 3 Life cycle of myiasis causing flies

can be secondarily infected with bacterial infections. Migratory forms can present as creeping lesions through burrows or tunnels in the skin.

Infections of various body cavities manifest as oculomyiasis, oral myiasis, nasal myiasis and aural myiasis depending on the site of location. Ocular myiasis manifests as unilateral foreign body sensation with the presence of the larva in various layers of the eye. In severe cases, anterior uveitis followed by posterior segment infection and retinal detachment can occur. Oral myiasis manifests as gingivitis, halitosis, pain and swelling of the mouth and internal areas inside the mouth. Foreign body sensation, pain and itching along with otorrhoea or anosmia are found in aural and nasal myiasis, respectively. Intestinal myiasis occurs in cases of accidental ingestion of eggs of myiasis causing flies causing abdominal pain, anorectal bleeding and anal pruritus. The urogenital myiasis may present as ureteral obstruction, or lumbar pain in chronic cases. Cerebral myiasis is extremely rare although it can have fatal outcomes.

Infection in Animals

As myiasis can occur in any vertebrate animal, infections and infestations simulating man are common. Though skin manifestations are commonest, depending upon the type of myiasis causing fly infecting the animal, ocular, nasal and

intestinal myiasis can occur in animals. Myiasis in cats and dogs and other domestic animals have been reported with symptoms like those in man.

Epidemiology and Public Health

Majority of the myiasis causing flies are found in the tropical and humid regions, though some important species show worldwide distribution. *D. hominis* is endemic in areas extending from Mexico to Northern Argentina, but not endemic in Chile. It is primarily found in warm, moist lowland regions. *C. anthropophaga* is found in tropical sub-Saharan Africa. Distribution of *Oestrus ovis* and *Hypoderma* spp. is worldwide (Fig. 4). *C. hominivorax* is endemic in Central America and South America, and *Chrysomya Bezziana* is primarily found in tropical regions in Africa, Indian subcontinent, Southeast Asia. *Wohlfahrtia magnifica* is distributed in areas of southeast Europe, Russia, Middle East and North Africa, whereas *Wohlfahrtia vigil* is mainly found in North America (Table 1).

Diagnosis

Microscopy

Specific identification of larvae is made by microscopic examination of the pair of dark-coloured chitinous plates on the posterior end of the larva (Fig. 5). Expertise is required for observing the slide under the microscope for definite diagnosis as numerous species are associated with the infestation. The pattern of the stigma plates containing the respiratory apertures and other morphological features helps in the identification of species of human myiasis causing flies (Table 2).

In Vitro Culture

Fly box containers with tight-fitting lids are used in *in vitro* culture of the larvae and other stages of flies. Animal excreta and waste food are used as the media for the growth and development with favourable conditions of temperature and humidity.

Serodiagnosis

Serological methods for the detection of larval antigens causing myiasis have been developed as an alternative to post-mortem examination and parasitological examination. Serodiagnosis allows cost-effective diagnosis of myiasis in living animals. An ELISA test prepared from the hypodermin C (HC) antigen of *H. lineatum* is used in many countries for serological detection of hypodermosis. The ELISA test performed using L1 crude somatic antigen in correlation with the clinical post-mortem examination has also been used for the serological diagnosis of myiasis. In humans, serodiagnosis can be helpful in the confirmation of the suspected cases, but due to the cross-reactivity between members of the subfamily, the identification of the species through serodiagnosis is not possible.

Molecular Diagnosis

Molecular methods are used for specific detection, and identifications of the larva PCR-RFLP targeting cytochrome oxidase I have been used for the molecular identification and differentiation of the most common species. A total of other 62 genes of Oestridae, Calliphoridae and Sarcophagidae have been studied for the molecular diagnosis of myiasis, which include COI, COII, tRNA-Leu, tRNA-Ile and 12S mtDNA, 16S rRNA and 18S rRNA (rDNA). Molecular diagnosis is the method of choice for the identification of the cutaneous myiasis.

Treatment

The treatment of myiasis, in principle, consists of (a) application of toxic substance to the egg or larva, (b) localized hypoxia for the emergence of the larva and (c) removal of the maggots through surgical or mechanical methods. The ivermectin,

Fig. 4 World map showing cases of human myiasis

Table 1 Epidemiology of flies causing myiasis

Species	Hosts	Distribution
Dermatobia hominis	Sheep, goats, humans, horses, donkeys, cattle, reindeer, deer, wild animals and birds	Mexico to Northern Argentina
Cordylobia anthropophagi	Sheep, cattle, humans, dogs and wild animals	Tropical sub-Saharan Africa
Oestrus ovis	Sheep, goats, llamas	Worldwide
Hypoderma spp.	Cattle, horses	Worldwide
Cordylobia hominivorax	Warm-blooded animals including humans	Central America and South America
Cordylobia bezziana	Large domesticated animals, native wildlife, occasionally humans	Africa, Indian subcontinent, Southeast Asia
Wohlfahrtia magnifica	Goats, sheep, poultry, wildlife occasionally humans	Southeast Europe, Russia, Middle East, North Africa
Wohlfahrtia vigil	Cats, dogs, rabbits, minks, foxes, humans	North America

given orally in a dosage of 150–200 μg/kg of body weight, is used for the treatment of myiasis in humans.

The treatment of cutaneous myiasis includes covering the breathing air holes on surface of the skin with thick petroleum jelly. Other occluding substances include bacon, petrolatum, adhesive tape and fingernails. Due to lack of oxygen, larvae move to the surface from where it is easily eliminated. Injection of 1% lidocaine can be used to paralyse the larva for easier extraction.

C. anthropophaga larva does not migrate deeper in the tissue, hence can be easily removed manually. Surgical excision is always recommended in migratory myiasis. It has been reported that the use of oral albendazole and ivermectin mobilizes the parasite towards the surface.

Treatment of myiasis in malignant wounds includes removal of maggots and surgical debridement of the malignant wound. This is followed by rigorous washing with the antibiotics and regular dressing changes. Other options include topical treatment like applying ivermectin containing propylene glycol solution to the affected area directly for 2 h.

Oral treatment is not recommended and the use of antibiotics is only advised if there is bacterial infection, for the treatment of furuncular myiasis.

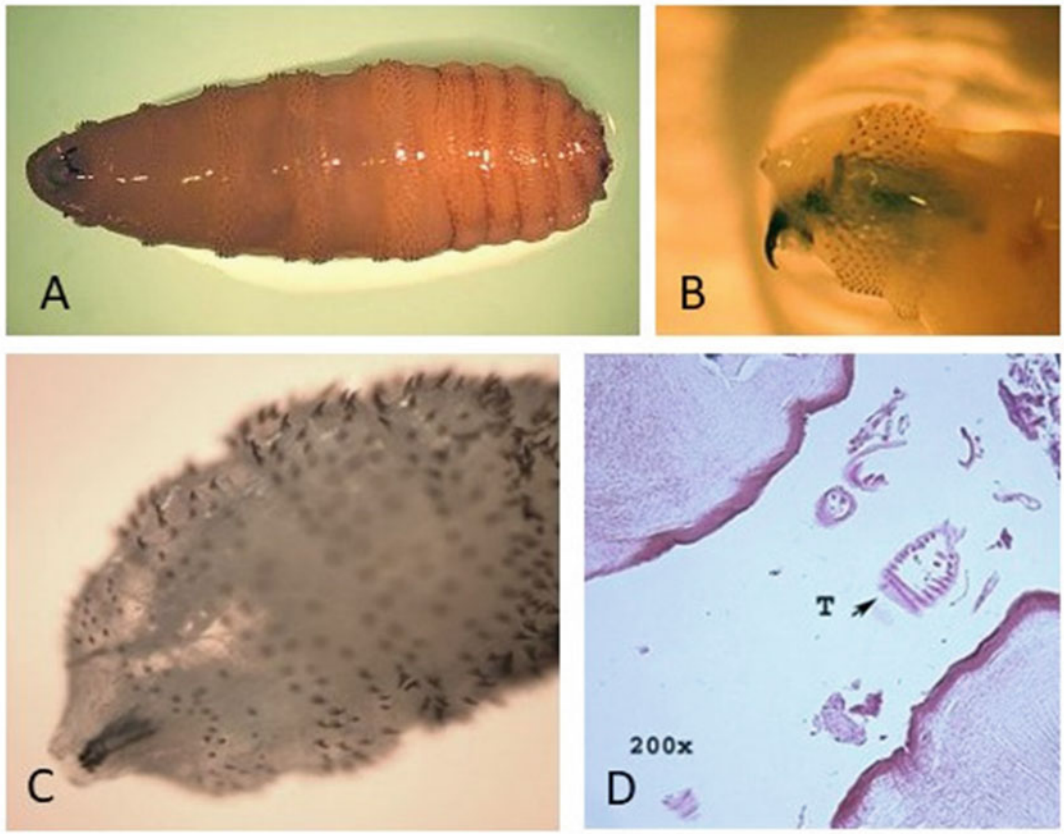

Fig. 5 (**a**) *Condylobia hominivorax* larvae isolated from forehead of a patient, (**b**) closer view showing the mandibles at the anterior end of a larva, (**c**) larva of *Dermatobia hominis* with prominent view of anterior end, (**d**) cross sections of a bot fly larva showing fragments of the trachea (courtesy: DPDx, CDC)

Table 2 Diagnostic methods in myiasis

S. no.	Diagnostic methods	Features
1.	Microscopy	Pattern of the stigma plates containing the respiratory apertures significant for identification Expertise required
2.	Morphological diagnosis	Specific knowledge necessary for the identification of the maggot's species Aspects of the papillae; position, shape, openings and structures of the posterior spiracles; shape and coloration of the dorsal tracheal trunks; the body surface (spines), the anterior spiracles considered for identification
3.	*In vitro* culture	Large-scale farm production 'Fly box' containers with tightly fitting lids used Food waste and animal excreta are used as media for growth Optimum temperature and humidity required
4.	Molecular diagnosis	Identification at the species level is possible Genes such as COI, COII, tRNA-Leu, tRNA-Ile and 12S mtDNA, 16S rRNA and 18S rRNA (rDNA) RFLP and RAPD

Topically, application of nitrofurazone over wounds 3 times per day for 3 days has proved to be useful for the treatment of oral myiasis. Irrigation of the ear with 70% ethanol, saline, 10% chloroform, oil drops, ivermectin drops, urea and dextrose helps in the removal of the larvae in aural myiasis. No specific treatment is available for the treatment of intestinal myiasis, although mebendazole, albendazole and levamisole have been reported to be successful in a few cases.

In animals, the treatment of choice is direct destruction of larva. The infected animals require antibiotic therapy along with the supportive treatments like fluid therapy. The larvae should be removed by surgical methods. Another method of treatment includes treating livestock in persistent insecticides to poison the larvae.

Prevention and Control

Preventive measures of myiasis in humans include improvement of sanitation, hygiene and eradication of flies by insecticides. Washing the clothes in hot water, drying the clothes under the sun, etc., are useful since the heat kills the eggs of myiasis causing flies.

For myiasis in animals, the preventive measures include (1) vector control (eradication of adult flies before any damage happens) and (2) insecticide spraying of organophosphorus, organochlorine insecticides in the place, where livestock are housed. Sterile insect treatment (SIT) is another method of control where overwhelming numbers of sterilized male flies are introduced into the wild. The females mate with the sterile males producing no offspring leading to reduction in the population in the next generation. Changing the favourable environment for the flies, for example by crutching and mulesing of sheep, is another method for the prevention of myiasis.

Case Study

A 28-year-old man alcoholic, living in slums, presented with acute upper lip swelling with an extensive necrotic area and fetid odour. The examination revealed a diffused indurated swelling nonfluctuant on palpation. The patient was drunk, with poor oral and general hygiene. The swelling revealed several orifices on its surface with live maggots involving intra-orally buccolabial fold.

1. What is the diagnosis?
2. What treatment can be done?

Research Questions

1. Is there any development of resistance of flies towards chemical insecticides?
2. Is it feasible to develop any vaccine against myiasis?

Further Readings

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Tungiasis

Sourav Maiti

Learning Objectives

1. To review the epidemiology of tungiasis.
2. To emphasize the serious nature of the infection that may lead to gangrene or bacterial sepsis needs to be emphasized.

Introduction

Tungiasis is a zoonotic ectoparasite in humans caused by the sand flea, *Tunga penetrans.* Tungiasis, as a neglected tropical disease, afflicts the populations with low socio-economic status. *T. penetrans* attaches commonly to the softer tissues in feet such as interdigital webs and periungual regions in the infected human hosts. The condition is characterized by single or multiple white or yellowish popular of nodular lesions with a brownish central aperture. Hypertrophy of the female jigger flea penetrating the skin is characteristic. Known by multiple names like *chigoe, chica, pulga de bicho, nigua, bicho de pe* and several others in multiple local languages, tungiasis remains a threat to the travellers owing to the expansion of ecotourism.

History

The earliest description of tungiasis synchronizes with the discovery of the Americas. G.de Oviedo y Valdes made the first mention of the sand flea in 1525 from Haiti. However, the first scientific description came from Alexo de Abreu in the early seventeenth century from Brazil. Slave trade introduced sand flea to West Africa several times during the seventeenth to nineteenth centuries. *T. penetrans* travelled to Angola in 1872 on a ship from Brazil through the ballast sands. Trades and military troops rapidly disseminated *T. penetrans* soon from Angola to the whole sub-Saharan Africa. Historical reports emerged describing native villages in South America with intense jigger infestations urging affected even to cut off their toes in sheer desperations. Several military operations stopped due to the excruciating walking difficulty among the soldiers. By 1899, *T. penetrans* reached the Indian subcontinent through the returning British troops, but possibly never got established.

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Taxonomy

The genus *Tunga* belongs to the family Tungidae; superfamily Pulicoidea; order Siphonaptera; class Insecta; and phylum Arthropoda.

The genus *Tunga* is divided into two subgenera: *Tunga* and *Brevidigita*. Subgenus *Tunga* contains six species—*T. penetrans*, *Tunga travassos*, *Tunga bondari*, *Tunga terasma*, *Tunga trimamillata* and *Tunga hexalobulata*. *Brevidigita* subgenus contains seven species including *Tunga caecata*, *Tunga caecigena*, *Tunga callida*, *Tunga libis*, *Tunga monositus*, *Tunga bossii* and *Tunga bonneti*.

T. penetrans and *T. trimamillata* are the two recognized human ectoparasites.

Genomics and Proteomics

Limited studies on genomic analysis of extracted gravid female and free-living both gender fleas show a low level of genetic variability in *T. penetrans*, particularly with the mitochondrial cytochrome oxidase II. Nuclear ITS2 analysis identified two genotypes—Atlantic (Brazilian and African samples) and Pacific (Ecuadorian samples). *T. trimamillata* displayed higher haplotype diversity. A relatively high level of gene flow across the Andean barrier suggests a high dispersal capacity of *T. penetrans*. Genomic pictures conform to the accidental introduction of *T. penetrans* in Africa in a single event during the nineteenth century and a complex evolutionary scenario.

SDS-PAGE and Western blot analysis have identified three immunodominant antigens of molecular weights 51.795, 23.795 and 15.38 kDa from *T. penetrans* isolates in Kenya. These proteins are possibly involved in acute pathological events; their role in immunization is yet to be investigated.

The Parasite Morphology

Adult Flea

Adult sand flea has three parts—head, thorax and abdomen. The adult female flea is smaller (about 1 mm in size) than the male. The flea contains branched tracheal rings, which help in identification. The body cuticle is relatively thick, which harbours the hypodermic cells. Gravid females are characterized by the ovaries enlarged to accommodate thousands of eggs. The protractible copulation organ at the posterior end compared to a groove distinguishes the male gender. The abdomen contains numerous segments. Enlargement occurs between segments II and III resulting in neosome. Gravid *T. trimamillata* is distinct from *T. penetrans* by the presence of 3 humps surrounding its head and thorax. Neosome of *T. trimamillata* is longer, 12 mm in length with similar breadth and height, approximately 5 mm each. Caudal disc in abdominal segments IV–X is wider than long and flattened in *T. penetrans* (Fig. 1), while it is conical in *T. trimamillata*.

Eggs

Eggs are yellow-white in colour, ovoid and measure 604 μm × 327 μm in size (Fig. 2).

Larva

Larva contains unevenly distributed bristles along with the head and the body. An eggshell breaker appears in the head, which disappears after moulting.

Pupa

Pupa appears U-shaped inside a thin-walled cocoon.

Fig. 1 Schematic diagrams of adult male and female, *Tunga penetrans*

Cultivation of Parasite

Experiments using Petri dishes filled with wet paperboard or sand permit egg development in vitro. Eggs hatch in 1–6 days. Feeding with nutrient flea breeding solution results in cocoon development and subsequent adult forms.

Laboratory Animals

Albino Wistar rats are used frequently in animal experimental study. Anaesthetization facilitates flea penetration and mating. Thus, the complete life cycle and biology studies are possible. They also aid in immunization research studies including cytokine kinetics.

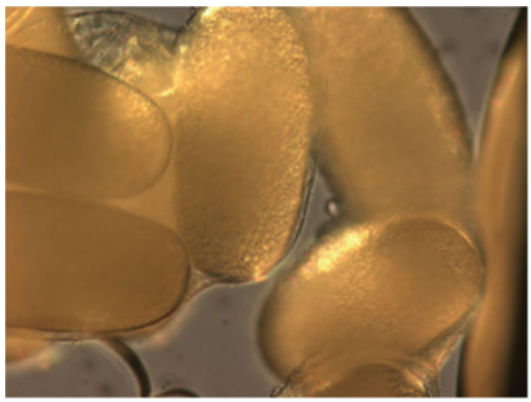

Fig. 2 A close-up view of the *Tunga* spp. eggs (image courtesy of DPDx, Centers for Disease Control and Prevention; https://www.cdc.gov/dpdx)

Life Cycle of *Tunga* Species

Hosts

Humans, dogs, cats, pigs, donkeys, monkeys and rodents.

Infective Agent

Adult female sand flea.

Transmission of Infection

Both the male and female sand fleas feed upon a warm-blooded host and prefer the parts with the soft and moist skin. The most frequent attachment sites are the regions that are in regular contact with the soil like the feet and the ventral part of the abdomen and also those parts difficult to reach by the host to dislodge like ears, tails and scrotum. After the blood meal, they mate and the female flea keeps the penetrating posture by attaching the mouthparts permanently with the stratum corneum of the epidermis. The gravid female flea thereafter burrows inside the stratum granulosum with its posterior part exposed to the exterior for the expulsion of eggs. Continued egg production causes impressive ten-fold hypertrophy of the abdomen (neosomy) reaching about 1 cm from less than 1 mm size. This hypertrophied abdominal segment is called neosome, which is characteristic of *Tunga* sp., and its shape and size vary among different species. The eggs are released in hundreds per day starting from the 8–10 days post-penetration until

4–6 weeks. The gravid female can remain attached for more than 5 weeks. Thereafter, the flea dies and the lesion begins to heal and sloughs out. The eggs hatch in the soil in 3–4 days releasing larvae. The larvae feed on organic debris and pass through two instars to become pupae encased in cocoons. The eggs, larvae and cocoons persist long in the environment. Usually, in 3–4 weeks from the hatching, the adults emerge from the cocoons and continue the cycle (Fig. 3).

Pathogenesis and Pathology

The fleas get themselves inside a pseudocyst cavity within the epidermis of the infected host, with their head thrust in the dermis to puncture blood vessels for nutrition. The caudal end moves towards the surface and communicates with the exterior via an orifice in the keratin layer aiding in breathing and egg expulsion. Neutrophillic and lymphocytic infiltration can occur around the lesion.

Acute inflammation in the host is characterized by redness, oedema and pain, which occur due to flea antigens and secondary bacterial infections. The wound is infected secondarily by aerobic and anaerobic bacteria carried by the flea and followed by scratching. Suppuration, ulceration, lymphangitis and gangrene formation occur commonly. After the expulsion of the eggs, the flea dies and gets sloughed away by the skin repair mechanisms.

Eisele et al. (2003) described five-staged pathogenesis of infection caused by the fleas. Stage I begins with the penetration resulting in an erythematous spot. Hypertrophy begins in stage II with more visibility of the lesion in the form of a pearly-whitish nodule with a black dot denoting the posterior cone of the parasite. Abdominal enlargement (neosomy) maximizes in the third stage in about 2–3 weeks of penetration. This stage typically demonstrates the egg expulsion with excruciating pain and pruritus. This follows black crusting and involution of the lesion (stage IV). A residual scar features the final stage (stage V). However, in practice, frequent super added infections hinder visualization of these stages. Hyperkeratosis, parakeratosis, acanthosis and deformed nails are frequent in the chronic stages through an unknown mechanism. Progression to disfigurement and mutilation of the toe can occur.

Immunology

Although an ectoparasite, complex non-specific and multifaceted immunological reactions result due to (a) antigens released from the stationary flea, (b) lipopolysaccharide of the endosymbiont *Wolbachia pipientis* and (c) accompanying and superinfecting bacterial pathogens.

Tungiasis induces interferon-gamma, interleukin-4 and tumour necrosis-alpha in the circulation. Disruption of the flea body, particularly during unskilled manipulation to remove the flea, induces severe inflammatory reactions to endosymbiont *W. pipientis* antigens including the surface proteins. Superinfections with other soil-transmitted bacterial pathogens also induce more immune responses.

Infection in Humans

T. penetrans attaches commonly to the softer tissues of the interdigital webs and periungual regions in the feet. Involvement of hands, soles, elbows, buttocks, anal region, genitalia, groin, neck and face has been reported. Single or multiple white or yellowish papular nodular lesions with a brownish central aperture are characteristic.

Symptoms begin with intense pain and itching and progress to ulceration, loss of toenail, deep fissures in the skin and walking difficulties. Superinfections with *Streptococcus* spp., Enterobacteriaceae and *Clostridium* spp. lead to suppuration, pustule formation and sepsis. Tetanus has been reported apart from other complications like lymphadenitis and gangrene formation. *T. trimamillata* lesions are reportedly more painful. Differential diagnoses include verruca, myiasis, melanoma, mycosis, insect bite, paronychia and foreign body impaction.

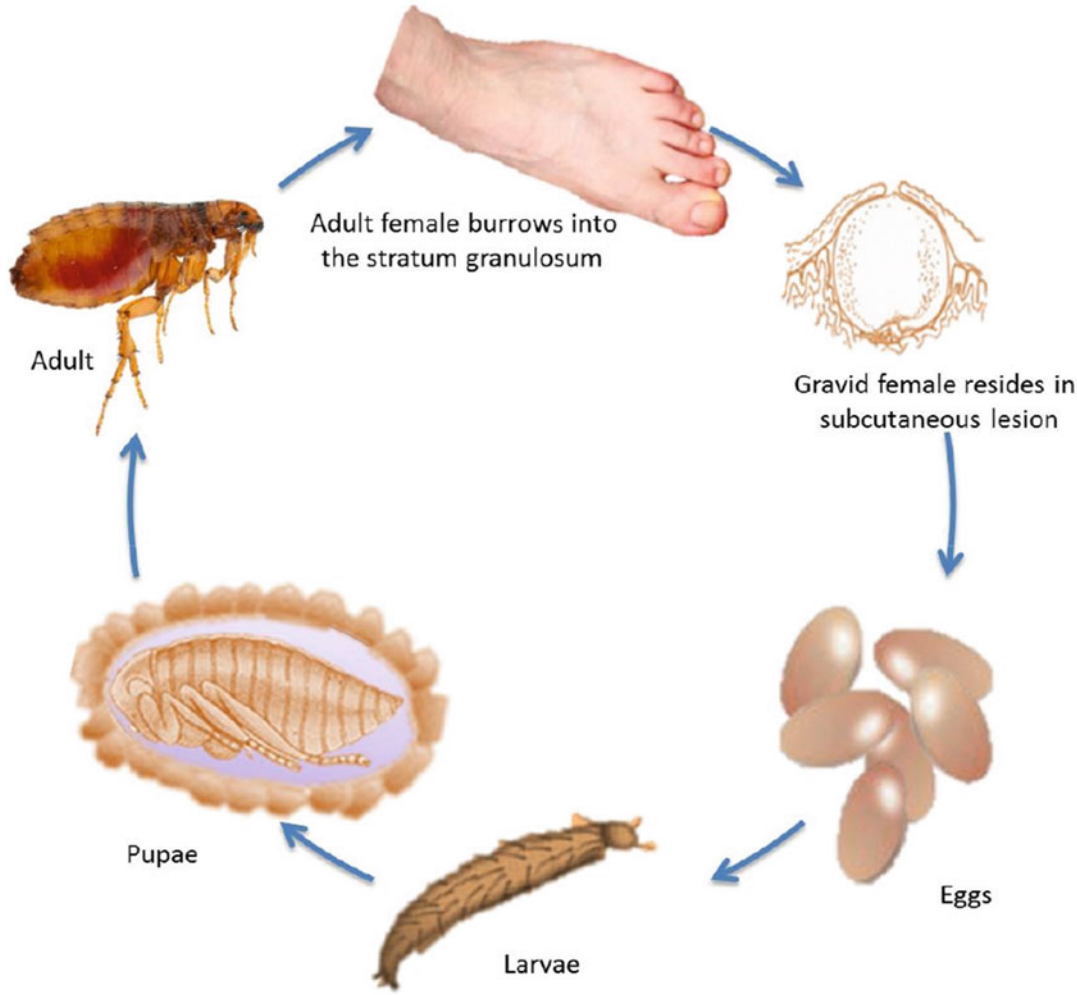

Fig. 3 Life cycle of *Tunga penetrans*

Infection in Animals

In animals, neosomes occur commonly around the claws and pads, causing walking difficulties. *T. penetrans* infection in pigs involves feet, snout, mammary glands and scrotum. Fleas-infested dogs change their position, lick the pads and avoid standing up. *T. trimamillata* affects the coronary band, coronary and digital cushions in the cattle and calcaneal regions, scrotum and udder in pigs. Crippling, inability to sustain own weight and involution of the mammary glands are notable features of the infection in these animals.

Epidemiology and Public Health

Tungiasis afflicts the economically deprived and low-hygiene areas including slums, fishing villages and thatched houses. Out of at least 13 different species, *T. penetrans* and *T. trimamillata* infest humans. *T. penetrans* is endemic in the neotropical realm including South America, Central America and the Caribbean. Poor communities show 16–54% prevalence in Brazil, Nigeria and Trinidad. *T. trimamillata* is found mainly in southern Ecuador and Peru. Sao Paulo and Minas Gerais of Brazil have documented the presence of

T. trimamillata. The west coast of India has reported a few tungiasis cases (Fig. 4, Table 1).

Highest disease burden falls upon the elderly and disabled persons with significant involvement of children in endemic areas. High flea burden in a single person causes immobilization, nutritional deficiency and cachexia. In children, the loss of concentration results in poor academic performance. Loss of overall productivity adds an economic burden in the impoverished communities.

Both the species are polyxenous involving hosts from multiple families. Promiscuity of *T. penetrans* involves at least eight different orders of mammals including cingulates, carnivores, rodents and primates. Pig appears the prominent reservoir for *T. penetrans*. Humans and dogs could be secondary or essential hosts. Domestic animals like cow, goat, sheep and pig can carry *T. trimamillata* ectoparasites along with the rodents. As the reduced pleural arch limits high jumps, *Tunga* sp. preferentially attaches to the regions that regularly touch the soil (feet) and are difficult to dislodge from ears and tails.

The immature stages of the fleas develop in the soil, near the residence of the host. The natural habitats for *T. penetrans* include sandy and warm soils of beach and dessert. Larvae of *T. penetrans* can be found in 2–5 cm depth of sand. Horse manure used as soil fertilizer contains both *T. penetrans* and *T. trimamillata* living in stables.

Laboratory Diagnosis

A wide variety of diagnostic methods are available in tungiasis (Table 2).

Microscopy

Clinical inspection of a watch-glass type patch with inflammation or crust is often sufficient to diagnose tungiasis. Dermoscopy reveals pigmented parasite with light brown to black rings and a central aperture. Eggs (Fig. 2) attached to the skin along with the faecal coil release are characteristic. Ectopic sites and atypical features need a biopsy to demonstrate the fragments of the female flea with eggs in its abdomen. The absence of characteristic spines differentiates fleas from the fly larvae and scabies mite. The globular head of the flea differentiates it from the human flea (*Pulex irritans*) with an angular head. The presence of three lobes anterior to the globular head and conical caudal disc is diagnostic of *T. trimamillata*.

Serodiagnosis

Serodiagnosis tests are not available for tungiasis.

Molecular Diagnosis

Molecular diagnosis helps in differentiating non-neosomic forms and identification from preserved samples where the morphological examination is difficult and time-consuming. A pre-PCR step is necessary to amplify the small quantity of DNA; restricted fragment length polymorphism (RFLP) and multiple displacement amplification (MDA) are promising. For *T. penetrans* ITS2 sequence, two different enzymes are used—MspI and RsaI. The former gives three bands (346, 115 and 51 bp) and the latter cleaves into two (266 and 246 bp). Similar steps in *T. trimamillata* result in two bands (396 and 115 bp) and three bands (266, 161 and 84 bp), respectively. MDA uses a bacteriophage, φ29 DNA polymerase, which replicates DNA at 30 °C and exonuclease-resistant, thiophosphate-modified degenerate hexamers to increase the yield for subsequent PCR.

Treatment

Surgical extraction of the whole flea followed by topical antibiotic application and tetanus prophylaxis constitutes the best treatment. Careful extraction is needed; any damage to the flea gives rise to severe inflammation. The use of sterile instruments is necessary to avoid blood-borne infections such as HBV, HCV and HIV,

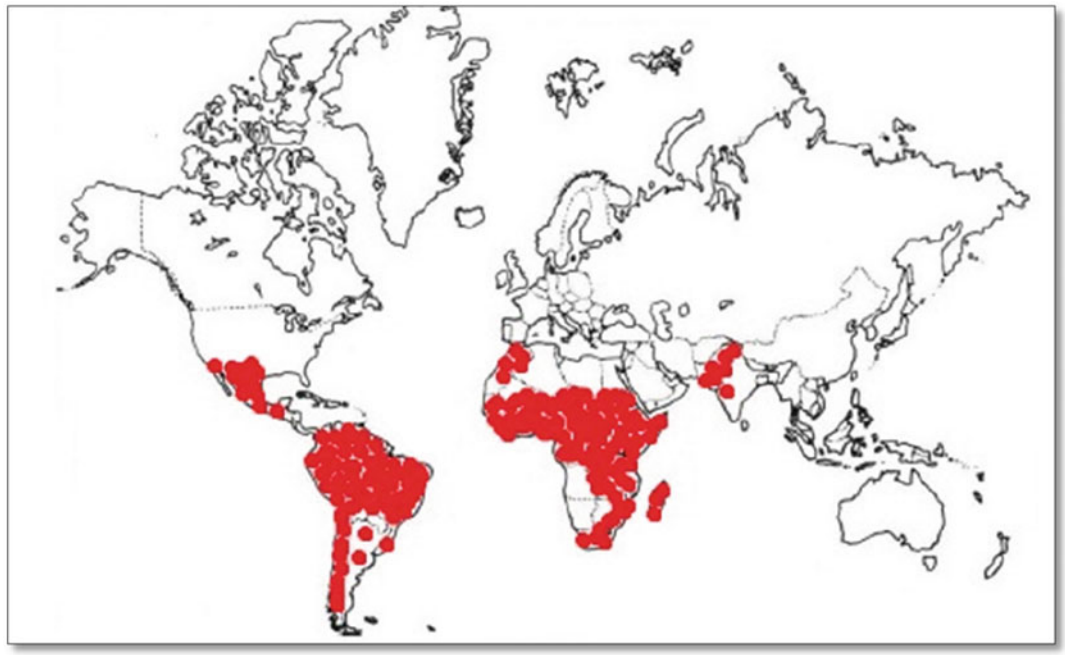

Fig. 4 Major endemic and sporadic distributions of tungiasis cases

since embedded flea removal is an invasive procedure. Oral thiabendazole and oral and topical ivermectin usages have no efficacy data. Aerobic and anaerobic bacterial superinfections need additional antimicrobial therapy.

Prevention and Control

Avoiding trips to endemic regions, particularly the beaches and areas with sandy soils, banana plantations and tropical forests, are helpful. Thick socks and closed shoes protect but to a certain degree only. Regular inspection of the feet can identify early lesions. Avoiding sleeping on the floor, particularly near the animal habitat, reduces the chance of infestation.

Control of tungiasis is difficult due to the presence of domestic and sylvatic animal reservoirs. Cemented flooring, paved streets, improved sanitation, regular waste collection and health education are the suggested effective methods to control tungiasis. Spraying of insecticides such as DDT sprays does help in the reduction of the flea population.

Case Study

A 34-year-old man comes to you with few warty lesions adjacent to the right toenail. He noticed those lesions due to intense itching. You observed the lesions with a magnifying lens to discover papular lesions with a central black spot. The

Table 1 Distribution of *Tunga* spp. of importance for humans

Species	Major distribution	Host
Tunga penetrans	Neo- and palaeo-tropical areas, sub-Saharan Africa	Wide range including humans, dogs, cats, pigs, donkeys, monkeys and rodents
Tunga trimamillata	Southern Ecuador, Peru	Humans, cows, goats, sheep, pigs and rodents

Table 2 Diagnostic methods for tungiasis

Diagnostic approaches	Sample	Methods	Targets	Comment
Dermoscopy	Skin lesion	Observation	Eggs, faecal coil, posterior part of ectoparasite through the opening	Clinically diagnostic
Microscopy	Biopsy	Histology	Chitinous fragments, eggs, globular head, caudal disc	Differentiation between *Tunga penetrans* and *Tunga trimamillata*
Molecular	Preserved samples, field samples	RFLP-PCR MDA-PCR	ITS2	Highly specific and discriminatory; used mainly for research

patient had a leisure trip to beaches in Brazil last month.

1. What are the suggestive points in support of tungiasis?
2. How would you confirm?
3. What microscopic findings would aid in differentiation from myiasis?
4. What will be your advice for the next trip to the same location?

Research Questions

1. What is the mechanism of hyperkeratosis and nail deformity in the chronic stages of tungiasis?
2. What low-cost effective preventive measures may be suggested for tungiasis?
3. What is the extent of tungiasis problem among animals?

Further Reading

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