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Rafael Toledo
Bernard Fried *Editors*

Digenetic Trematodes

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Rafael Toledo • Bernard Fried
Editors

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Editors
Rafael Toledo
Departamento de Biología Celular y Parasitología
Facultad de Farmacia, Universidad de Valencia
Burjassot, Valencia, Spain

Bernard Fried
Department of Biology
Lafayette College
Easton, PA, USA

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Preface

Digenetic trematodes are a major group of parasites of humans and animals and are distributed worldwide. They have a complex life cycle and require at least two hosts, one of which is usually a mollusk. Over 100 species of digenetic trematodes have been recorded infecting humans and the list is very extensive considering the trematode species that parasitize animals. The morbidity and mortality caused by several species in humans can be considered mild or even benignant, though other species have important implications for human health. For example, schistosomiasis affects more than 200 million people and causes almost 300,000 deaths per year. Moreover, it is well established that several species of trematodes act as promoters of malignancy. Despite these facts, human trematode infections have been neglected for years, probably in relation to their limited distribution to low- and middle-income countries. However, this picture has been changing in recent years. Factors such as the migration flows, increased international tourism, changes in alimentary habits, and the globalization of food markets are expanding their geographical limits and the population at risk worldwide.

In this book, we draw attention to trematode infections that cause disease in humans and other trematodes of interest in veterinary and wildlife. To this purpose, the book has been divided into three parts. The first part is devoted to analyze the modern concepts on the biology and systematics of trematode. The second part focuses on the groups of trematodes that have important implications for human health. Each of the six major groups of human trematodes and the corresponding diseases (schistosomiasis, fascioliasis, paragonimiasis, opisthorchiasis, and clonorchiasis and the intestinal trematodes) are dealt with under separate chapters. Moreover, the section is completed with two chapters dealing with the epidemiology and diagnoses of trematode infections. In these chapters, emphasis is placed on recent advances and gaps in our understanding that must be filled to complete the knowledge of these trematodes. In the third part of the book, the main groups of trematodes of veterinary and wildlife interest are analyzed. As mentioned above, the list of potential trematodes that might be discussed in this section is vast.

For convenience, we have chosen to focus the chapters on those groups of trematodes that are also recognized to have implications for human health.

The main goal of the book is to present the trematodes and the corresponding diseases in the framework of modern parasitology, considering matters such as the application of novel techniques and analysis of data in the context of host–parasite interactions and to show applications of new techniques and concepts to the studies on digenetic trematodes.

In summary, in this book the most recent information of the major groups of digenetic trematodes is compiled with the aim of providing an update of the current status of knowledge on these important parasites in the context of modern parasitology.

Rafael Toledo
Bernard Fried

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Part III Trematodes of Interest in Veterinary and Wildlife Disease

Contributors

Ross H. Andrews Department of Parasitology and the Liver Fluke and Cholangiocarcinoma Research Centre Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Faculty of Medicine, St. Mary's Campus, Imperial College London, London, UK

Lawrence R. Ash Infectious & Tropical Diseases, Fielding School of Public Health, University of California, Los Angeles, CA, USA

M. Dolores Bargues Departamento de Biología Celular y Parasitología, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain

David Blair James Cook University, Townsville, QLD, Australia

Jong-Yil Chai Department of Parasitology and Tropical Medicine, College of Medicine, Seoul National University, Seoul, South Korea

J. Guillermo Esteban Departamento de Biología Celular y Parasitología, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain

M. Carmen Ferreras Facultad de Veterinaria, Departamento de Sanidad Animal, Universidad de León (ULE), León, Spain

Bernard Fried Department of Biology, Lafayette College, Easton, PA, USA

Sudeep Ghatani Department of Zoology, North-Eastern Hill University, Shillong, Meghalaya, India

Petr Horák Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Jane E. Huffman Northeast Wildlife DNA Laboratory, Department of Biological Sciences, East Stroudsburg University, East Stroudsburg, PA, USA

Nadda Kaitsopit Department of Parasitology and the Liver Fluke and Cholangiocarcinoma Research Centre, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Libuše Kolářová Institute of Immunology and Microbiology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic

Aneta Kostadinova Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic

Fred A. Lewis Department of Schistosomiasis, Biomedical Research Institute, Rockville, MD, USA

M. Yolanda Manga-González Departamento de Sanidad Animal, Instituto de Ganadería de Montaña, Consejo Superior de Investigaciones Científicas (CSIC)—ULE, León, Spain

Santiago Mas-Coma Departamento de Biología Celular y Parasitología, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain

Libor Mikeš Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

Carla Muñoz-Antoli Departamento de Biología Celular y Parasitología, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain

Robert C. Peoples Schistosomiasis Lab, Biomedical Research Institute, Rockville, MD, USA

Ana Pérez-del-Olmo Unitat de Zoologia Marina, Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Parc Científic, Universitat de València, Valencia, Spain

Trevor N. Petney Department of Ecology and Parasitology, Institute of Zoology, Karlsruhe Institute of Technology, Karlsruhe, Germany

Bishnupada Roy Department of Zoology, North-Eastern Hill University, Shillong, Meghalaya, India

Weerachai Saijuntha Walai Rukhavej Botanical Research Institute, Mahasarakham University, Maha Sarakham, Thailand

Jollin Andrea Shylla Department of Zoology, North-Eastern Hill University, Shillong, Meghalaya, India

Paiboon Sithithaworn Department of Parasitology and the Liver Fluke and Cholangiocarcinoma Research Centre, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Veena Tandon Department of Zoology, North-Eastern Hill University, Shillong, Meghalaya, India

Rafael Toledo Departamento de Biología Celular y Parasitología, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain

Matthew S. Tucker Department of Schistosomiasis, Biomedical Research Institute, Rockville, MD, USA

M. Adela Valero Departamento de Biología Celular y Parasitología, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain

Part I
General Aspects of the Trematodes

Chapter 1
Form and Function in the Digenea

Robert C. Peoples and Bernard Fried

1.1 Introduction

For our coverage on form and function in the Digenea, we have relied heavily on the earlier chapters on this topic in Fried and Haseeb [1] and in Fried [2]. We have considered herein the major organ systems covered in those two earlier works. Therefore, for background information on the subject matter covered in this chapter, those two earlier works should be consulted. In regard to our literature search, the words "Digenea" and "Trematoda" were each paired with the following search terms: female reproductive system, male reproductive system, neuromuscular system, nervous system, lymph system, lymphatic system, alimentary tract, sense organs, sensory structures, tegument, tegumentary system, parenchyma, holdfast organs, excretory system, osmoregulatory.

Emphasis in this chapter is based on the newer literature, i.e., since the coverage by Fried [2] and places emphasis on salient literature between 1997 and 2012. The layout of this chapter will proceed in the following pattern: a description and overview of the digenean body system of interest, a discussion of the body system as it applies to adult digeneans, a discussion of said body system as it pertains to various digenean larval stages (if applicable).

R.C. Peoples (✉)
Schistosomiasis Lab, Biomedical Research Institute,
12111 Parklawn Drive, Rockville, MD 20851, USA
e-mail: rpeoples@afbr-bri.com

B. Fried
Department of Biology, Lafayette College, Kunkel Hall,
Room 204, Easton, PA 18042-1778, USA

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1.2 Tegumentary System

Digenetic trematodes have a syncytial tegument. The epidermis is composed of a distal anucleate cell layer. Cell bodies containing the nuclei, called cytons, lie beneath a superficial layer of muscles, connected to the distal cytoplasm by way of channels, called internuncial processes. These processes are sometimes adorned with microvilli [3]. Vesicles within the distal cytoplasm serve an unknown purpose although some authors believe they may contribute to the peripheral surface of the tegument as they move outwards, perhaps replacing membrane damaged by host antibodies [4].

During the metamorphosis of a miracidium into either a sporocyst or redia, ciliated epithelial cells are shed and the distal cytoplasm originating at the intracellular ridges spreads over the organism's surface. Well-developed microvilli are present on the surface of both sporocyst and redia. The luminal surface of tegumental cells in rediae may be thrown into a large number of flattened sheets that extend to other cells in the body wall and to cercarial embryos contained in the lumen. Nutritive molecules such as glucose can pass through the tegument to developing cercariae [5]. Amino acids and hexoses can be absorbed through the tegument. Depending on the species, a variety of molecules can be absorbed either through the tegument and/or the lining of the gut [6].

Early embryos of cercariae are covered with a primary epidermis. A definitive epithelium forms underneath this primary epidermis. Nuclei of this secondary epithelium sink into the parenchyma, and in its final form cercarial tegument has an organization similar to that of adult worms. During the process of encystment, cystogenic cells found within the parenchyma secrete cyst material. The wall of the developing metacercarial cyst is completed when the cercarial tegument sloughs off and the cyst material it contains undergoes chemical and/or physical changes, in turn enveloping the worm within a completed cyst. A thin coating of cytoplasm, originating from cystogenic cells in the parenchyma encloses the metacercaria within its cyst and gives rise to the finished adult worm tegument [3].

Hacariz et al. [7] found an abundance of fatty acid-binding protein 1 in a surface protein fraction taken from the fasciolid *Fasciola hepatica*. Hong [8] compared the surface tegument of metacercariae and adult trematodes of the plagiorchiid *Plagiorchis muris*. The distribution of papillae and tegumentary spines was similar except for a reduced density of tegumentary spines on the posterior portions of the adults. The author attributed this to the development of reproductive organs.

Han and coauthors [9] studied the surface ultrastructure of *Cercaria caribbea*. It is the first record describing a cyathocotylid cercaria from a brackish water gastropod in the Republic of Korea.

Podvyaznaya and Galaktionov [10] made observations on the branching sporocysts of the bucephalid *Prosorhynchoides borealis* found within the bivalve *Abra prismatica*. The authors noted two different types of terminal regions in the sporocysts: one specialized for the investigation and penetration of host tissues and the other containing a high degree of germinal cells specialized for cercarial

production. The authors hypothesize that this two-pronged approach to both host invasion and cercarial production help explain why sporocysts of this species are not only able to survive for long periods of time within their bivalve hosts, but also produce numbers of cercariae comparable to those observed in other digeneans utilizing rediae and/or daughter sporocyst infrapopulations within the first intermediate host. Pinheiro and collaborators [11] described the unique nuclear morphology of the surface epidermal cells of the echinostomatid *Echinostoma paraensei* miracidia. Many mitochondria and vesicles contained in these epidermal cells were attached to the interepidermal ridges by a septate junction.

1.3 Sensory System

Trematodes exhibit a wide variety of sensory adaptations, including photosensory, chemosensory, and mechanosensory structures. Depending on the species and particular life stage, various structures may be observed at distinct times throughout the organism's life cycle.

Filippi et al. [12] observed two different types of sensory structures on the surface tegument of the hemiurid *Lecithochirium musculus*. Four dome-like fusiform structures containing nerve bulbs filled with electron-lucent material and mitochondria were present on the anterior dorsal surface of the tegument. Hemispherical electron-dense collars were observed at the top of the nerve bulbs. Striated rootlets were found just beneath the hemispherical electron-dense collars. The second type of sensory receptor observed existed in two variations: a bulb-like mono-lobed structure and a bulb-like bi-lobed structure. Both of these variations consisted of a nerve bulb containing mitochondria, electron-lucent material, and a conical electron-dense collar from which extended a striated rootlet. These sensory structures were observed mainly around the ventral sucker.

Abdul-Salam and Sreelatha [13] identified more than 13 types of sensory receptors on the surface tegument from cercariae of the trematode *Austrobilharzia* sp. The ciliated receptor types differed in cilium length and structure of the surrounding collar and tegumentary base. The authors described for the first time a multi-ciliated receptor found on a strigeid cercaria. Bogea and Caira [14–16] found that most cercarial sensory receptors on four different species of trematodes examined—*Allassogonoporus* sp. (Allassogonoporidae) [14], *Allopodocotyle* sp. (Opecoelidae) [15], *Crepidostomum* sp. (Allocreadiidae), *Bunodera* sp. (Allocreadiidae) [16]—were mechanoreceptors in nature. Podhorsky and coauthors developed an identification key based on papillary patterns for trichobilharzid cercariae. Some sensory receptors did not stain well with silver nitrate making visualization of all sensory receptors difficult [17]. Sopott-Ehlers and collaborators [18] studied the photoreceptors of cercariae of the schistosomatid *Trichobilharzia ocellata*. They described the presence of pigment cup ocelli, a special type of unpigmented rhabdomeric photoreceptor, as well as three unicellular photoreceptors that were found arranged in a three dimensional configuration. The special type of lensing observed in the

pigmented eyes of *T. ocellata* is believed to be exclusive to members of the genus *Trichobilharzia*, whereas the other phaosomous-like receptors observed on the cercariae are probably more widespread amongst members of the family Schistosomatidae. The absence of any mitochondrial lensing in *T. ocellata* and probably all other digeneans is believed to be a result of the evolution of endoparasitism [18].

Bogea [19] compared the ultrastructure and chaetotaxy of sensory receptors as well as the neuromorphology of cercariae belonging to three separate families: Allocreadiidae, Lecithodendriidae, and Opecoelidae. The results of the study indicate that the major categories of cercarial sensory receptors are nonciliated (including sheathed and subtegumentary types) and ciliated (including uncollared and collared types) receptors. Taxon-specific chaetotaxic patterns and receptor types observed in cercariae may aid in sorting out phylogenetic relationships between trematode families.

1.4 Neuromuscular System

Muscles that occur most consistently throughout the subclass Digenea are circular muscles lying just beneath the basal lamina of the tegument, with longitudinal and diagonal layers underlying the circular muscles [20]. These muscles envelop the rest of the body like a sheath.

Muscles are often most prominent in the anterior regions of the worm. Strands of muscles that connect dorsal to ventral superficial muscles are usually found in lateral areas. Muscle fibers are smooth and their nuclei occur in cytons called myoblasts connected to fiber bundles and located in various sites around the body, often in syncytial clusters [3].

The digenean nervous system consists of longitudinal nerve cords connected at intervals by transverse ring commissures. Several nerves run anteriorly originating from the cerebral ganglion. The dorsal, lateral, and ventral nerve cords supply posterior parts of the body. Ventral nerves are usually the most developed [3].

An important excitatory neurotransmitter is 5-hydroxytryptaine, and acetylcholine is apparently the major inhibitor of neuromuscular transmission [21]. Neuropeptides serve a relatively unknown role within the nervous systems of trematodes. Some authors believe that they may serve as messenger systems that regulate and control a variety of bodily processes. There is evidence that some neuropeptides help coordinate complex muscular activities involved in the formation of eggs in the oogenotop [21]. Stewart et al. [22] showed that all three classes of neuroactive substances were present in both developing progenetic metacercariae and adult worms of the bucephalid *Bucephaloides gracilescens*. Staining patterns for cholinergic and peptidergic substances showed significant overlap while the serotoninergic system was confined to a separate set of neurons. The most abundant neuropeptide known in trematodes is the pancreatic polypeptide-like neuropeptide F [23]. FMRFamide-related peptides (FaRPs) are also present in

trematodes. With respect to neuropeptide diversity, trematode species possess only one or two distinct FaRPs. FaRP bioactivity in trematodes appears to be restricted to myoexcitation [23].

Halton and Maule [24] analyzed many aspects of the morphology and function of the neuromuscular system in trematodes. The trematode nervous system consists of an archaic brain and associated pairs of longitudinal nerve cords cross-linked as an orthogon by transverse commissures. These structures are in continuity with an array of peripheral nerve plexuses that innervate a well-differentiated gridwork of somatic muscle as well as a complexity of myofibers associated with organs of attachment, feeding, and reproduction. Neuronal cell types are mainly multi- and bipolar and highly secretory in nature. The contents of these secretions have been identified cytochemically to include all three major types of cholinergic, aminergic, and peptidergic messenger molecules. A landmark discovery in flatworm neurobiology was the biochemical isolation and amino acid sequencing of two groups of native neuropeptides: neuropeptide F and FaRPs. Both families of neuropeptides are abundant and broadly distributed in platyhelminths, occurring in neuronal vesicles in representatives of all major flatworm taxa. Dual localization studies have revealed that peptidergic and cholinergic substances occupy neuronal sets separate from those of serotoninergic components. FaRPs and 5-HT are myoexcitatory, while cholinomimetric substances are generally inhibitory. There is immunocytochemical evidence that FaRPs and 5-HT have a regulatory role in the mechanism of egg assembly. Muscle responses to FaRPs are mediated by a G-protein-coupled receptor. The signal transduction pathway for contraction involves the second messenger cAMP and protein kinase C.

Neuropeptides are ubiquitous intercellular signaling molecules in all metazoans with nervous systems. Immunochemistry studies have revealed that neuropeptides are widespread and abundant in the nervous systems of helminth parasites. FaRPs have been identified in trematodes and are strongly myoactive. The absence of FaRPs from vertebrates suggests that compounds with a high affinity for FaRP receptors are likely to have selective effects against helminths and, if protected against degradation, could have therapeutic potential [25].

Tolstenkov and co-workers [26] found that most morphological features of the opisthorchiid *Opisthorchis felineus* remain constant between cercariae, metacercariae, and adult worms. However, the diameter of individual muscle fibers increases distinctly in the adult worms. The general pattern of 5-HT IR fibers in cercariae, metacercariae, and adult *O. felineus* remains the same. Despite the large increase in body size, the number of 5-HT IR neurons remains almost the same in cercariae and metacercariae and only a modest increase in the number of neurons was observed in the adult worms. Sebelova and coauthors [27] compared the neuromuscular system ultrastructure and innervation patterns amongst various life stages of the echinostomatid *Echinostoma caproni*. The authors found that FaRP expression in the innervation of the ootype wall in adult worms was demonstrated only in post-ovigerous worms and not in pre-ovigerous worms. These findings suggest that FaRP neuropeptides may be involved in the process of egg assembly. Muscle organization and innervation patterns in trematodes appear to be highly conserved.

Stewart et al. [28] observed that the male reproductive tract was established in advance (day 3) of the female system (day 4) in the two strigeids *Apatemon cobitidis* and *Cotylurus erraticus*. The authors also observed that the longitudinal muscle fibers of the female tract appeared prior to the outer and more densely arranged circular muscles. FaRPs are believed to regulate egg assembly in platyhelminths.

Tolstenkov and co-workers [26] found that the proportion of 5-HT IR neurons in comparison to body mass was greatest in actively moving cercariae of the opisthorchiid *Opisthorchis felineus* when compared to metacercariae and adult worms of the same species. Tolstenkov et al. [29] studied and compared the neuromuscular morphologies of seven different cercariae. Specialized muscle fibers found in the cercariae of the psilostomatid *Sphaeridiotrema globulus* allow it to change the shape of its tail. In the seven species studied—*Echinostoma revolutum* (Echinostomatidae), *Cathaemasia hians* (Cathaemasiidae), *Psilochasmus oxyurus* (Psilostomatidae), *Sphaeridiotrema globulus* (Psilostomatidae), *Paramphistomum cervi* (Paramphistomatidae), and *Diplodiscus subclavatus* (Diplodiscidae)—no correlation between body size and the number of 5-HT immunoreactive neurons was observed. However, the size of the neurons followed the body size. Bogea [30] describes the process and benefits of the Sevier-Munger method for studying the innervation of tegumentary receptors in heterophyid cercariae. First, the cercariae were fixed in hot 5 % formalin. They were then developed in a solution of ammoniacal silver and 2 % formalin under the microscope for 10 min. Nerve cells were found to stain black against a pale gold background. Fine nerve fibers of the subsurface nerve plexus were observed. The fine nerve fibers sent distal branches from the plexus to the cercarial tegument. The branches, in turn, became fine nerve endings, projecting as receptors in the cephalic (5CIV(5), 2CV(2), and 2CV(4)), anterior (4AIL, 3AIIL, 2AIIIL), midbody (1ML, 3MV), posterior (1PIL, 1PIIL, and 1PIIIL), and caudal (2UD) regions of the cercaria. Visually, the Sevier-Munger method adequately demonstrates the association of cercarial tegumentary receptors with the subsurface nerve plexus.

Leksomboom and coauthors [31] used the acetylcholinesterase (AChE) reaction to show that the nervous system was very similar amongst metacercariae, migrating juveniles, and adult worms of the opisthorchiid *Opisthorchis viverrini*. The main components of the nervous system consisted of three pairs (dorsal, ventral, and lateral) of bilaterally symmetrical longitudinal nerve cords and two cerebral ganglia. Stewart et al. [28] found that 5-HT and FaRP (the flatworm FaRP GYIRFamide) were localize immunocytochemically in both the central and the peripheral nervous systems of developing worms.

1.5 Alimentary System

Within digeneans, the process of digestion is primarily extracellular in the caeca. Although their caeca do not apparently bear any gland cells, gastrodermal cells of trematodes may in certain species secrete some digestive enzymes. Proteases,

dipeptidases, an aminopeptidase, lipases, acid phosphatase, and esterases have all been reported [6]. Depending on the species, the gastrodermis of trematodes may be syncytial or cellular [6].

Ferrer et al. [32] used monoclonal anti-actin antibody immunoelectron microscopy to detect for the first time the presence of actin in the apical cytoplasmic projections of the digestive cell of the adult brachylaimid *Brachylaima* sp. Jones and co-workers [33] studied the digestive tract of the gyliauchenid *Gyliauchen nahaensis* and found that unlike most digeneans which possess expanded caeca for nutrient absorption, *G. nahaensis* possesses a reduced caecum but an unusually large foregut. Unusual morphological features of the alimentary tract include large apical projections present in the posterior regions of the pharynx that are believed to serve as filtration structures and elongate flask-shaped invaginations of the apical cytoplasm in the anterior and middle esophagus for surface amplification. The cell bodies associated with these flask-shaped invaginations are rich in secretory vesicles. It is proposed that these regions of increased surface area promote extracellular digestion. The atypical gut morphology of *G. nahaensis* coupled with the arrangement of its suckers is believed to be an adaptation to the predominantly herbivorous diets of its definitive hosts.

Podvyaznaya [34] described the development of the alimentary tract using cercariae of the bucephalid *Prosorhynchoides borealis*. The foregut and caecum primordial arise in early cercarial embryos as two additional cellular cords. The primordial pharynx appears as a cluster of myoblasts in the mid part of the foregut primordium whose proximal end abuts onto the ventral embryonic tegument. Later, a lumen develops within the gut primordial and their component cells form the embryonic cellular epithelium with an essentially similar structure in the foregut and caecal regions. The foregut epithelial cells merge to form a syncytium. The most proximal foregut area remains cellular for the longest period of time. The syncytial lining of the foregut establishes syncytial connections with secretory cytons differentiating in the surrounding parenchyma. These cytons produce secretory granules, which are transported through cytoplasmic connections to the foregut syncytium. Before cercariae reach maturity, their foregut epithelium becomes anucleate and continuous with the external tegument. By the end of cercarial development, numerous short lamellae appear on the luminal surface of the caecal epithelium. The caecal cells become involved in secretory activity as indicated by the presence of Golgi-derived secretory bodies in their cytoplasm. Podvyaznaya [35] described the development of the alimentary tract using cercariae of the diplostomid *Diplostomum pseudospathaceum*. The foregut primordium appears in early cercariae as a cellular cord. Later, the lumen develops within the foregut primordium, and its cells give rise to the cellular epithelium, limiting this lumen. During subsequent development, the lateral plasma membranes separating the cells disappear from the primary foregut epithelium, as do nuclei and most of the cellular organelles. Eventually the foregut lining becomes the thin anucleate syncytial layer. Each of two caecal branches appears to arise from a row of large cuboid cells. The primordial gastrodermal cells are involved in synthetic and secretory activity and give rise to numerous secretory inclusions. These inclusions release their

contents into the cavities that develop between adjacent primordial caecal cells. The intercellular cavities gradually increase in size and fuse to eventually form a single caecal lumen. In mature cercariae, the large caecal lumen packed with electron-dense secretory material is limited by a thin cellular gastrodermis, displaying no secretory features.

Ramasamy and collaborators [36] observed the unusual structure of the digestive tract from metacercariae of the clinostomid *Euclinostomum multicaecum*. The digestive tract has several main lateral caeca that divide further posteriorly and give rise to numerous smaller branches that are distributed throughout the fluke. The multicaeca are believed to aid in nutrient absorption during rapid and prolonged feeding directly following encystment. The caecal wall consists of a syncytial gastrodermal epithelium, which bears loop-like lamellae that extend into the lumen and enclose spherical inclusion bodies, perhaps increasing the potential absorptive surface area. Fibrous basal lamina and an underlying layer of muscle fibers support the gut caeca. Parenchymal cells occupy much of the extracellular space.

1.6 Respiratory System

Takamiya and co-workers [37] found that the troglotrematid *Paragonimus westermani* possesses three different types of mitochondria that function selectively, rather than obligatorily, and are distributed throughout its tissues. Small mitochondria that possessed both cytochrome c oxidase activity and many cristae were localized in the tegument and tegumental cells. Two types of larger parenchymal mitochondria, some with many cristae and others with lesser amounts, exhibited fumarate reductase activity.

1.7 Excretory System

The protonephridia of digeneans are termed flame bulbs (or flame cells). They are enclosed flask-shaped units each containing a tuft of fused cilia. The fused cilia beat rapidly, encouraging excretory fluid to flow into and out of the flame bulb by way of a pore. The flagella are surrounded by rodlike extensions of the flame cell, which extend between similar projections of the proximal tubule cell [38]. These interlacing rods form a lattice-like weir, which serves as a filter. Additionally, a thin membrane can often be found between the rods of the lattice structure. The beating of the flagella in turn creates a pressure gradient that draws fluid through the weir and into the collecting tubule [3]. Aggregations of flame cells join collecting ducts that eventually feed into an excretory bladder. In adult trematodes the contents of the excretory bladder leave through a pore. In digeneans, the excretory pore is almost always located on the posterior end of the worm [3]. In addition to waste removal by the excretory system, wastes are also removed via diffusion across the tegument and

epithelial lining of the gut. Exocytosis of vesicles within the cytoplasm also takes place, flushing waste products produced within the organism outwards [3]. Urea, uric acid, and predominantly ammonia are the nitrogenous waste products reported from trematodes [3].

Hanna and coauthors [39] identified intracytoplasmic hydrolytic activity of the sustentacular tissue, proposing that scavenging of effete cells and cytoplasmic debris most likely aids in osmoregulation within the tubules of the fasciolid *Fasciola hepatica*. The presence of numerous mitochondria and smooth endoplasmic reticula suggests an increased amount of micromolecular nutrients, metabolites, and excretory products being moved both inward and outward of the sustentacular tissue. Poddubnaya et al. [40] reported for the first time, multiple contact sites (septate junctions and zonulae adherents) between the membranes of the terminal and adjacent canal cells in the protonephridial terminal organ of a digenean. Septate junctions were observed to traverse the epithelial cytoplasm of the canal wall. Similar septate junctions were also observed within the cytoplasmic cord at the level of the tip of the flame tuft in both longitudinal and oblique sections. The similarities observed in the morphology of the protonephridial terminal organ between both the digeneans studied—*Azygia lucii* (Azygiidae) and *Phyllodistomum angulatum* (Gorgoderidae)—and the aspidogastrean *Aspidogaster limacoides* (Aspidogastridae) suggests a close relationship between these two subclasses. Sue and Platt [41] observed several distinct morphological features within the excretory bladder of *Thrinascotrema brisbanica* n. g., n. sp. in turn erecting the new family Thrinascotrematidae (Digenea: Plagiorchiida) for this species. The shape and extent of the excretory bladder, the stenostomate arrangement of the excretory collecting ducts within the adult worm, metacercaria and cercaria, as well as the cercarial protonephridial formula $2(12+12+12)+(12+12+12)$ makes this species morphologically distinct.

Niewiadomska and Czubaj [42] described for the first time heterocellular gap junctions (nexus) between tegumental cytons and paranephridial canal walls in metacercariae of the diplostomid *Diplostomum pseudospathaceum*.

1.8 Reproductive System

1.8.1 Male Reproductive System

Most trematodes (excluding members of the family Schistosomatidae) are hermaphroditic, with some species being capable of self-fertilization. Cross-fertilization, however, is the most ubiquitous form of reproduction. A few cases of parthenogenesis have also been reported [43–45]. Within the host adult worms find each other by means of chemoattractants and, except in schistosomes, the active compound appears to be a free sterol, presumably cholesterol, or a closely related steroid [3].

The male reproductive system of most trematodes usually consists of two testes. Some species have only one testis, while others may possess up to a dozen. The shape of the testes also varies depending on species, with some being round and others being more branchlike. Each testis has a vas efferens that connects with others to form a vas deferens, eventually connecting to a genital pore located within the genital atrium, usually located on the midventral surface. An internal seminal vesicle, whose purpose is to store sperm, can be found along the vas deferens. The male copulatory organ, called the cirrus, is located near the internal seminal vesicle. It can be constricted to form an ejaculatory duct and be evaginated to transfer sperm to the female reproductive system. The ejaculatory duct is often surrounded by prostate gland cells [3].

Yang et al. [46] examined the ultrastructure of the sperm and the process of fertilization in the schistosomatid *Schistosoma japonicum* [46]. The authors observed the sperm tail to be a single flagellum with a unique axoneme, originating from a centriole, whose structure includes two types—a $9\times2+1$ in the main part of the flagellum and $9\times2+0$ design near the end of the flagellum. The sperm ultrastructure of *S. japonicum* is unusual when compared to that of other Digenea, in that the layout of the sperm axoneme houses two different configurations coupled with the fact that the striated root is absent in this species. This suggests that the evolution of *S. japonicum* is quite distant from other digeneans. According to a study, mating in the schistosomatid *S. mansoni* worms occurs before the maturation of the sexual organs. At 3 weeks post-infection, a majority (59 %) of the male worms had begun to form their gynaecophoric canals, although testicular lobes and tegumental tubercles were absent. At 4 weeks post-infection, 77.2 % of the *S. mansoni* male worms studied had developed testicular lobes with active germinative cells and 26 % of these male worms had begun developing tegumental tubercles [47]. Souza and collaborators [48] examined the reproductive system of the echinostomatid *Echinostoma paraensei*. It was found to be fully functional in hamsters by day 14-post-infection. Skirnisson and coauthors [49] outlined the morphology of the reproductive system of the schistosomatid *Trichobilharzia regenti*. Male worms were found in greater abundance than females. Specimens collected from experimentally infected hosts were smaller than those obtained from naturally infected hosts [49]. Foata and coauthors [50] are the first workers to describe the ultrastructure of spermatozoa and the process of spermiogenesis within a trematode belonging to the family Deropristidae. Spermiogenesis in the species studied, *Deropristis inflata*, was found to follow that of most other trematodes with the exception of an electron-dense region on the centriole. The authors determined that the external ornamentations of the plasmic membrane, as well as the distal part of the nucleus in front of those of the mitochondria, could be used to help identify the species. Seck et al. [51] studied the ultrastructure of the spermatozoan and the process of spermiogenesis in the trematode *Carmyerius endopapillatus*. This is the first study to use transmission electron microscopy to observe a member of the family Gastrothylacidae. Hanna and co-workers [39] identified intracytoplasmic hydrolytic activity of the sustentacular tissue, proposing that this

mechanism of recycling useful molecules may aid in the maturation of the spermatozoan in the fasciolid *Fasciola hepatica*. Quilichini and collaborators [52] were the first researchers to perform an ultrastructural study of spermiogenesis and the spermatozoan morphology of the allocreadiid *Crepidostomum metoecus*. The spermatozoan was characterized by an anteriorly located lateral expansion, the presence of spinelike bodies, and the unusual attribute of possessing two mitochondria instead of one (as is the case with most digenean spermatozoa). It has been noted that spermiogenesis in the opecoelid *Helicometra fasciata* undergoes unusual flagellar rotation, greater than 90°. Similar to the morphological characteristics reported by Quilchini et al. [52], an anteriorly located lateral expansion of the spermatozoan was observed. Additionally, a centriolar derivative was also reported [53]. It has been found that male specimens of the schistosomid *Schistosoma mansoni* possessed different testicular lobe morphologies based on whether it was a unisexual infection or a mixed infection. Male worms in unisexual infections would often partner up with one another. Male worms found within the gynaecophoric canals of other male worms expressed smaller testicular lobes, suckers, and overall body length and width when compared to their partners [54]. Levron et al. [55] studied the process of spermiogenesis and the ultrastructure of the spermatozoan in the opecoelid *Poracanthium furcatum*. Within the spermatozoan the posterior part of the centriole is unusual in that it comprises a central element. Levron and co-workers [56] initiated the first ultrastructural study of spermiogenesis and the spermatozoan of a digenetic trematode belonging to the family Zoogonidae, *Diphterostomum brusinae*. Distinguishing characteristics of the spermatozoan of *D. brusinae* are external ornamentations of the plasma membrane and the anterior and posterior extremities [56]. Levron et al. described the distinguishing characteristic of a centriolar extension on the spermatozoan of the monorchiid *Monorchis parvus*. Another unusual characteristic of the spermatozoan in *M. parvus* which sets it apart from the spermatozoan of other digenetic trematodes is the presence of two mitochondria instead of one [57]. Ndiaye et al. [58] described the first time the simultaneous presence of dorsolateral cytoplasmic expansion, external ornamentation of the plasma membrane, and spinelike bodies in the spermatozoan of the fasciolid *Fasciola gigantica*, were observed during spermiogenesis. The same observations were also made in an earlier report [59], focusing on *F. hepatica*. Ndiaye and coauthors [59] described the phenomenon of the mitochondrion migrating to the median cytoplasmic process before the fusion of the second axoneme during spermiogenesis in the brachylaimid *Scaphiostomum palaearcticum*. Seck et al. [60] described the characteristic traits of the spermatozoan of the paramphistomatid *Paramphistomum microbothrium*—external ornamentations located anteriorly and a lateral expansion exhibiting a "spine-like body." This study marks the first instance in which a spinelike body has been described within the spermatozoan of a digenetic trematode. Neves et al. [61] reported the occurrence of reproductive system abnormalities in both male and female *Schistosoma mansoni* (Schistosomatidae) worms. The cause of these deformities was unknown but was believed to be genetic in nature.

1.8.2 Female Reproductive System

The female reproductive system of most trematodes consists of a single ovary connected to a ciliated oviduct with a proximal sphincter or ovicapt, regulating the passage of ova. A seminal receptacle forms as an outpocketing of the wall of the oviduct. At its base is a slender tube, termed the Laurer's canal, which may either end blindly in the parenchyma or open through the tegument. It is believed that the Laurer's canal is a vestigial vagina [3]. The vitelline glands are located in chambers adjacent to the ovary and connect by way of the oviduct. Vitelline cells produced within the vitelline glands provide oocytes with yolk. Following the inclusion of vitelline cells, the oocyte then continues along the egg-forming apparatus, or oogenotop, to another chamber termed the ootype. Within the ootype, tiny unicellular glands termed Mehlis' glands deposit their products onto the oocyte. The Mehlis' glands feed into the ootype through many tiny ducts within the lining of the oogenotop. Following this step, the oocyte continues along towards the uterus. During this time, shell granules begin to coalesce around the oocyte and accompanying vitelline cells and secretions. The distal end of the uterus is often quite muscular, termed the metraterm, serving as an ovijector and as a vagina. The female genital pore opens near the male genital pore, usually together within the genital atrium [3].

Neves et al. observed both the male and female reproductive systems of adult *Schistosoma mansoni* (Schistosomatidae). They found that in female worms the ootype is lined by thick cuboidal epithelial cells with plaited bases and nuclei with flabby chromatin, marking a clear distinction between the ootype epithelium and that of the uterus. This feature suggests that each cell lining the ootype represents an individual gland [54]. Biolchini and co-workers observed that by week three, post-infection in Swiss Webster mice, ovaries were beginning to develop within one third of all the female *Schistosoma mansoni* (Schistosomatidae) worms examined. Developing ovaries were observed in 69 % of the female worms examined on week four. Maximum growth of the suckers in female worms was observed at week four [47]. D'Avila et al. were the first to describe the general morphology of the gametes as well as the cells of the glands associated with the reproductive apparatus of the two eucotylids *Tanaisia bragai* and *T. inopina* [62]. Yang and collaborators [46] examined the process of fertilization in the schistosomatid *Schistosoma japonicum*. Fertilization occurred in the posterior part of the oviduct in an area of the oviduct where lamellae were absent. Some cortical granules were observed to fuse with the plasma membrane and discharge their contents onto the surface of the fertilized ovum. The remaining cortical granules were seen to dissolve into the cytoplasm. By the secondary mature division, the secondary oocyte divided to form a female pronucleus. Soon after, the female pronucleus and the male pronucleus came together to form a zygote. Swiderski and coauthors described in detail the process of vitellogenesis and egg production in the microphallid *Maritrema feliui*. The authors listed the different types of glycogen and lipids present throughout the process [63]. Stewart et al. [22] showed that FaRPs were responsible for neuronal control of the muscles of

the female reproductive tract during egg assembly in the bucephalid *Bucephaloides gracilescens*. Poddubnaya and coauthors [64] compared the ultrastructure of the uterus of the aspidogastrid *Aspidogaster limacoides* to those of two digenetic trematodes, the gorgoderid *Phyllodistomum angulatum* and the azygiid *Azygia lucii*. The uterine linings were quite thin within all three species examined except for the perinuclear region of the epithelial cells. Septate junctions occurred between adjacent epithelial cells within the uterine wall. The similarity amongst the uterine walls of all three species was postulated as an ultrastructural marker potentially showing a close phylogenetic relationship between the Aspidogastrea and the Digenea. *Azygia lucii* (Azygiidae) exhibited a high level of vesicular exocytotic activity in the epithelial cytoplasm when compared to that of *Phyllodistomum angulatum* (Gorgoderidae), perhaps a symptom of the level of egg emission as well as the presence of non-ciliated, non-free swimming, and non-free living miracidia within this species [64].

Greani et al. gives a generalized description of vitellogenesis. A generalized description of vitellogenesis is as follows: (1) Vitellocytes have a cytoplasm mainly filled with ribosomes and very few mitochondria (2) An increase in endoplasmic reticula and Golgi complexes occurs (3) Shell globules are produced and coalesce into clusters; some glycogen particles are also present (4) Mature vitellocytes are filled with shell globule clusters and generally contain a large lipid droplet. Glycogen particles are grouped at the periphery of the cell [65].

Southgate and collaborators [66] showed that development of the female reproductive system in some dioecious trematodes belonging to the genus *Schistosoma* does not depend on species-specific pairing. In mixed infections worms may change mates and when the opportunity arises heterospecific pairs of worms will change partners to conspecific pairs. Interspecific pairing in adult schistosomes will lead to either hybridization or parthenogenesis. Although hybridization does occasionally occur in mixed infections within the definitive host, certain isolating mechanisms have been proposed: specific mate recognition, site selection within the host, heterologous immunity. Pre-zygotic isolating mechanisms are geographical isolation and host specificity.

1.9 The Effects of Drug Treatment on Trematode Body Systems

Due to the undesired affects, both potential and observed, caused by parasites on their hosts, many drug treatments have been tested and used successfully on trematodes. These drug treatments frequently induce both chemical and in turn physical changes to the trematode. Digenean body systems affected by drug treatments do not function in their normal manner. Alterations to trematode body systems induced by drug treatment are described below.

It has been observed that certain proteins isolated from a surface protein fraction taken from the fasciolid *Fasciola hepatica* showed strong homology with the

following proteins: AKT-interacting protein (*Xenopus tropicalis*), sterol *O*-acyltransferase 2 (*Homo sapiens*), and integrin beta 7 (*Mus musculus*). The authors suggest that these proteins could be possible candidates for future control strategies [7]. Shalaby et al. demonstrated irregular tegumental distortion and ruptured sensory papillae in the paramphistomid *Paramphistomum microbothrium* when treated in vitro with the antimalarial drug artemether [67].

Ferraz et al. [68] tested the effects of varying doses of the anthelmintic drug praziquantel on the echinostomatid *Echinostoma paraensei* when developing inside a mouse host. The doses of 50 and 100 mg/kg of praziquantel eliminated all the worms. Lesser doses caused contraction of the body morphology with vacuolization of the parenchyma, retraction of tegumental spines and the peristomic collar, disorganization of the vitelline glands, as well as the development of vesicles and peeling of the tegument.

Bhardwaj and collaborators used RNA interference to suppress the expression of the tegumental phosphodiesterase SmNPP-5 in schistosomules and adults of the schistosomatid *Schistosoma mansoni*. Injecting parasites into mice tested the effects of suppressing expression of the SmNPP-5 gene in vivo. It was found that, unlike controls, parasites whose SmNPP-5 gene was demonstrably suppressed at the time of host infection were greatly impaired in their ability to establish infection. The results of this experiment show that SmNPP-5 is a virulence factor in schistosomes [69].

Veerakumari and coauthors [70] tested the effects of *Acacia arabica* on the surface ultrastructure and morphology of the paramphistomid *Cotylophoron cotylophorum*. Severe vacuolization in the parenchyma and oral sucker, lesions on the testis, and craterlike distortions on the tegument were observed.

De Oliveira et al. [71] tested the effects of various concentrations of the essential oil of *Baccharis trimera* on adult *Schistosoma mansoni* (Schistosomatidae) worms. Male worms were found to be more susceptible to drug treatment when compared to female worms. A decline in worm motility and eventual mortality was observed after 30 h of exposure to the highest concentration of the essential oil, 130 μg/mL. At lesser concentrations, peeling of the tegument, destruction of tubercles, tegumentary spines, and the oral and acetabular suckers were observed in both male and female worms.

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Chapter 2
The Systematics of the Trematoda

Aneta Kostadinova and Ana Pérez-del-Olmo

2.1 Introduction

The Trematoda Rudolphi, 1808 are a class of the phylum Platyhelminthes that comprises two subclasses, the Aspidogastrea Faust & Tang, 1936 and the Digenea Carus, 1863. The subclass Aspidogastrea is a small group (4 families, 12 genera considered valid, *c*. 80 species) parasitic in molluscs, fishes and chelonians [1, 2]. Aspidogastreans like the digeneans use molluscs as first obligate hosts but are characterised by being external rather than internal parasites of these hosts, and by having a single-generation life-cycles lacking asexual reproduction and a stage comparable to the cercaria [2–4]. Key information on the aspects of morphology, life-cycles, taxonomy, systematics and phylogeny of the aspidogastreans can be found in Rohde [1, 2, 5, 6], Gibson [3], Gibson and Chinabut [7] and Zamparo and Brooks [8].

The subclass Digenea comprises a large and diverse group (*c*. 2,500 nominal genera, *c*. 18,000 nominal species; see [9]) of cosmopolitan platyhelminths that are obligatory parasitic in invertebrate intermediate and vertebrate definitive hosts. Digeneans are found in all vertebrate classes but are less diverse in agnathans and chondrichthyans [10, 11]. The subclass is characterised by a number of autapomorphies, associated with the unique complex digenean life-cycle: (i) acquisition of a

A. Kostadinova (✉)
Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic
e-mail: aneta.kostadinova@uv.es; kostadinova@paru.cas.cz

A. Pérez-del-Olmo
Unitat de Zoologia Marina, Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Parc Científic, Universitat de València, Aptdo 22085, 46071 Valencia, Spain
e-mail: ana.perez-olmo@uv.es

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vertebrate host as a terminal addition to the life history; (ii) alternation of sexual and asexual reproductive generations; (iii) a series of asexual generations within the first intermediate host (typically mollusc); (iv) free-swimming cercaria with a tail; (v) tiers of ectodermal cells on the miracidium; (vi) lack of digestive system in the miracidium and mother sporocyst [3, 4, 12]. For details and apomorphies at lower taxonomic levels see the review by Cribb et al. [4]. Although the complexity of digenean life-cycles may have influenced the expansion of the Digenea rendering it the most speciose group among Platyhelminthes [12], the mainstay of digenean systematics has been the information obtained from examination of the sexual generation, i.e. the adults from vertebrates [13].

The classification of the Digenea has long been a challenge especially because of the difficulties in establishing relationships and finding diagnostic characters for identification keys of the higher taxa [3, 14, 15]. Thus whereas most groupings established at lower taxonomic levels using adult morphology have been widely accepted, the search of apparent non-homoplasious morphological characters at the higher taxonomic levels has been the subject of debate and (sometimes heated) discussions (for details, see Gibson [3], Pearson [16], Gibson and Bray [14]).

The early attempts for classification of the digeneans relied upon sucker arrangements initially at the generic level, i.e. *Monostoma* Zeder, 1800, *Distoma* Retzius, 1786, *Amphistoma* Rudolphi, 1801 and *Gasterostomum* von Siebold, 1848, were unsatisfactory [15] whereas later treatments have incorporated more morphological characters including features of the daughter sporocyst/redia and/or cercaria, and life history patterns [17–23]; see Gibson [3] for a detailed discussion on the aspects of the evolution of the Trematoda.

2.2 Keys to the Trematoda

Perhaps one of the most important endeavours of this century in the field of digenean taxonomy is the publication of the *Keys to the Trematoda*, a series on the systematics and identification of the platyhelminth class Trematoda [24–26]. The three volumes provide detailed historical background and novel concepts for the systematics and taxonomy at the generic and suprageneric levels and a reappraisal of the generic diagnoses via re-examination of type- and/or other representative species. Considering just these two aspects makes the series an essential unique source of information on the Trematoda well into the twenty-first century. Furthermore, although the superfamily was treated as the basic unit of classification, the editors have made a substantial effort towards a classification reflecting a natural system of the Digenea considering morphological evidence in conjunction with phylogenies inferred from molecular data. This provides a sound basis for future molecular studies addressing phylogenetic relationships at the suprageneric level.

There are 148 families with 1,577 genera considered valid in the *Keys to the Trematoda*. An examination of the distribution of generic diversity (estimated as the number of valid genera) across digenean superfamilies related to their complexity

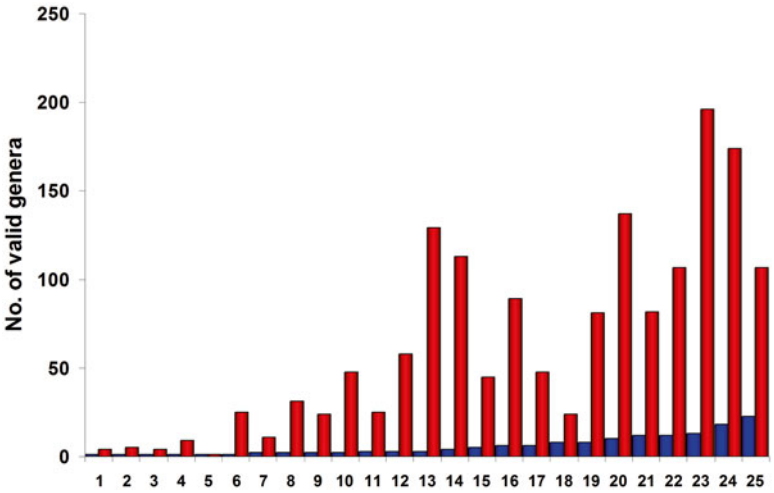

Fig. 2.1 The distribution of digenean generic diversity (assessed as the number of the genera considered valid, *red bars*) along a gradient of increasing superfamily complexity (assessed as the number of constituent families, *blue bars*). Data extracted from the *Keys to the Trematoda* [24–26]. *Order of superfamilies*: 1, Azygioidea; 2, Bivesiculoidea; 3, Transversotrematoidea; 4, Haplosplanchnoidea; 5, Heronimoidea; 6, Bucephaloidea; 7, Clinostomoidea; 8, Haploporoidea; 9, Microscaphidioidea; 10, Monorchioidea; 11, Cyclocoeloidea; 12, Schistosomatoidea; 13, Opisthorchioidea; 14, Allocreadioidea; 15, Gymnophalloidea; 16, Diplostomoidea; 17, Pronocephaloidea; 18, Brachylaimoidea; 19, Echinostomatoidea; 20, Lepocreadioidea; 21, Gorgoderoidea; 22, Paramphistomoidea; 23, Hemiuroidea; 24, Microphalloidea; 25, Plagiorchioidea

(estimated as the number of families) illustrates a general trend of association between the two estimates (Fig. 2.1). The lower extreme of the complexity gradient is represented by six monotypic superfamilies [1–6, characterised by poor generic richness (1–9 genera with only superfamily Bucephaloidea Poche, 1907 containing 25 genera)]. The upper extreme comprises the most complex superfamilies, i.e. the Hemiuroidea Looss, 1899, Microphalloidea Ward, 1901 and Plagiorchioidea Lühe, 1901 (comprising 13–23 families), with generic richness varying between 107 and 196 genera. The variability in the middle range is due to two patterns indicating the need of further systematic work. Thus two superfamilies, the Opisthorchioidea Looss, 1899 and the Allocreadioidea Looss, 1902, are characterised by a large number of genera (129 and 113, respectively) whose familial affiliations require further scrutiny. Notably, these are among the superfamilies recovered to contain paraphyletic taxa, i.e. Heterophyidae Leiper, 1909 + Opisthorchiidae Looss, 1899 (see Olson et al. [27], Thaenkham et al. [28]) and Opecoelidae Ozaki, 1925 + Opistholebetidae Fukui, 1929 [27], respectively, and this supports our suggestion (also see below).

The second pattern observed in Fig. 2.1 is associated with a relatively low generic richness that is unequally distributed among the families: Gymnophalloidea Odhner, 1905 (42 genera among 5 families); Pronocephaloidea Looss, 1899 (48 genera among 6 families); and Brachylaimoidea Joyeux & Foley, 1930 (24 genera

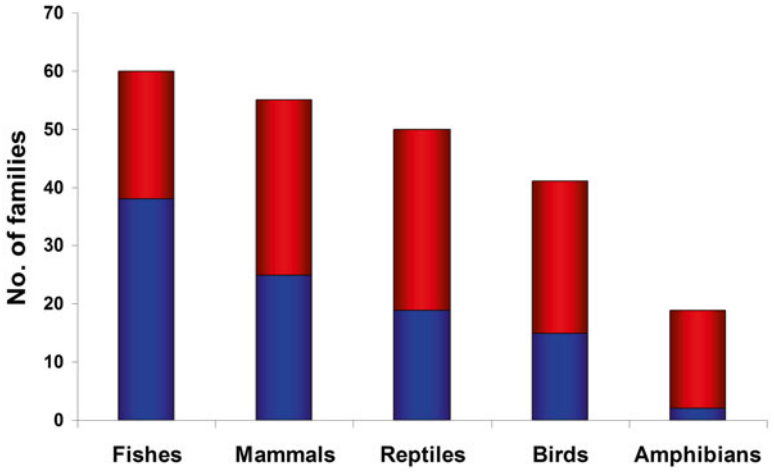

Fig. 2.2 The distribution of digenean diversity (assessed as the number of families) among the major definitive host groups (data from Bray [9]). Highlighted in *blue* are the digenean families found exclusively in a given host group, the remaining (highlighted in *red*) are found in more than one group

among 8 families). The large number (relative to content) of the families within these higher taxa indicates higher rates of "splitting" and the molecular phylogeny of Olson et al. [27] has demonstrated that this is the case on at least one occasion, i.e. the Brachylaimidae Joyeux & Foley, 1930 was recovered as paraphyletic, with the Leucochloridiidae Poche, 1907 nested. However, a molecular-based hypothesis based on denser taxon sampling within these superfamilies is required to test this suggestion.

Regarding the distribution of digenean taxonomic diversity among the major definitive host groups, 99 (67 %) of the digenean families are parasitic in a single vertebrate group (highlighted in blue in Fig. 2.2) whereas the remaining are found in more than one host group (highlighted in red in Fig. 2.2). Data from Bray [9] plotted in Fig. 2.2 illustrate that the highest number of the digenean families that occur in fishes are exclusively fish parasites whereas the number of families found only in amphibians is the lowest; the remaining vertebrate groups occupy intermediate position with respect to their exclusive associations with the digenean families.

2.3 Molecular Approaches to Digenean Phylogeny: Higher Taxa

Molecular data from nucleic acid sequences represent a useful source of independent data for phylogenetic inference. As predicted by Gibson and Bray [14], our understanding of the diversity, systematics and evolutionary relationships of the Digenea

has substantially advanced as a result of the expansion of molecular-based studies in the past 15 years. Ribosomal RNA (rRNA) genes (and their spacer regions) and mitochondrial (mt) genes have been the most popular markers used in the systematic studies of digeneans at several nested taxonomic scales; this is associated with the varying rates of evolution of the gene regions. Whereas rRNA genes have been used for inferring relationships at higher taxonomic levels, the internal transcribed spacer (ITS1 and ITS2) separated by the 5.8S rRNA gene has been utilised for exploring species boundaries in a range of studies related to 155 species of 19 digenean families (see Nolan and Cribb [13] for an exhaustive review). Another relatively recent review on the advances and trends in the molecular systematics of the parasitic platyhelminths covers comprehensively studies on the Digenea at several taxonomic scales [29]. Here we highlight selected examples with significant contribution towards the development of the molecular phylogeny of the Digenea at the higher taxonomic levels rather than provide an account of the investigations at the lower levels.

The first studies of the relationships within the Digenea at the suprageneric scale used the most conserved of the nuclear rRNA genes, the gene encoding the 18S subunit [30–33]. Soon focus has been placed on the 5′ variable domains (D1–D3) of the 28S rRNA gene as suitable data source for inferring relationships at several levels, i.e. among species, genera and closely related families [34], and the first studies at the suprageneric level proved to be influential.

Tkach et al. [35] developed a molecular phylogeny of one of the most diverse digenean groups, the formerly recognised suborder Plagiorchiata, based on partial 28S rDNA sequences for 28 species of 13 families. They demonstrated the derived position of the Plagiorchiata in relation to all major digenean lineages considered in their analysis. Tkach et al. [35] also found that Plagiorchiata of the hypotheses based on morphological and life-cycle characters [22, 36–38] is paraphyletic and suggested as a solution the exclusion of the superfamilies Opecoeloidea, Dicrocoelioidea and Gorgoderoidea. These authors considered the Plagiorchiata *sensu stricto* to comprise the superfamilies Plagiorchioidea (including the Plagiorchiidae Lühe, 1901, Haematoloechidae Freitas & Lent, 1939, Telorchiidae Looss, 1899, Brachycoeliidae Looss, 1899 and Leptophallidae Dayal, 1938) and Microphalloidea (including the Microphallidae Ward, 1901, Prosthogonimidae Lühe, 1909, Lecithodendriidae Lühe, 1901 and Pleurogenidae Looss, 1899). Their analyses also indicated close relationships between the genera *Macrodera* Looss, 1899 and *Paralepoderma* Dollfus, 1950, *Leptophallus* Lühe, 1909 and *Metaleptophallus* Yamaguti, 1958, and *Opisthioglyphe* Looss, 1899 and *Telorchis* Lühe, 1899. The first four genera were later (in the *Keys to the Trematoda*) placed in the family Leptophallidae [39] and the latter two were accommodated within the family Telorchiidae [40].

In an updated analysis of partial 28S rDNA sequences using a larger number of diverse taxa (51 species belonging to 27 families), Tkach et al. [41] assessed the relationships of Plagiorchiata with 14 digenean families. The results of their study confirmed the main groupings (and their content), i.e. the Plagiorchioidea and Microphalloidea, found in Tkach et al. [35] and revealed a basal position of the families Schistosomatidae Stiles & Hassall, 1898, Diplostomidae Poirier, 1886, Strigeidae Railliet, 1919, Brachylaimidae and Leucochloridiidae and a sister-group

relationship between the Renicolidae Dollfus, 1939 and Eucotylidae Cohn, 1904, both associated with the superfamily Microphalloidea.

Tkach et al. [42] used partial 28S rDNA sequences to explore the phylogenetic interrelationships of 32 species belonging to 18 genera and four families of the superfamily Microphalloidea with members of the Plagiorchioidea (eight species of six genera) as outgroups. They demonstrated that the representatives of the Microphalloidea form three main lineages corresponding to the families Lecithodendriidae, Microphallidae and Pleurogenidae + Prosthogonimidae and suggested synonymies at the generic level (*Floridatrema* Kinsella & Deblock, 1994 with *Maritrema* Nicoll, 1907, *Candidotrema* Dollfus, 1951 with *Pleurogenes* Looss, 1896, and *Schistogonimus* Lühe, 1909 with *Prosthogonimus* Lühe, 1899). Whereas the first synonymy was not accepted by Deblock [43], the latter two were considered in the *Keys to the Trematoda* [44, 45].

All of the above mentioned studies concern solving pieces of the puzzle of digenean relationships at higher taxonomic levels. The first step to a more inclusive analysis of digenean phylogeny is that of Cribb et al. [10] who attempted a combined evidence approach using morphological characters for all stages of the digenean life-cycle and complete 18S rDNA sequences for 75 digenean species of 55 families. Analyses of this first morphological dataset with a published character matrix identified the Bivesiculidae Yamaguti, 1934 + Transversotrematidae Witenberg, 1944 as the sister group to the remainder of the Digenea and the Diplostomoidea Poirier, 1886 + Schistosomatoidea Stiles & Hassall, 1898 as the next most basal taxon. The combined evidence solution of Cribb et al. [10] was found to exhibit greater resolution than morphology alone with the predominant effect of the molecular data on tree topology. Analyses of the combined data found no support for a basal position of the Heronimidae Ward, 1917 and revealed that the earliest divergent digeneans include the Diplostomoidea (Diplostomidae and Strigeidae) and Schistosomatoidea (Sanguinicolidae and Schistosomatidae) with the Transversotrematidae and Bivesiculidae progressively less basal.

Although Cribb et al. [10] found poor resolution of higher digenean taxa, the relationships at the superfamily level were well resolved. These include the superfamilies:

- The Hemiuroidea, with the Azygiidae Lühe, 1909 as basal, the Sclerodistomidae Odhner, 1927, Accacoeliidae Odhner, 1911, Syncoeliidae Looss, 1899, Derogenidae Nicoll, 1910 and Didymozoidae Monticelli, 1888 in one clade, and the Hemiuridae Looss, 1899 (recovered as paraphyletic) and Lecithasteridae Odhner, 1905 in the other.
- The Paramphistomoidea Fischoeder, 1901 [including the Paramphistomidae Fischoeder, 1901, Diplodiscidae Cohn, 1904, Microscaphidiidae Looss, 1900 (as Angiodictyidae Looss, 1902) and Mesometridae Poche, 1926].
- The Opisthorchioidea (including the Cryptogonimidae Ward, 1917, Heterophyidae and Opisthorchiidae).
- The Echinostomatoidea Looss, 1899 (including the Echinostomatidae Looss, 1899, Fasciolidae Railliet, 1895, Philophthalmidae Looss, 1899 and Cyclocoelidae Stossich, 1902).

- The Acanthocolpoidea Nahhas & Cable, 1964 (including the Acanthocolpidae Lühe, 1906, Campulidae Odhner, 1926 and Nasitrematidae Ozaki, 1935).
- The Lepocreadioidea Odhner, 1905 [with the Lepocreadiidae Odhner, 1905 (recovered as paraphyletic), Enenteridae Yamaguti, 1958 and Gyliauchenidae Fukui, 1929 but not the Apocreadiidae Skrjabin, 1942 which grouped with the Haploporoidea Nicoll, 1914 and Monorchioidea]; there was no support for a close relationship between the superfamily Haploporoidea and the Haplosplanchnidae Poche, 1926.
- The Haploporoidea (the Haploporidae Nicoll, 1914 and Atractotrematidae Yamaguti, 1939).

Cribb et al. [10] found weak support for Fellodistomoidea (containing the Tandanicolidae Johnston, 1927 and Fellodistomidae Nicoll, 1909) and the Plagiorchioidea (containing a subgroup formed by the Plagiorchiidae, Brachycoeliidae and Cephalogonimidae Looss, 1899; and Microphallidae, Pachypsolidae Yamaguti, 1958, Zoogonidae Odhner, 1902 and Faustulidae Poche, 1926). On the other hand, the Opecoelidae and Opistholebethidae Fukui, 1929 were strongly related as well as there was a strong sister relationship between the Monorchiidae Odhner, 1911 and the enigmatic genus *Cableia* Sogandares-Bernal, 1959 which has variously been placed in the Lepocreadiidae, Opecoelidae, Enenteridae and the Acanthocolpidae.

The most comprehensive phylogeny of the Digenea to date is that of Olson et al. [27]; it is also the first re-evaluation of relationships at higher taxonomic levels that has affected digenean classification. These authors estimated digenean relationships after adding a substantial number of novel sequences for complete 18S and partial (variable domains D1–D3) 28S rRNA genes (80 and 124, respectively). Their combined dataset which was found to yield the most strongly supported results thus comprised a rich and diverse array of taxa representing all major digenean groups (163 species of 77 families) (see Table 2.1). One important outcome of this study is the first molecular-based classification proposed based on the results from Bayesian analysis of the combined dataset; the authors went further by considering in association of putative synapomorphies that add morphological or ontological support to the molecular data.

Generally the molecular phylogenetic analyses of Olson et al. [27] supported the most recent classification of the Digenea provided in the *Keys to the Trematoda* at the familial and superfamilial levels (but see differences in superfamilial placements highlighted in Table 2.1) but provided strong evidence for a different subdivision (and membership in some cases) at the higher taxonomic levels. This has led to the recognition of a number of new taxa at the ordinal and subordinal levels (one order and nine suborders; see Table 2.1).

Important in the new classification is the reflection that the molecular phylogeny of the Digenea does not support its traditional division into three groups at the ordinal level, i.e. the Strigeida La Rue, 1957, the Echinostomida La Rue, 1957 and the Plagiorchiida La Rue, 1957 [14, 18, 46]. Olson et al. [27] split the subclass Digenea into two major groups, the order Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003 and the order Plagiorchiida La Rue, 1957 (these were referred to

Table 2.1 Classification of the Digenea of Olson et al. [27] and of the *Keys to the Trematoda* (Gibson et al. [24], Jones et al. [25] and Bray et al. [26])

Olson et al. [27]		Keys to the Trematoda
Superfamily	Family	Different superfamilial placements and/or additional families
Order Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Suborder Diplostomata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Brachylaimoidea Joyeux & Foley, 1930	Brachylaimidae Joyeux & Foley, 1930 + Leucochloridiidae Poche, 1907[a]	Hasstielsiidae Hall, 1916; Leucochloridiomorphidae Yamaguti, 1958; Moreauiidae Johnston, 1915; Ovariopteridae Leonov, Spasskii & Kulikov, 1963; Panopistidae Yamaguti, 1958; Thapariellidae Srivastava, 1953
Diplostomoidea Poirier, 1886	Diplostomidae Poirier, 1886 + Strigeidae Railliet, 1919	Bolbocephaloididae Strand, 1935; Brauninidae Wolf, 1903; Cyathocotylidae Mühling, 1898; Proterodiplostomidae Dubois, 1936
Schistosomatoidea Stiles & Hassall, 1898	Schistosomatidae Stiles & Hassall, 1898 Clinostomidae Lühe, 1901 Sanguinicolidae von Graff, 1907 Spirorchiidae Stunkard, 1921	**Clinostomoidea Lühe, 1901** (also including Liolopidae Odhner, 1912)
Order Plagiorchiida La Rue, 1957		
Suborder Apocreadiata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Apocreadioidea Skrjabin, 1942	Apocreadiidae Skrjabin, 1942	**Lepocreadioidea Odhner, 1905**
Suborder Bivesiculata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Bivesiculoidea Yamaguti, 1934	Bivesiculidae Yamaguti, 1934	
Suborder Bucephalata La Rue, 1926		
Bucephaloidea Poche, 1907	Bucephalidae Poche, 1907	
Gymnophalloidea Odhner, 1905	Fellodistomidae Nicoll, 1909 Tandanicolidae Johnston, 1927	Gymnophallidae Odhner, 1905; Botulisaccidae Yamaguti, 1971

Suborder Echinostomata La Rue, 1926		
Echinostomatoidea Looss, 1899	Echinostomatidae Looss, 1899 + Fasciolidae Railliet, 1895	Calycodidae Dollfus, 1929; Cathaemasiidae Fuhrmann, 1928; Rhopaliidae Looss, 1899; Rhytidodidae Odhner, 1926
	Philophthalmidae Looss, 1899	
	Psilostomidae Looss, 1900	
	Cyclocoelidae Stossich, 1902	**Cyclocoeloidea Stossich, 1902** (also including Typhlocoelidae Harrah, 1922)
Suborder Haplosplanchnata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Haplosplanchnoidea Poche, 1926	Haplosplanchnidae Poche, 1926	
Suborder Hemiurata Skrjabin & Guschanskaja, 1954		
Azygioidea Lühe, 1909	Azygiidae Lühe, 1909	
Hemiuroidea Looss, 1899	Hemiuridae Looss, 1899 + Lecithasteridae Odhner, 1905	Bathycotylidae Dollfus, 1932; Dictysarcidae Skrjabin & Guschanskaja, 1955; Hirudinellidae Dollfus, 1932; Isoparorchiidae Travassos, 1922; Ptychogonimidae Dollfus, 1937; Sclerodistomoididae Gibson & Bray, 1979
	Accacoeliidae Odhner, 1911	
	Derogenidae Nicoll, 1910 (*Hemiperina* Manter, 1934; *Derogenes* Lühe, 1900)[b]	
	Didymozoidae Monticelli, 1888	
	Sclerodistomidae Odhner, 1927	
	Syncoeliidae Looss, 1899	
Suborder Heronimata Skrjabin & Schulz, 1937		
Heronimoidea Ward, 1917	Heronimidae Ward, 1917	
Suborder Lepocreadiata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Lepocreadioidea Odhner, 1905	Lepocreadiidae Odhner, 1905	Deropristidae Cable & Hunninen, 1942; Liliatrematidae Gubanov, 1953; Megaperidae Manter, 1934
	Enenteridae Yamaguti, 1958	
	Gorgocephalidae Manter, 1966	
	Gyliauchenidae Fukui, 1929	

(continued)

Table 2.1 (continued)

Olson et al. [27]		Keys to the Trematoda
Superfamily	Family	Different superfamilial placements and/or additional families
Suborder Monorchiata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Monorchioidea Odhner, 1911	Monorchiidae Odhner, 1911	
	Lissorchiidae Magath, 1917	
	Cableia Sogandares-Bernal, 1959	
Suborder Opisthorchiata La Rue, 1957		
Opisthorchioidea Looss, 1899	Heterophyidae Leiper, 1909 + Opisthorchiidae Looss, 1899	
	Cryptogonimidae Ward, 1917	
Suborder Pronocephalata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Pronocephaloidea Looss, 1899	Pronocephalidae Looss, 1899	Nudacotylidae Barker, 1916
	Labicolidae Blair, 1979	
	Notocotylidae Lühe, 1909	
	Opisthotrematidae Poche, 1926	
	Rhabdiopoeidae Poche, 1926	
Paramphistomoidea Fischoeder, 1901	Cladorchiidae Fischoeder, 1901	Paramphistomidae Fischoeder, 1901; Balanorchiidae Stunkard, 1925; Brumptiidae Stunkard, 1925; Choerocotyloididae Yamaguti, 1971; Gastrodiscidae Monticelli, 1892; Gastrothylacidae Stiles & Goldberger, 1910; Olveriidae Yamaguti, 1958; Stephanopharyngidae Stiles & Goldberger, 1910; Zonocotylidae Yamaguti, 1963; Zygocotylidae Ward, 1917
	Microscaphidiidae Looss, 1900 + Mesometridae Poche, 1926	**Microscaphidioidea Looss, 1900**
	Diplodiscidae Cohn, 1904	

Suborder Transversotremata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Transversotrematoidea Witenberg, 1944	Transversotrematidae Witenberg, 1944	
Suborder Xiphidiata Olson, Cribb, Tkach, Bray & Littlewood, 2003		
Allocreadioidea Looss, 1902	Opecoelidae Ozaki, 1925 + Opistholebetidae Fukui, 1929	Allocreadiidae Looss, 1902; Batrachotrematidae Dollfus & Williams, 1966
	Acanthocolpidae Lühe, 1906[b]	**Lepocreadioidea Odhner, 1905**
	Brachycladiidae Odhner, 1905	**Lepocreadioidea Odhner, 1905**
Gorgoderoidea Looss, 1899	Gorgoderidae Looss, 1899	Anchitrematidae Mehra, 1935; Braunotrematidae Yamaguti, 1958; Collyriclidae Ward, 1917; Cortrematidae Yamaguti, 1958; Mesocoeliidae Dollfus, 1929; Prouterinidae Foreyt, Schell & Beyer, 1996
	Callodistomidae Odhner, 1910	**Gymnophalloidea Odhner, 1905**
	Dicrocoeliidae Looss, 1899	
	Encyclometridae Mehra, 1931	**Plagiorchioidea Lühe, 1901**
	Haploporidae Nicoll, 1914 + Atractotrematidae Yamaguti, 1939	**Haploporoidea Nicoll, 1914**
	Orchipedidae Skrjabin, 1913	
	Paragonimidae Dollfus, 1939	
	Troglotrematidae Odhner, 1914	
Microphalloidea Ward, 1901	Microphallidae Ward, 1901 (*Maritrema* Nicoll, 1907, *Microphallus* Ward, 1901)	Anenterotrematidae Yamaguti, 1958; Diplangidae Yamaguti, 1971; Eumegacetidae Travassos, 1922; Exotidendriidae Mehra, 1935; Gyrabascidae Macy, 1935; Leyogonimidae Dollfus, 1951; Phaneropsolidae Mehra, 1935; Renschetrematidae Yamaguti, 1971; Stomylotrematidae Poche, 1926; Taiwantrematidae Fischthal & Kuntz, 1981
	Eucotylidae Cohn, 1904	**Cyclocoeloidea Stossich, 1902**

(continued)

Table 2.1 (continued)

Olson et al. [27]		Keys to the Trematoda
Superfamily	Family	Different superfamilial placements and/or additional families
	Lecithodendriidae Lühe, 1901	
	Pachypsolidae Yamaguti, 1958	
	Pleurogenidae Looss, 1899	
	Prosthogonimidae Lühe, 1909	
	Renicolidae Dollfus, 1939	
	Zoogonidae Odhner, 1902 + Faustulidae Poche, 1926	
Plagiorchioidea Lühe, 1901	Plagiorchiidae Lühe, 1901 (*Glypthelmins* Stafford, 1905, *Skrjabinoeces* Sudarikov, 1950, *Haematoloechus* Looss, 1899)	Dolichoperoididae Johnston & Angel, 1940; Echinoporidae Krasnolobova & Timofeeva, 1965; Gekkonotrematidae Yamaguti, 1971; Glypthelminthidae Cheng, 1959; Haematoloechidae Freitas & Lent, 1939; Leptophallidae Dayal, 1938; Meristocotylidae Fischthal & Kuntz, 1964; Mesotretidae Poche, 1926; Ocadiatrematidae Fischthal & Kuntz, 1981; Opisthogonimidae Travassos, 1928; Orientocreadiidae Yamaguti, 1958; Reniferidae Pratt, 1902; Styphlotrematidae Baer, 1924; Thrinascotrematidae Jue Sue & Platt, 1999; Urotrematidae Poche, 1926
	Auridistomidae Stunkard, 1924	
	Brachycoeliidae Looss, 1899	**Gorgoderoidea Looss, 1899**
	Cephalogonimidae Looss, 1899	
	Choanocotylidae Jue Sue & Platt, 1998	
	Macroderoididae McMullen, 1937	
	Omphalometridae Looss, 1899	
	Telorchiidae Looss, 1899	

Different superfamilial placements are indicated in bold; underlined are families for which molecular data are required

[a]Paraphyletic relationships in the analysis of Olson et al. [27] indicated with a +

[b]Polyphyletic in Olson et al. [27]

as superorders by Cribb et al. [4]) thus confirming the prediction of Gibson and Bray [14] and the results of Cribb et al. [10]. The Diplostomida comprises three superfamilies whereas the Plagiorchiida has a more complex structure with 13 suborders (referred to as orders by Cribb et al. [4] and Littlewood [12]) containing a total of 19 superfamilies (see Table 2.1 for details). The four more inclusive suborders in the phylogeny of Olson et al. [27] are:

- The Hemiurata Skrjabin & Guschanskaja, 1954 represented by two superfamilies, the Azygioidea Lühe, 1909 (monotypic) and the Hemiuroidea (seven families as in Cribb et al. [10], see above).
- The Bucephalata La Rue, 1926 represented by two superfamilies, the Bucephaloidea (monotypic) and the Gymnophalloidea (including two families).
- The Pronocephalata Olson, Cribb, Tkach, Bray & Littlewood, 2003 represented by two superfamilies, the Paramphistomoidea (including four families) and the Pronocephaloidea (including five families).
- The Xiphidiata Olson, Cribb, Tkach, Bray & Littlewood, 2003 represented by four superfamilies, the Gorgoderoidea (monotypic), the Microphalloidea (including nine families), the Allocreadioidea (including four families) and the Plagiorchioidea (including eight families; Table 2.1).

An important outcome of the development of a molecular phylogeny of the Digenea is that inferences can be made on the origins and evolution of the digenean life-cycle. Cribb et al. [4] used the hypothesis and the classification of Olson et al. [27] and life-cycle traits derived from a large database (*c*. 1,350 species) of information on the life-cycles for the Digenea to explore the evolution of the digenean life-cycle. Cribb et al. [4] inferred that gastropods were the basal host group for the Digenea, parasitism of bivalves being a result of host-switching that has occurred multiple times, and found no convincing evidence for a deep level coevolution between the major digenean clades and their molluscan hosts. Regarding the second intermediate hosts, these authors illustrated a great diversity with discontinuous distributions on the phylogeny of different host types and concluded that three-host life-cycles have been derived from two-host life-cycles and adopted repeatedly. With respect to definitive hosts, these authors suggested an origin for the Digenea in association with teleosts followed by host-switching into chondrichthyans and provided alternative explanations for parasitism in tetrapods based on the topologies of relationships within the Xiphidiata and Diplostomida.

2.4 Towards Greater and Focused Representation of Digenean Diversity in Phylogenies

The development of the molecular phylogeny of the Digenea coincided with that of the *Keys to the Trematoda* so that a full consensus with the classification based on the molecular results of Cribb et al. [10], Olson et al. [27] and Tkach et al. [35, 41, 42]

has been reached in the treatment of the five superfamilies in the third volume [9]. Overall, a comparison between the two classifications of the Digenea summarised in Table 2.1 shows a considerable congruence. The superfamilial placement of 12 families (8 %, highlighted in bold in Table 2.1) in the *Keys to the Trematoda* departed from that inferred from the molecular phylogeny of Olson et al. [27].

Although the analysis of Olson et al. [27] represents the broadest sampling of the Digenea to date (52 % of a total of 148 currently recognised digenean families), a number of omissions (families highlighted in Table 2.1) were depicted [27, 29]. We here comment upon the taxa involved in the molecular phylogeny of the Digenea in association with the content of the *Keys of the Trematoda* focusing on additional important omissions rather than on those previously identified in an attempt to outline the suprageneric taxa that require further exploration in a molecular phylogeny.

Olson et al. [27] did not include in their analysis representatives of the type-families of the Allocreadioidea (the Allocreadiidae Looss, 1902), Gymnophalloidea (the Gymnophallidae Odhner, 1905) and Paramphistomoidea (the Paramphistomidae). Therefore, the basis of each of these superfamilies was not actually established and should not be considered definitive; also see [47]. At the lower taxonomic level, the same problem exists, i.e. lack of data from the type-genera of the families Cryptogonimidae, Opisthorchiidae, Strigeidae and Plagiorchiidae. Notably, the first three taxa were recovered in clades in which paraphyly was detected [27]. The Plagiorchioidea represents a special case. Formally, the type-family has been sampled at the time of the study of Olson et al. [27]. However, the three genera whose representatives have been sequenced (*Glypthelmins* Stafford, 1905, *Skrjabinoeces* Sudarikov, 1950 and *Haematoloechus* Looss, 1899) were transferred to different families, recognised in 2008, i.e. the Glypthelminthidae Cheng, 1959 and the Haematoloechidae [48, 49]. Therefore, the Plagiorchioidea also needs re-establishment preferably based on molecular data from representatives of the type-genus *Plagiorchis* Lühe, 1899 of the type-family Plagiorchiidae.

A number of superfamilies characterised by high taxonomic diversity at the generic and suprageneric levels have been underrepresented in the broad phylogeny of the Digenea by Olson et al. [27]. These are (in order of increasing generic richness, data from the *Keys to the Trematoda*; see also Fig. 2.1): Echinostomatoidea, Diplostomoidea, Paramphistomoidea, Plagiorchioidea, Allocreadioidea, Opisthorchioidea, Lepocreadioidea and Microphalloidea.

Using only a small fraction of the actual generic/familial diversity in the phylogeny of the Digenea by Olson et al. [27] has typically led to problems in resolving relationships. Thus the family Echinostomatidae (correct name given in the taxonomic listing of the taxa studied and the trees but referred to as "Echinostomidae"(sic) elsewhere in the text; see [27]) was represented by just two genera, *Echinostoma* Rudolphi, 1809 and *Euparyphium* Dietz, 1909 (sequence for *Euparyphium melis*, a synonym of *Isthmiophora melis* (Schrank, 1788), see [50, 51]) and found to be paraphyletic. The family represents a diverse and complex group comprising 43 genera belonging to 10 subfamilies [51] and it is likely that denser sampling would lead to better resolution of the relationships within the superfamily Echinostomatoidea

(molecular data for 7 out of 81 genera currently available [27]); effort should also be focused on representation of the four families not sampled to date (Table 2.1).

The superfamily Diplostomoidea was represented by five out of 89 genera, two diplostomid (*Alaria* Schrank, 1788 and *Diplostomum* Nordmann, 1832) and three strigeid genera (*Apharyngostrigea* Ciurea, 1927, *Cardiocephaloides* Sudarikov, 1959 and *Ichthyocotylurus* Odening, 1969) and the members of these genera were found intermingled in the clade of Diplostomoidea; the type-genus of the Strigeidae was not sampled [27]. The assessment of the relationships within the superfamily therefore, requires further exploration based on a wider array of taxa including the type-genus of the family Strigeidae, *Strigea* Abildgaard, 1790; we also mark as important omissions the families Cyathocotylidae Mühling, 1898 and Proterodiplostomidae Dubois, 1936 (Table 2.1).

Although the Opisthorchioidea and one of its constituent families, the Cryptogonimidae, were resolved in the phylogeny of Olson et al. [27], the remaining two families were not since the Heterophyidae was recovered as paraphyletic with the Opisthorchiidae nested within it. Seven out of a total of 129 genera of the superfamily were sampled in their study but none of the type-genera of the three families; their re-establishment in a molecular phylogeny is therefore still in the pending state. Recently, Thaenkham et al. [28] added 18S rDNA sequences for species of three genera, *Haplorchis* Looss, 1899, *Procerovum* Onji & Nishio, 1916 and *Metagonimus* Katsurada, 1912, and examined the relationships within the Opisthorchioidea based on a wider generic representation (including a sequence for a species of *Opisthorchis* Blanchard, 1895, the type-genus of the Opisthorchiidae) but their analysis also recovered a paraphyletic relationship between the Heterophyidae and Opisthorchiidae, the latter nested within the former.

The diversity of the family Microphallidae, the type of the Microphalloidea, was underrepresented (2 out of 47 genera, i.e. *Microphallus* Ward, 1901 and *Maritrema*) in the study of Olson et al. [27]. These authors found that in some analyses the representatives of the two subfamilies, the Microphallinae Ward, 1901 and the Maritrematinae Nicoll, 1907, were split among different clades. Tkach et al. [42] added sequences for more representatives of the two genera and recovered the Microphallidae as monophyletic. Nevertheless, the complex structure and diverse content of the family still awaits exploration of relationships based on a much wider taxon sampling.

2.5 Integrated Approaches to Digenean Diversity, Taxonomy and Systematics

A review of the history of the development of studies on the most comprehensively studied digenean superfamilies (Hemiuroidea, Lepocreadioidea, Plagiorchioidea and Schistosomatoidea) indicates a framework that would lead to robust estimates of phylogeny: (i) systematic inventory of the group; (ii) detailed understanding of morphology; (iii) taxonomic revision; (iv) classification system; (v) molecular

phylogeny; (vi) revised classification. In this section, we shall illustrate the progress within this framework focusing on the superfamilial level.

Historically, the most extensively studied digenean higher-level taxon appears to be the Hemiuroidea, a highly diverse group of parasites found predominantly in marine teleosts but also in freshwater teleosts, elasmobranchs and occasionally in amphibians and reptiles [52]. The knowledge on species diversity accumulated over two centuries and focused sampling and revisionary work based on material from the North-East Atlantic have largely contributed to an improved classification of this large and heterogeneous group (13 families and 196 genera recognised in the *Keys to the Trematoda*). In a series of monographs Gibson and Bray provided original descriptions, detailed comments on the morphology and life-cycles, host-parasite records (including larval stages) and identification keys for all of the hemiuroid species recorded from the North-East Atlantic; these included representatives of the families Accacoeliidae, Azygiidae, Hemiuridae, Hirudinellidae Dollfus, 1932, Ptychogonimidae Dollfus, 1937, Sclerodistomidae and Syncoeliidae [53–55]. Gibson and Bray [56] revised the superfamily and proposed a classification and a hypothesis for the evolution of the Hemiuroidea based on the functional morphology of the adults; these authors also provided detailed definitions of hemiuroid structures and analysis on their systematic value and possible function based on original data. According to Gibson and Bray's [56] classification the Hemiuroidea is divided into 14 families: Accacoeliidae (with two subfamilies), Azygiidae (with two subfamilies), Bathycotylidae Dollfus, 1932, Bunocotylidae Dollfus, 1950 (with four subfamilies), Derogenidae (with three subfamilies), Dictysarcidae Skrjabin & Guschanskaja, 1955 (with three subfamilies), Hemiuridae (with nine subfamilies), Hirudinellidae, Isoparorchiidae Travassos, 1922, Lecithasteridae (with six subfamilies), Ptychogonimidae, Sclerodistomidae (with three subfamilies), Sclerodistomoididae Gibson & Bray, 1979 and Syncoeliidae (with two subfamilies). The studies of Gibson and Bray thus provided a much needed systematic framework to be evaluated with the aid of molecular evidence.

The first molecular phylogeny of the Hemiuroidea was based on the V4 variable domain of the 18S rRNA gene for 33 species representative of ten hemiuroidean families after the concept of Gibson and Bray [56] plus the Didymozoidae [31]. Analyses of Blair et al. [31] supported the monophyly of the Hemiuroidea as represented by the taxa sampled and revealed two main groups, one containing all members of the Hemiuridae and the lecithasterinae lecithasterids and one comprises the members of Derogenidae, Didymozoidae, Hirudinellidae, Sclerodistomidae, Syncoeliidae and Accacoeliidae whereas the Isoparorchiidae and the hysterolecithinae lecithasterids appeared separately close to the base of the hemiuroid tree and the Azygiidae fell outside the hemiuroid clade. Hemiuroids were well represented although with a lower number of taxa (18 species belonging to 7 families) in the phylogeny of the Digenea of Olson et al. [27]. Their analyses strongly supported the distinct status of the Hemiurata with Hemiuroidea and Azygioidea as separate superfamilies. Within the Hemiuroidea, the Derogenidae was recovered as polyphyletic and a paraphyletic relationship of the Hemiuridae and the Lecithasteridae was depicted (as in [10, 31]). Consequently the results of

the molecular phylogenies were considered in the *Keys to the Trematoda*: the Azygiidae was recognised at the superfamily level [57] and the Didymozoidae was included within the Hemiuroidea [52].

Recently, Pankov et al. [58] described a new bunocotyline genus *Robinia* Pankov, Webster, Blasco-Costa, Gibson, Littlewood, Balbuena & Kostadinova, 2006 and presented a phylogenetic hypothesis for the Bunocotylinae Dollfus, 1950 and the Hemiuroidea based on sequence data analyses of an increased number of taxa (from 22 species for complete 18S and partial 28S rRNA genes and from 37 species for the V4 domain of the 18S rRNA gene). Both molecular analyses confirmed the monophyly of the Hemiuroidea, its division into two major clades and the polyphyly of the Derogenidae, as in previous studies [10, 27, 31], and suggested that the Gonocercinae Skrjabin & Guschanskaja, 1955 (with two genera, *Gonocerca* Manter, 1925 and *Hemipera* Nicoll, 1913), may require a distinct familial status. The authors found poor support for the distinct status of the Lecithasteridae and Hemiuridae, following previous suggestions based on different sequence data sets [10, 27, 31]. The results of this study also indicated that increased taxon sampling for and analysing the V4 domain of the 18S rRNA gene separately, failed to resolve many monophyletic hemiurid subfamilies thus adding little to the study of Balir et al. [31]. Pankov et al. [58] suggested that much greater taxon sampling for both 18S and 28S genes is needed in order to test the consistency of the present classification system of the Hemiuroidea with the evolutionary relationships of its members.

The Lepocreadioidea is one of the complex and problematic digenean superfamilies. Ten families and 137 genera are recognised in the *Keys to the Trematoda* but molecular studies have demonstrated that three of these families (Acanthocolpidae, Apocreadiidae and Brachycladiidae Odhner, 1905) are not closely related to the Lepocreadiidae ([10, 27, 59]; see Table 2.1). Bray, Cribb and colleagues devoted a comprehensive series of studies (*c*. 50 papers) on the diversity of the Lepocreadioidea in marine teleosts, predominantly in the Indo-West Pacific and the North-East Atlantic, which resulted in detailed descriptions of a vast number of species (including many new), erection of new and/or reassessment of the existing genera and construction of identification keys to species and parasite-host and host-parasite lists (see Bray et al. [60] for a list of the most inclusive references). These data provided a sound basis for revisory work [61–66]. On the other hand, extensive sampling for molecular studies carried out in parallel with morphological assessments has supplied an admirable number of sequences for species from a wide range of genera. Bray et al. [60] assessed the phylogenetic relationships of representative species of the superfamily Lepocreadioidea using partial 28S rDNA and *nad*1 sequences for members of the families Lepocreadiidae (42 species), Enenteridae (6 species), Gyliauchenidae (6 species) and Gorgocephalidae Manter, 1966 (1 species), along with 22 species representing eight other digenean families. The study recovered the Lepocreadioidea as monophyletic, comprising six groups: three well-recognised families (Enenteridae, Gorgocephalidae and Gyliauchenidae) and three groups resulting from the partitioning of the Lepocreadiidae in the phylogenetic tree. The latter were recognised as families by Bray & Cribb [67] who also provided amended family diagnoses.

A similar increased effort to collect and characterise morphologically and/or molecularly representatives of the members of the Plagiorchioidea and Microphalloidea by Tkach and colleagues [68–71] has contributed significantly to our understanding of the relationships and family structures of these large taxa (see above). The results of the molecular phylogenies [35, 41, 42] are partially reflected in the family level classifications in the *Keys to the Trematoda* [39, 48, 49, 72]. However, the two superfamilies are far too large and still require sustained systematic research.

Augmented representation of the species/genera of blood flukes has also resulted in advancing the knowledge on the relationships within the superfamily Schistosomatoidea. Snyder & Locker [73] examined phylogenetic relationships among ten genera (*Austrobilharzia* Johnston, 1917, *Bilharziella* Looss, 1899, *Dendritobilharzia* Skrjabin & Zakharow, 1920, *Gigantobilharzia* Odhner, 1910, *Heterobilharzia* Price, 1929, *Orientobilharzia* Dutt & Srivastava, 1955, *Ornithobilharzia* Odhner, 1912, *Schistosoma* Weinland, 1858, *Schistosomatium* Tanabe, 1923 and *Trichobilharzia* Skrjabin & Zakharow, 1920) of the family Schistosomatidae using 28S rDNA sequences (variable domains D1–D2) and found two major clades, one comprising the genera *Schistosoma* and *Orientobilharzia* parasitic in mammals and one consisting of predominantly bird parasites. These authors suggested an Asian origin of *Schistosoma*. Snyder [74] expanded the data on the Schistosomatoidea by generating 18S and 28S rDNA sequences for species belonging to eight genera of the Spirorchiidae Stunkard, 1921. Phylogenetic analyses involving representatives of the order Diplostomida recovered Spirorchiidae as paraphyletic with three genera from marine turtles exhibiting a sister-group relationship with the Schistosomatidae whereas five genera from freshwater turtles were found to occupy basal positions in the phylogeny of the tetrapod blood flukes. This coupled with the basal position within the schistosomatid clade of the genera *Austrobilharzia* and *Ornithobilharzia*, both comprising species with marine life-cycles, led to a suggestion that schistosomatids arose after a marine turtle blood fluke ancestor successfully colonised birds [74]. Lockyer et al. [75] presented the most comprehensive phylogeny of the Schistosomatidae to date, based on the sequences of three genes, complete 18S and 28S rRNA and mitochondrial cytochrome *c* oxydase subunit 1 (COI), for 30 species representing ten of the 13 known genera and almost all species of *Schistosoma*. The phylogeny provided evidence for the validity of two of the four currently accepted subfamilies [76], the Gigantobilharziinae Mehra, 1940 (comprising the genera *Dendritobilharzia* and *Gigantobilharzia*) and the Schistosomatinae Stiles & Hassall, 1898 (including *Austrobilharzia*, *Heterobilharzia*, *Orientobilharzia*, *Ornithobilharzia*, *Schistosoma* and *Schistosomatium*) but not for the subfamily Bilharzeillinae Price, 1929 since the representatives of the *Bilharziella* and *Trichobilharzia* did not form a monophyletic clade. The study of Lockyer et al. [75] confirmed an Asian origin for *Schistosoma* and the position of *Orientobilharzia* within the *Schistosoma*. The nomenclatural change has recently been formally justified by Aldhoun and Littlewood [77] who transferred to *Schistosoma* the four species of *Orientobilharzia* they considered valid [as *Schistosoma bomfordi* Montgomery, 1906, *S. turkestanicum* Skrjabin,

1913, *S. dattai* (Dutt & Srivastava, 1952), *S. harinasutai* (Kruatrachue, Bhaibulaya & Harinasuta, 1965)] and provided an amended generic diagnosis of *Schistosoma* and a revised key to the subfamily Schistosomatinae.

2.6 Future Research Prospects

In conclusion, molecular phylogenetics appears key to understanding the evolution of the Digenea. Although there is an agreement that further effort is needed towards achieving an improved representation of digenean taxonomic diversity in molecular phylogenies [27, 29], challenges in selection of gene loci exist and a direction of efforts appear to have been clarified recently. It is apparent that molecular analyses of digenean relationships at higher taxonomic levels will continue to rely upon the 18S and 28S rDNA sequences because a rich database has already been acquired.

However, evidence has been accumulating recently that promotes the utility of complete 28S rRNA gene as phylogenetic marker and illustrates the benefits of improved phylogenetic signal when used in combination with 18S rRNA gene at different levels within and between metazoan taxa including platyhelminths, e.g. [75, 78, 79]. Lockyer et al. [79] examined the utility of this approach in resolving the interrelationships between the major flatworm clades and stressed that Bayesian inference and maximum likelihood appear to give more congruent trees than maximum parsimony with respect to traditional concepts [75]. Mallatt and colleagues [80, 81] have evaluated the phylogenetic relationships in Ecdysozoa (molting animals) using likelihood-based Bayesian inference on nearly complete 18S + 28S rDNA sequences and suggested that this may prove to be a combination of best genes and a tree-building method for reconstruction of ecdysozoan phylogenies. Waeschenbach et al. [82] used nearly complete 28S rDNA sequences (4,047–4,593 nt) in combination with complete 18S rDNA sequences (1,940–2,228 nt) and Bayesian analyses, to resolve cestode interrelationships at the ordinal level. They demonstrated that the addition of domains D4–D12 of 28S rRNA gene contributes to a substantial improvement of phylogenetic signal resulting in overall better nodal support, topology stability and greater resolution compared with previous molecular estimates of cestode interrelationships based on 18S + partial (domains D1–D3) 28S rRNA genes. With regard to digenean interrelationships, the pioneer study of Lockyer et al. [79] is a promising start especially because it is the first phylogeny inferred from a combination of three independent datasets (i.e. for 18S, 28S and COI).

Mitochondrial genomes may offer a wealth of homologous markers for both systematics and diagnostics, but in contrast to nuclear ribosomal genes, few mitochondrial genes have been tested because of the limited availability of PCR primers and the higher rates of evolution thus rendering them more suitable for resolving more recent radiations; see, e.g. [83] for a review. However, whole mitochondrial genome sequences have been shown to resolve deep-level relationships in many metazoan groups [84] and the use of mtDNA spanning multiple genes has been

considered promising [83]; also see Philippe et al. [85] for an in-depth focus on the use of genome-scale data in phylogenies. At the less inclusive taxonomic levels, modern genomic approaches may also provide an in-depth understanding of the patterns of speciation and construction of robust phylogenies as illustrated by the recent developments in the genetic research on species of the genus *Schistosoma*; see, e.g. [86–90].

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Part II
Trematodes of Interest in Human Health

Chapter 3
Schistosomiasis

Fred A. Lewis and Matthew S. Tucker

3.1 Introduction

In humans, several species of the trematode genus *Schistosoma* cause the disease schistosomiasis, or snail fever. The disease is also frequently referred to as bilharzia (and bilharziasis) to recognize Theodor Bilharz, the physician who first described the parasite in humans in 1851. Human schistosomiasis is an ancient disease. Reference to it has been found in Egyptian papyri, and calcified eggs have been discovered in Egyptian mummies from around 1200 BC. Schistosomiasis is one of the most important parasitic diseases of man, and it causes significant morbidity and mortality on several continents. The World Health Organization (WHO) estimates that schistosomiasis is transmitted in over 70 countries, throughout a wide belt of the tropics and subtropics [1]. The three major schistosomes infecting humans are *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*. It is difficult to estimate the number of schistosomiasis cases in the world, but the number is considered to be greater than 200 million, mostly in Africa [2]. By species, *S. haematobium* causes 130 million cases, *S. mansoni* causes 73 million cases, and *S. japonicum/mekongi* cause two million cases. Mortality rates are difficult to assess for schistosomiasis, although it has been estimated that schistosomes cause approximately 280,000 deaths per year [3]. As many as 150,000 of these infections can be attributed to *S. haematobium*, with the majority of these in sub-Saharan Africa. In addition to active infections, it is estimated that almost 800 million people worldwide are at risk for schistosomiasis [4].

Aside from death, schistosome infections can lead to numerous health problems, among them chronic disability, cognitive impairment, chronic anemia, abdominal pain, diarrhea and, for *Schistosoma haematobium* infections, urogenital

F.A. Lewis • M.S. Tucker (✉)
Department of Schistosomiasis, Biomedical Research Institute,
12111 Parklawn Drive, 20852 Rockville, MD, USA
e-mail: flewis@afbr-bri.com; mtucker@afbr-bri.com

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problems, including bladder dysfunction, hematuria, and correlation with bladder cancer. Schistosomiasis commonly presents itself as a chronic, gradually debilitating illness. Because of this feature and frequent coinfections with other helminths, it has always been difficult to determine the total public health impact of schistosomiasis. It may be more appropriate to measure the effect of schistosomiasis on human health by examining morbidity using the disability-adjusted life year (DALY) ranking system. One WHO report showed that schistosomiasis ranked fairly low on the DALY scale [5]. However, when researchers began taking into account the myriad health problems posed by schistosomiasis, estimates of disease burdens ranked schistosomiasis considerably higher [6], and now most investigators believe that the community impact of schistosomiasis is usually underestimated. Regardless of where schistosomiasis falls in the DALY and other ranking estimates, there is no doubt that it is a serious disease. Among parasitic diseases, it is probably second only to malaria in importance as a public health menace in tropical areas of the world.

The three major *Schistosoma* species infecting humans have different distributions [7]. *S. mansoni* is distributed widely throughout Africa; transmission also occurs in the Middle East and several countries of South America and some of the Caribbean islands. *S. haematobium* is widely distributed throughout Africa, frequently overlapping with *S. mansoni*, and also found in areas of the Middle East. *S. japonicum* is localized to Indonesia, China, and parts of Southeast Asia. Species of lesser importance are *S. mekongi* (Cambodia, Laos) and *S. intercalatum* (central and west Africa). Overall, around 80–90 % of the schistosomiasis cases worldwide occur in sub-Saharan Africa.

Schistosomiasis is often endemic in areas where other serious infectious diseases also have a huge impact. For example, diseases such as malaria and HIV/AIDS often have a much higher community profile, and consequently more resources devoted to their control. Schistosomiasis is usually grouped into that category of diseases called the neglected tropical diseases (NTDs), which also include numerous diseases caused by soil-transmitted helminths, protozoa such as trypanosomes, and a few other organisms [8]. The NTDs predominantly affect the human population that live in the poorest conditions and where treatment is usually difficult to acquire.

Being a waterborne disease, schistosomiasis is acquired by direct contact with the infective larvae (cercariae) that emerge from freshwater snail hosts. Where schistosomiasis is endemic, human contact with parasite-contaminated water is an unavoidable event of daily life. As a result of human manipulation of the local environment, such as the development of new irrigation projects or building of dams, the range of the disease can also spread easily by the establishment of new breeding sites for the snails. Also, man-made environmental modifications may benefit one schistosome species over that of another. A prime example of this can be seen in the recent history of schistosomiasis in Egypt [9]. There, with major water projects and the completion of the Aswan Dam in the mid-twentieth century, schistosomiasis caused by *S. mansoni* largely replaced that caused by *S. haematobium* along major

sections of the Nile river, probably reflecting ecological changes favoring those of *Biomphalaria alexandrina* (regional host of *S. mansoni*) over those of the host snails for *S. haematobium* (*Bulinus truncatus*).

There is a vast research literature devoted to schistosomiasis. These studies amply demonstrate the complexity of the parasite's life cycle, and point to why schistosomiasis is the challenging health problem that it is in developing countries. A single chapter on schistosomiasis cannot cover, in depth, all aspects of this complex disease, but we have tried to highlight those areas of particular interest that may be important for future research in the continuing efforts to control this devastating disease.

3.2 Systematics

Schistosomes, usually referred to as blood flukes, are members of the phylum Platyhelminthes (flatworms) of the class Trematoda and family Schistosomatidae. The name *Schistosoma* (i.e., split body) refers to the appearance of the adult male worm whose lateral edges fold to form a groove (gynecophoral canal) where the female worm resides. Schistosomes differ from other trematodes in that they are dioecious parasites. Interestingly, at the adult stage, there is an obvious sexual dimorphism between male and female worms. Schistosomes and related flukes infect a wide variety of definitive hosts, and currently 21 species are recognized within the genus *Schistosoma*. Of the five schistosome species most often associated with human infections, the most important clinically and most studied species are *S. mansoni*, *S. haematobium*, and *S. japonicum*. The two others are *S. intercalatum*, which is closely related to *S. haematobium*, and *S. mekongi*, a close relative of *S. japonicum*. For this review, we will concentrate on discussions of schistosomiasis caused by *S. mansoni*, *S. haematobium*, and *S. japonicum*.

The geographic distribution of schistosomiasis is necessarily limited to the distribution of the particular intermediate snail hosts the parasite utilizes. Since so much of the life history of schistosomes involves a mollusc, we should briefly mention the systematics of these snails. Within the phylum Mollusca there are two large families, in the class Gastropoda, that are important for our discussions. These families are the Pomatiopsidae and Planorbidae. The taxonomy of gastropods has been in a state of flux for the last several years, but for the purposes of our discussion, *S. japonicum* infects members of the Pomatiopsidae (genus *Oncomelania*). These snails have gills, many have an operculum, and the sexes are separate.

The planorbid snails that serve as intermediate hosts for *S. mansoni* and *S. haematobium* do not have gills or an operculum, and they are hermaphroditic. Members of the genus *Biomphalaria* are intermediate hosts for *S. mansoni*, and *Bulinus* spp. are intermediate hosts for *S. haematobium*.

As shown in Fig. 3.1, the mature planorbid *Biomphalaria* is typically larger than *Bulinus*, and both are a great deal larger than *Oncomelania. Biomphalaria* and *Bulinus* occupy some of the same ecological niches and are nearly always submerged in water, while *Oncomelania* is an amphibious snail, spending much of its time attached to vegetation or on soil above the water surface.

Fig. 3.1 Examples of intermediate snail hosts for the three major schistosomes infecting humans. From *left* to *right*, *Bulinus truncatus truncatus* (host for *S. haematobium*), *Biomphalaria glabrata* (host for *S. mansoni*), and *Oncomelania hupensis hupensis* (host for *S. japonicum*)

3.3 Biology of the Parasite and Life cycle

Historically, one of the major problems in defining the schistosome life cycles was discovering the suitable snail intermediate hosts in which they were transmitted. In fact, around 1900, there was some question whether a snail host was even involved [10]. Knowing that the trematode *Fasciola hepatica* required an intermediate snail host, workers began searching for a snail involvement for schistosomes as well. Of the three major human schistosome species, the life cycle of *S. japonicum* was the first to be fully described (1914, by Miyairi and Suzuki). Around that time, there was considerable controversy whether or not *S. mansoni* and *S. haematobium* were even two distinct species, but this argument was laid to rest by Leiper in 1915.

The life cycles of the major schistosomes are quite similar—the differences chiefly consist of the intermediate snail species involved, and differences in the tissue distribution within the definitive hosts. A life cycle schematic of schistosomiasis is given in Fig. 3.2. The life cycle stages will be discussed in more depth in the following sections, but a short summary is appropriate here. The schistosomes have an infective, free swimming larval stage (cercaria) that gains entry into the mammalian, definitive host by skin penetration. The parasites pair in the liver and migrate to either the mesenteric veins draining the intestines (*S. mansoni* and *S. japonicum*) or veins of the urogenital system (*S. haematobium*). Eggs laid by the mature female worm then pass from the body through feces or urine. Another larval stage (miracidium) hatches from the egg in fresh water and infects the intermediate snail host, whereby asexual reproduction occurs resulting in the infective cercariae. Figures 3.3 and 3.4 show electron micrographs of different life cycle stages of *S. mansoni*.

For reference, Table 3.1 lists sizes of the various life cycle stages for *S. mansoni*, *S. haematobium*, and *S. japonicum*.

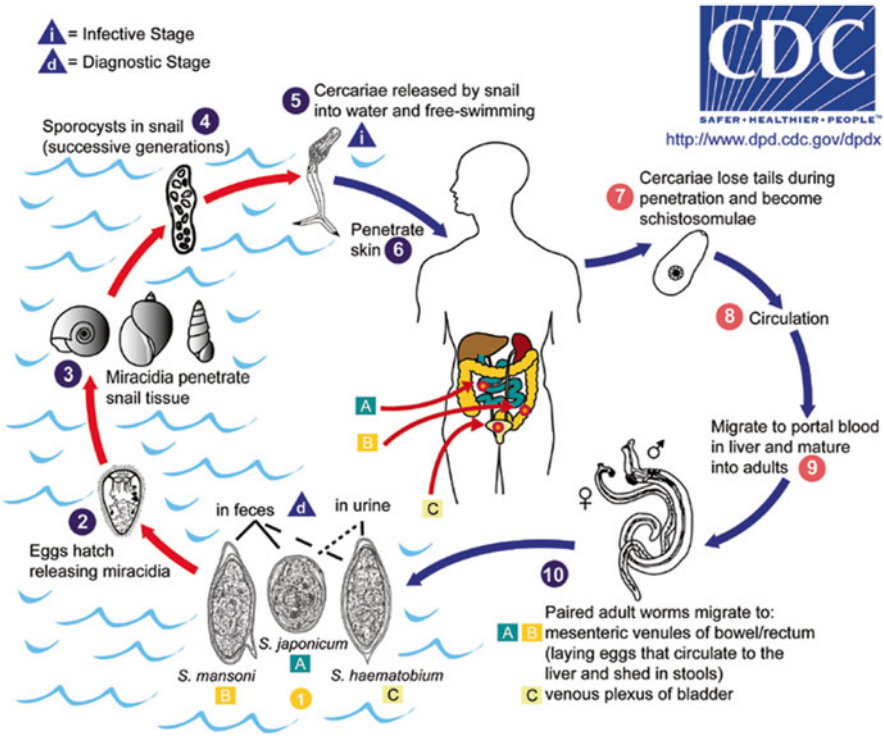

Fig. 3.2 The life cycle of human schistosomes (image courtesy of the Centers for Disease Control and Prevention, DPDx). The figure depicts several life cycle stages that are mentioned in the text. Key stages mentioned in the text include: miracidia, cercariae, schistosomules, adult worms, and eggs

3.3.1 Biology of the Various Life cycle Stages

3.3.1.1 Cercariae

The schistosome cercaria is a free-living, actively swimming stage with a relatively short infectious life span (24–48 h) whose sole purpose is to infect a suitable definitive host. The mature cercaria has two main segments. The first (body) is the progenitor of the adult worm, and is attached to an extremely muscular, bifurcated tail. The tail serves to propel the organism through the water column, and it can serve as a fulcrum to assist the body in gaining entrance into the skin of the definitive host. The length of a *S. mansoni* cercaria (Fig. 3.3a) is approximately 500 μm, but it contracts and elongates almost continuously. Among the many schistosome species, there are usually species-specific morphological and behavioral differences in the cercariae. For example, *S. mansoni* cercariae exhibit a discontinuous swimming pattern throughout the water column, never resting but for more than a few seconds

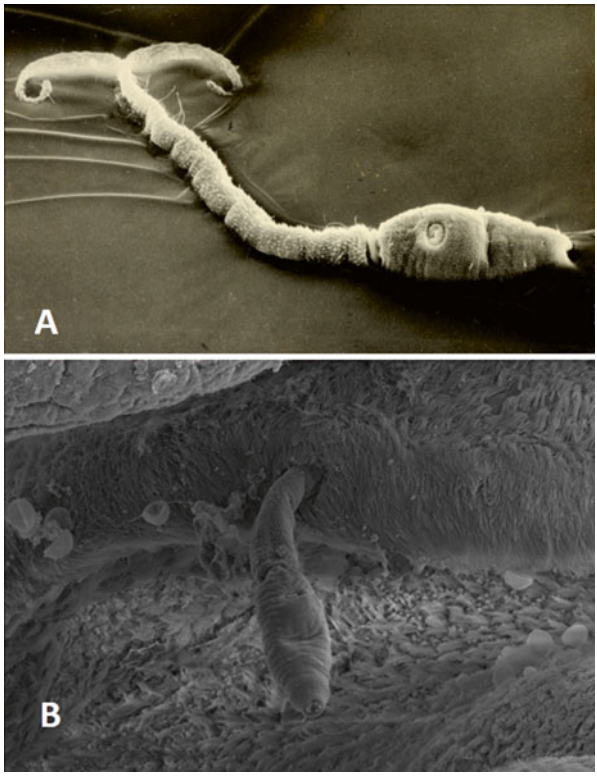

Fig. 3.3 Scanning electron micrographs of *Schistosoma mansoni* cercariae. (**a**) Complete cercaria showing body and tail portions. Since both body and tail are contractile, the overall length of this stage varies considerably, usually between 300 and 500 μm. The acetabulum (ventral sucker) is a circular structure in the body portion of the organism. From this position, the pre- and post-acetabular glands are defined. (**b**) Cercaria emerging from mantle collar tissue of *Biomphalaria glabrata*

before swimming again. In contrast, *S. japonicum* cercariae may swim to the surface and remain there, quiescent for several minutes at a time, yet still be fully infectious.

The cercaria of the most studied species (*S. mansoni*) possesses about 1,000 cells, with numerous cell types, ranging from sensory cells, muscle cells, nerve cells, support cells and others [11]. Many of the organ systems found in the adult worm are already formed, in miniature, in the body of the cercaria. The tail is heavily muscular, with an excretory duct running its entire length. This duct forks at the base of the tail furcus and empties out at terminal excretory pores.

A substantial part of the body volume is taken up by the acetabular glands and their contents. These glands (pre- and post-acetabular glands) are so named due to their relative position to the acetabulum (ventral sucker). These glands provide secretions that are involved in host skin penetration, and are exhausted soon after penetration, so they do not have counterparts in the adult worm. The morphology and various structures of this stage are well represented in various publications [11–13].

Upon emergence from a snail (see Fig. 3.3b), cercariae must find a suitable host within minutes. Fatty acids such as linoleic acid and amino acids such as arginine in the skin are important chemoattractants for the cercariae. When a cercaria comes in contact with the human skin surface, it will probe for an entrance site, often finding

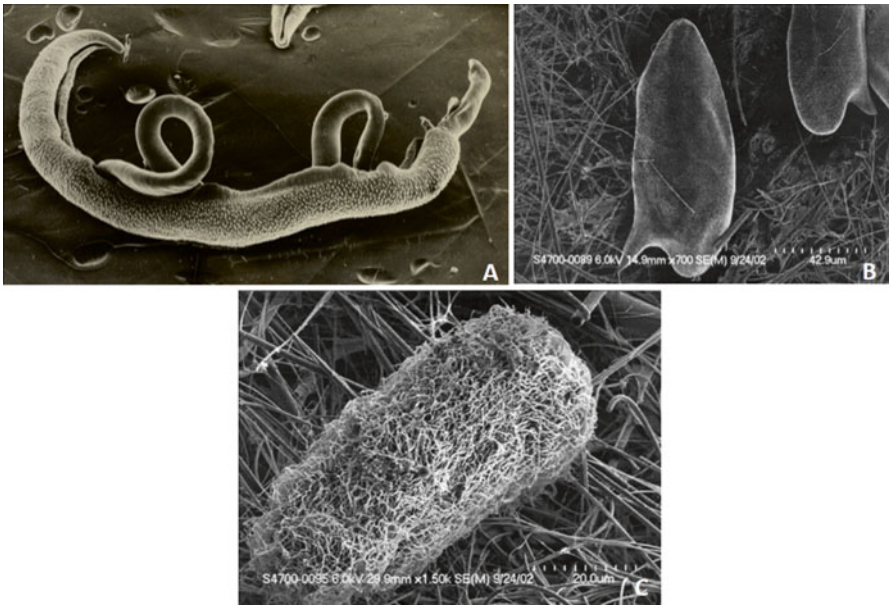

Fig. 3.4 Scanning electron micrographs of other *Schistosoma mansoni* life stages. (**a**) Adult male and female worms in copula. The female is residing in the gynecophoral canal of the male worm. Length of males ranges from 6 to 13 mm; that of females ranges from 10 to 20 mm. (**b**) Egg with characteristic lateral spine. Approximate dimensions are 140 μm (length) by 60 μm (width). (**c**) Miracidium with epidermal plates covered with cilia. Dimensions approximately 135 μm×55 μm

Table 3.1 Sizes of the various life cycle stages for *S. mansoni*, *S. haematobium*, and *S. japonicum*

	Adult male (mm)	Adult female (mm)	Cercaria (μm)	Egg (μm)
S. mansoni length	9–12	12–16	~500	140
S. mansoni width	1–1.2	0.15–0.20	30–40	60
S. haematobium length	12–14	16–20	~500	144
S. haematobium width	0.8–1.0	0.2–0.3	30–40	58
S. japonicum length	12–20	18–25	~500	81
S. japonicum width	0.5	0.2–0.3	30–40	63

one at the surface skin irregularities associated with hairs, pilosebaceous follicles, or other ridges or wrinkles [14, 15]. Droplets of secretion from the post-acetabular glands help serve as adhesive anchors for the body. The cercaria uses strenuous muscle activity to burrow into the crevice, as seen by a series of vigorous tail thrusts and expansion and contraction of the body. This entry is no doubt aided by the secretions of the penetration glands, in which numerous penetration enzymes are found. Often, multiple cercariae can be seen following one another into a breached opening. The tail detaches as the cercaria enters the outer layer of skin.

3.3.1.2 Schistosomules

Soon after entering mammalian skin, the body of the cercaria has to adapt from a fresh water environment to one bathed in tissue fluid. In order to do this, it undergoes a series of complex morphological and physiological changes. At this point, it is referred to as a schistosomule (or schistosomulum) [12, 16]. Among the more prominent changes is the formation of a double-layered, or heptalaminate, membrane, loss of the carbohydrate-rich glycocalyx, and becoming water intolerant. Depending on the species, the schistosomule resides in the skin from one to several days before entering the blood vasculature in the dermis. A small percentage can enter the lymphatics, then the bloodstream via the thoracic duct. Once in the bloodstream, it is thought that their migration to the blood capillaries of the lung is entirely passive. Due to the small diameter of the alveoli vessels, for the schistosomule to migrate from the venous to the arterial side, it undergoes a lengthening of the body and loses some mid-body spines. At this point, the organism has not appreciably changed in volume from that of a cercaria, but it is more elongated and morphologically appears worm-like.

Much of what we know about the schistosomule's early migration pathway is derived from studies in mice. Early studies of parasite recovery depended on dissecting tissues and counting the emerging worms at various time points after cercarial exposure. Due to the nature of this rather crude process, many organisms could not be accounted for. It was not until the early 1980s, when radiographic tracking techniques were developed, that a much clearer picture emerged regarding the relative lengths of time schistosomules could be found in the various tissues en route to the liver [17, 18]. Figure 3.5 shows a summarized pattern of the percentage of penetrating *S. mansoni* cercariae that were found in the skin, lungs and liver over time (data are the results of several published and unpublished studies). About 40 % of the cercariae result as adult worms in the mouse, with the remainder probably never reaching the liver, but recirculating in the blood and becoming trapped in other tissues. Whether a similar pattern of migration and percentage of mature worms (from invading cercariae) occurs in humans is a matter of conjecture.

3.3.1.3 Adult Male and Female Worms

In the mouse, the length of time for *S. mansoni* and *S. japonicum* to finally reach the liver differs; *S. japonicum* migrates there several days before those of *S. mansoni*. It is not until they do reach the liver, however, that they begin to feed and grow to the adult worm stage. Once they begin to grow the sexes can be easily distinguished (Fig. 3.4a). The male can develop fully in the absence of the female, but the female is stunted and does not achieve sexual maturity in the absence of the male. In a mature bisexual infection the male and female lie *in copula*, with the female lying within the gynecophoral canal of the male. This pairing is necessary for the physical and reproductive development of the female. It is believed that the worms pair in the liver before migrating to their final destination, that of the mesenteric veins

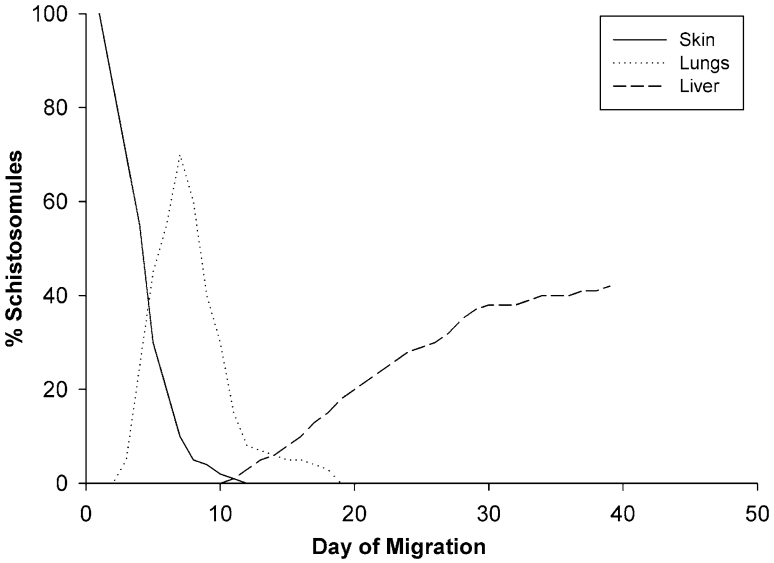

Fig. 3.5 Diagrammatic representation of a tracking experiment of *S. mansoni* schistosomules in the skin, lungs, and liver of mice. Cercariae were labeled with (^{75}Se)-selenomethionine, and tissues processed for autoradiography at various times after exposure to cercariae

(*S. mansoni* and *S. japonicum*), or the veins of the urogenital system (*S. haematobium*). The size of the adult worms varies somewhat between species and age of the worms (see Table 3.1). Male worms are shorter than the slender and elongated female worms, but they are very muscular and robust, with powerful suckers. This morphology allows the male worm to both carry the female and anchor the pair at the egg laying site in the mesenteric venules. Unpaired female worms will not reach the preferred egg laying destination without being paired with a male worm.

Adult worms can live in humans for several years. Based on several epidemiologic studies, the average life span of the adult worm is thought to be about 5 years. However, there have been several case reports demonstrating adult worms living 30 years or more, as shown by continued egg passage from infected individuals who no longer lived in endemic areas.

3.3.1.4 Eggs

Female schistosomes produce eggs continuously, and it has been reported that each *S. mansoni* mature female worm can produce up to 300 embryonated eggs/day. The egg morphology is diagnostic for each species. *S. mansoni* eggs are elongated with a prominent lateral spine near the posterior end. *S. japonicum* eggs are oval and smaller with a rudimentary (not always observable) lateral spine. Those of *S. haematobium* are elongated and possess a terminal spine at the posterior end.

Sizes of the egg for *S. mansoni* and *S. haematobium* are approximately the same, while *S. japonicum* eggs are not quite as large (Table 3.1). Each egg contains an embryo, the miracidium, which fills nearly the entire interior space of the egg. Figure 3.4b shows an electron micrograph of a *S. mansoni* egg.

For the egg to pass from the body and reach freshwater, it has to gain access from the venous blood system to the intestinal lumen (*S. mansoni* and *S. japonicum*) or the urogenital tract or bladder (*S. haematobium*). The female worm deposits eggs near the venous wall, and since the eggs are not motile, it is believed that they traverse the tissue walls by one or a combination of mechanisms. Egg-released enzymes probably play a large role in their migration through the tissue. Also, there is good evidence that the host's own inflammatory immune response (granulomas) serves to speed this migration along. What happens during this process is the trigger that sets the stage for development of the disease, schistosomiasis. The root of the problem is that not all of the eggs are excreted, and a great many are trapped in various tissues in the body. For *S. mansoni* and *S. japonicum*, these sites are the intestinal wall and liver. For *S. haematobium*, it would mainly be tissues of the urogenital tract. Most of the pathology in a schistosome infection results from the deposition of eggs in the tissues and the host's response to them. Later in this chapter we will devote more attention to the egg and the processes of this pathology.

3.3.1.5 Miracidia

Once the egg is eliminated through the feces or urine and reaches freshwater, the egg shell ruptures and the actively motile, multi-ciliated miracidium emerges. What causes egg hatching is not completely defined, but it is believed to be affected by changes in osmotic pressure and other factors [19]. The miracidium is one of the two free-living stages of the schistosome (the other being the cercaria). After hatching from an egg, the developmental success of the miracidium (Fig. 3.4c) depends on finding a suitable snail host before its energy stores (glycogen) are depleted, which occurs roughly 12 h after hatching.

The ability of miracidia to infect snails in large bodies of water, sometimes referred to as their "scanning power," has been studied in the lab and the field—often leading to conflicting results. Much of the success of the miracidium may have to do with the combination of its positive phototaxis and the particular dispersion of snails, which, for *Biomphalaria* spp., are often more concentrated near the shallow edges of water bodies. However, there is evidence that some chemoattractive response of the miracidium exists when it is in close proximity to the snail. The miracidium will probe the soft tissues of the snail, usually the margins of the head-foot or the tentacles, to search for a site through which it can penetrate most easily. With the aid of penetration enzymes and vigorous burrowing activity, it can gain complete entry into the soft tissues within minutes.

During entry into the snail, the miracidium loses its multi-ciliated plates (Fig. 3.4c) and soon thereafter stops migrating through the tissue. Comparable to the situation in which a cercaria needs to transform to a schistosomule, the miracidium must also undergo changes to adapt to the tissue environment of the snail.

The miracidium transforms into the stage referred to as the primary (mother) sporocyst. From this point forward, the reproduction of the schistosome in the snail is asexual. The primary sporocyst stage is essentially a sac-like, breeding chamber within which numerous secondary (daughter) sporocysts begin forming. Around 2 weeks after miracidial penetration, secondary sporocysts escape from the primary sporocyst and migrate to the hepatopancreas and gonads. Around 2 weeks after settling in these nutrition-rich locations the secondary sporocysts can each give rise to thousands of cercariae. Once cercariae become fully developed, they emerge from the secondary sporocysts, migrate to the anterior end of the snail, and burst into the surrounding water from the margins of the soft tissues, usually the head-foot, mantle collar, and pseudobranch (at least for *B. glabrata*). Under optimum conditions, an infected snail can release hundreds of thousands of cercariae over its lifetime. The astounding productivity of the schistosome in the snail drives home the point that it may only take a small number of infected snails in a local water body to maintain that area as an active transmission site.

What we know about genetics of the snail and parasite is important here, since their interrelationship has a tremendous impact on the epidemiology of the disease. Most of the research on the development of the parasite in the snail host has been conducted with the *S. mansoni*/*B. glabrata* combination. It is during the period of miracidial transformation that a crucial hurdle must be overcome by the parasite for full development to proceed. In the 1950s, Newton [20] developed the groundwork for looking at the genetic underpinnings of the snail in controlling the parasite's development. His snail crossing studies, using resistant and susceptible *B. glabrata*, and testing the known F1 offspring (determined by pigmentation patterns) for parasite susceptibility clearly showed a genetic control for susceptibility to the parasite. The subsequent, and enormous, contributions by Richards led to the conclusion that the compatibility of host snail and parasite is controlled by a series of genes of both parasite and snail [21, 22]. The interaction ranges from total resistance, or non-susceptibility of the snail, to full compatibility, resulting in a fulminating schistosome infection. The number of genes and multiple alleles involved give some testament to the idea that snail and parasite have co-evolved over millennia. Although a great deal is known about the genetic influence of the snail on parasite development, comparatively less is known about the parasite's genetics in determining infectivity. The populations of parasites in the field are almost certainly enormously diverse, owing to the multiple alleles that must be involved in infectivity. An evaluation of the schistosome population genetics is an important focus in the recently developed schistosomiasis control program in Zanzibar (http://score.uga.edu/).

3.4 Epidemiology

Humans contract schistosomiasis when they come into contact with water sources contaminated with the infectious form of the parasite (cercaria). The degree of human waste contamination to the local water bodies is the driving force that

dictates the prevalence of schistosomiasis in a community, given the fact that the sanitary disposal of human wastes, as seen in most developed countries, is often totally absent in the regions of poverty where schistosomiasis is prevalent.

It is important that control programs must take into account the epidemiology of the disease in the local region. Knowing the prevalence of disease, whether transmission is seasonal or year-round, and how effective are the post-treatment evaluations, all play large roles in the direction an effective control program should take.

A great many things contribute to any discussion in the epidemiology of schistosomiasis, and several of these, such as diagnosis, treatment, and control are being discussed more fully in other sections of this chapter.

It is difficult to make sweeping statements about the epidemiology for schistosomiasis, considering the different schistosome species involved. For instance, the fact that *S. japonicum* also uses reservoir hosts complicates the transmission pattern, since both *S. mansoni* and *S. haematobium* are primarily transmitted by humans. That being said, some generally accepted statements about a few components of the epidemiology of schistosomiasis are the following.

3.4.1 Prevalence

Most age–prevalence curves display a peak in schistosomiasis prevalence in school-age and young adult populations, with a gradual decline later in life (Fig. 3.6). Not surprisingly, the major contributors to egg contamination to the environment also fall in the school-age/young adult groups. The disease takes a devastating toll on young children (school-aged), who are most at risk because of the high frequency of time spent swimming or bathing in water containing infectious cercariae. What contributes to the decline of prevalence in the older populations may be one or a combination of events: (1) the die-off of the worms after the peak is reached, given the estimate of a mean of around 5 years (on average) that the parasite can live in humans; (2) decline in water contact as the population ages; and (3) evidence that partial immunity may develop over time [23].

There is usually a skewness, or over-dispersion, of egg counts within the community. Thus, the majority of eggs being excreted come from a minority of the residents. It may be too simple to attribute this to a direct reflection of worm burdens, since there is increasing evidence that a variety of other things may come into play, such as host genes that might contribute to a role in the pattern of egg excretion [24].

In high transmission areas, some workers have reported schistosomiasis prevalence as high as 100 % of the population, although lower figures are more commonly reported. Incidence, commonly measured after chemotherapeutic campaigns (e.g., 1 year post treatment), can sometimes rise to near pretreatment levels within 1–2 years after treatment, emphasizing the need for periodic retreatment of the population.

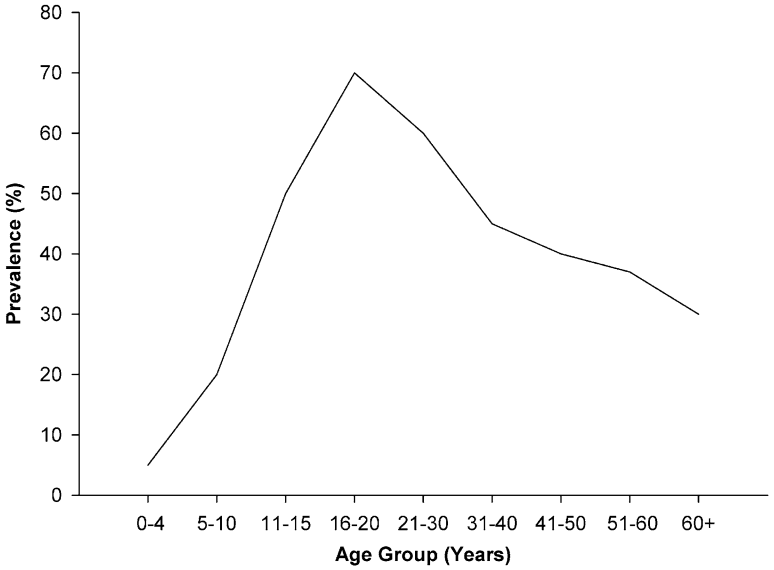

Fig. 3.6 Diagrammatic representation of schistosomiasis prevalence by age in a typical population where there is a moderate risk of contracting schistosomiasis

3.4.2 Infection Intensity

On a community-wide basis, there is a rough correlation between intensity of infection and prevalence. For *S. mansoni* and *S. japonicum*, intensities of infection in a community have historically been assessed as eggs/gram of feces. For *S. haematobium* infections, figures of eggs/unit of volume of urine are most often used. The practical difficulties of obtaining these numbers can be imagined, since egg counting requires considerable manual labor. Also, repeated examinations may have to be done to rule out the problem with the discontinuous nature of egg excretion. Considerable efforts have been made to develop other means of assessing prevalence and intensity, usually based on serum or urine analysis.

Males often tend to have higher worm burdens than females. This may have more to do with social customs than any sex-related factor, for example those that dictate the greater degree of occupational or recreational water contact of males over that of females.

The question of how intensity, egg counts, and worm burdens interrelate in humans was the subject of a series of studies conducted by Cheever and colleagues on autopsies of scores of cadavers in Brazil and Egypt [25, 26]. Among the many significant conclusions from these studies was that, in general, the stool egg counts in *S. mansoni* patients had an almost linear relationship with worm burden. In one study, roughly 1 egg/g of feces equated to one worm pair. These autopsies were, for the most part, conducted on individuals who previously presented with significant

disease, so we do not know how representative this would be for those with low egg yields. Nevertheless, these and follow-up studies helped cement our knowledge of tissue egg burdens as it correlates with disease in humans.

3.4.3 Snails at Transmission Sites

Surveys of snail populations in endemic areas are essential for defining areas of transmission. It is often found that only a small percentage of snails in an active transmission site are actively shedding cercariae. In addition to examining snails at potential transmission sites, methods have been developed to determine the presence of free-swimming cercariae by mechanical cercariometry devices, or placing sentinel animals (mice) in the water, followed by mouse dissection or hepatic portal perfusion several weeks later, looking for the presence of adult worms.

In some geographic regions, such as northeastern Brazil, transmission sites may dry up for several months of the year. A small percentage of the snails, however, are known to be able to withstand these dry conditions by burrowing into the mud and reemerging once the sites contain water again. There have even been reports of snails with pre-patent infections being able to withstand drying, and then liberate cercariae once the transmission site contains water [27].

3.5 Clinical Aspects

Depending on the stage of the schistosome infection, a wide range of clinical symptoms may occur, and many of them are hard to distinguish from those of several other diseases. A mild or transient rash may develop soon after cercarial invasion of the skin, but this may be overlooked. Considering that only one or a few cercariae may enter the skin at any one time, and the fact that they are so small as to make them barely visible to the naked eye, their entry into the skin may go unnoticed.

Sometimes a febrile illness occurs around 3–6 weeks after cercarial exposure. This acute stage syndrome is commonly referred to as Katayama fever, named after the original location (Katayama district, Hiroshima, Japan) in which this was a prominent feature of the infection. Katayama fever is observed most often in individuals who have had no prior exposure to schistosomes. Rather than a reaction to schistosome eggs, its etiology—at least initially—is thought to involve circulating immune complexes, and its severity may increase upon oviposition. Aside from fever, there may be anorexia, prostration, bloody diarrhea, hepatosplenomegaly, and eosinophilia.

The clinical picture of chronic infections in humans varies somewhat with the schistosome species. A large number of those people infected may be asymptomatic. Those with *S. mansoni* or *S. japonicum* infections, though, can develop a condition referred to as intestinal schistosomiasis. This is characterized by abdominal pain, lethargy, ascites, chronic diarrhea, and hepatosplenomegaly. Infection can lead

to serious liver damage in chronic cases. This is to be distinguished from urinary, or urogenital schistosomiasis, caused by *S. haematobium* infections. Blood in the urine (hematuria) is a hallmark of this disease, and in chronic phases infected patients may exhibit a host of urinary tract complications, ranging from urinary tract obstruction, kidney involvement, hydronephrosis, bladder calcification, genital lesions, renal failure, and bladder cancer. Compounding the symptomatology, co-infection with *S. mansoni* and *S. haematobium* is often seen in regions where the two species overlap geographically.

In the case of *S. haematobium* infections, one issue that has recently drawn a great deal of interest is the growing body of evidence supporting an association between urogenital *S. haematobium* infections and an increased risk to HIV/AIDS [28]. There may be as much as a three-fold to four-fold increase risk of having HIV in women with a preexisting *S. haematobium* infection. Several causative factors may be involved in this scenario. Among them are (1) a breach in the integrity of the epithelium of the female genital tract, caused by the parasitic infection, that may facilitate HIV viral entry, and (2) increased susceptibility to the viral infection may result from the predominantly Th2-type immune response with the schistosome infection. There is also the possibility that inflammation in the genital tract of men with *S. haematobium* infections may increase the risk of male-to-female transmission of the virus.

Apart from the more classical symptoms of schistosomiasis described above, scientists are now beginning to better recognize the role that the disease contributes to chronic anemia, and the negative impact it has on cognitive development and malnutrition in children. Since coinfection with other helminths, such as hookworm, often occurs in schistosomiasis endemic areas, the complication presented by schistosome-induced anemia has especially been overlooked.

Besides the liver, intestine and urogenital tract, schistosome eggs have been found in other (ectopic) sites, such as the lung, appendix, and central nervous system. Central nervous system involvement, although rare, can present as transverse myelitis, cerebral lesions, epileptic seizures, and paralysis.

A significant advance in assessing morbidity in the field has been through the use of ultrasound. Protocols for using ultrasound for schistosomiasis-related pathology were developed in the 1990s [29]. In particular, this can give a better measure of infection intensity in those who may have low egg burdens, but significant disease. It is also useful for assessing possible changes in pathology after drug cure. A good discussion on the use of ultrasound in assessing morbidity in schistosomiasis is given by Carlton et al. [30], who also describe some of the difficulties of its use with reference to *S. japonicum* infections.

3.6 Diagnosis

The definitive diagnosis for a schistosome infection is the microscopic detection of eggs in stool (for *S. mansoni* and *S. japonicum*) or urine (for *S. haematobium*) samples. Numerous techniques have been developed to detect the eggs, some involving

concentration/sedimentation of samples by such reagents as formal glycerine. For many years, the most common approach, at least for *S. mansoni*, has been the Kato-Katz thick smear assay, which uses cellophane and malachite green–glycerin to more easily detect the eggs from prepared fecal specimens. Although relatively quick and inexpensive, other direct assays it is complicated by the day-to-day variation in egg passage. With a sensitivity of about 30 eggs/g feces, active infections may be missed unless repeated samples are taken over time. Another method for the diagnosis of schistosomiasis is the detection of circulating anodic and cathodic antigens (CAA and CCA) released by adult worms into blood or urine. Monoclonal antibodies to these antigens have been developed and scaled up to test in various parts of the world. CCA detection in urine can be as sensitive as a single Kato-Katz test in areas that have a high intensity of infection. Some control programs are now incorporating a urine-based CCA assay for screening *S. mansoni* infections. Results from the field have found CCA tests for *S. mansoni* are comparable to Kato-Katz and other antibody tests [31, 32].

Tests for detecting circulating antibodies (IgG) have been developed for a variety of schistosome antigens, and have proven useful for some purposes. The Centers for Disease Control and Prevention (Atlanta, GA) utilize a FAST-ELISA test for diagnosis of *Schistosoma* spp. using *Schistosoma mansoni* adult microsomal antigen (MAMA). One drawback in this approach though is that schistosome-specific antibodies remain detectable even after drug treatment has cleared the infection. Also, test sensitivity with the FAST-ELISA is reduced for species other than *S. mansoni*, so a secondary test (immunoblot) must sometimes be used to distinguish between species.

3.7 Treatment

There is a long and interesting history in the search for effective drug treatments for schistosomiasis. In the early 1900s, antimony tartrate (tartar emetic) began to be used as an antischistosomal drug. Several serious toxic side effects with this drug made it less than ideal for treating the disease. Additionally, the need for extended stays in a clinic or hospital while undergoing treatment meant the loss of income for the patient during that time. It was not surprising, therefore, that many patients refused to complete the 2–4 week treatment regimen. In the 1920s, Fouadin (stibophen), a drug that could be given intramuscularly, became available. It also had toxic side effects, although perhaps not quite so severe as with antimony tartrate. In the late 1940s, a new drug was introduced that promised a new standard of treatment. This was Miracil D, an oral drug that was shown to have a better effective cure rate and required a less lengthy treatment regimen. The side effects though were alarming enough to continue the search for better drugs. Later, drugs such as metrifonate, hycanthone, and oxamniquine were developed, having their own strengths and weaknesses showing their own strengths and weaknesses for their use. For example, none of these drugs were shown to be effective against all three major schistosome species, some had safety/cost concerns, and/or less than stellar cure rates and concerns over drug resistance.

The greatest advance in the field of schistosomiasis chemotherapy came about by the development of Praziquantel (PZQ) in the laboratories of Bayer AG and Merck KGaA in Germany in the mid-1970s. The drug was moved into clinical trials and large-scale use by the mid-1980s. By 1985 the WHO reported that approximately one million people had been treated with PZQ [33]. PZQ is now the major component of schistosomiasis control programs that advocate mass drug administration to affected populations. The WHO recommends that PZQ be used at a single dose of 40 mg/kg [34]. In 2012, over 35 million people were treated with PZQ [35]. As impressive as this is, more work is required on the part of drug companies donating drugs and governments implicating an integrative solution to control and eventually eliminate schistosomiasis. Multiple features have made PZQ the drug of choice for treating schistosomiasis, including the following: (1) it is effective against all the three major species, *S. mansoni*, *S. haematobium*, and *S. japonicum*, (2) it has good pharmacologic properties (it can be given as a single oral dose and is usually well tolerated), and (3) its relatively low cost (<US$0.10 per tablet). With the price of PZQ having dropped the last few years, it has been made more available for use in numerous mass drug administration campaigns, with considerable success. Studies aimed at determining the effectiveness of PZQ focus on cure rates and/or egg reduction rates. Cure rates of PZQ are generally >50 %; 100 % cure rates are seldom reported [36]. This is likely due to re-infection in high-transmission areas and other parasite-related factors. PZQ is effective against most stages of schistosomes, but it is particularly ineffective against juvenile worms. It is theorized that the lack of susceptibility of juvenile worms may be related to poor PZQ cure rates and treatment failures that occur in some areas. Because of this feature, it is recommended that people be re-treated with PZQ weeks after the first dose to increase cure rates and decrease egg passage to the environment.

As with other drugs, re-infection is still a problem after treatment, and there is a lingering fear in the community that such widespread use of the drug may eventually lead to the development of drug resistance in the parasites. Drug resistant lines are reported to have been produced in the laboratory [37] and some studies have reported reduction in PZQ susceptibility as potential resistance in the field [36, 38]. However, some argue that the resistance in the field may be a result of the refractive character juvenile stage worms exhibit in response to PZQ. At the time of this writing, resistance has not been detected in field situations with any certainty, although there is continued effort to monitor this in mass drug campaigns. Needless to say, determining the mechanisms of drug action and potential drug resistance would have a huge impact on future discovery of anti-schistosome drugs.

A considerable amount of research has focused on deciphering the possible mode of action of PZQ. In early studies it became evident that PZQ causes damage to the worm tegument, greatly augmented by antibodies directed to tegumental antigens. It also causes a striking muscle paralysis in worms that is associated with an influx of calcium ions. Because of the alteration in ion transport after PZQ treatment, much effort has focused on ion transporters and channels as potential targets of PZQ. Currently, the best candidates for molecular targets of PZQ may be calcium ion channels (specifically beta subunits), but there is no consensus agreement. Just

as the molecular targets for PZQ remain unclear, potential resistance mechanisms have been elusive. Attempts to discover differences in protein sequences of calcium channels in resistant vs. susceptible isolates were inconclusive. Currently, efforts are underway to discover markers of PZQ resistance in *in vitro*-selected PZQ resistant lines and new generation molecular technologies are being applied to dissect transcriptome and genotypic changes in response to PZQ treatment. Clearly, more effort should be devoted to the molecular determination of putative resistance markers in light of the fact that PZQ is being relied on so heavily in control efforts.

For several years, investigators have also shown that artemisinin drugs also exhibit effectiveness against *Schistosoma* spp. These drugs are natural products derived from the sweet wormwood plant, *Artemisia annua*. These drugs have potent antimalarial activity and are the part of front-line therapies (artemisinin combination therapies) to treat *Plasmodium falciparum* on a global basis. Artemisinins began to be investigated as schistosomiasis drugs in the 1980s and have been used in patients since the 1990s [39]. Artemisinins are effective against all three major schistosomes infecting humans and they have a marked effect on immature worms, with less activity on adult worms (contrast against oxamniquine and PZQ). This property makes them good drug candidates for use as prophylaxis in areas of high schistosomiasis transmission [40]. The co-prevalence of malaria and schistosomiasis in certain areas brings up the possibility of increasing the risk of artemisinin-resistant malaria parasites if schistosomes are being targeted. The emergence of artemisinin-resistant malaria in Southeast Asia has brought much concern to the judicious use of these drugs and may reduce the chance that they can be used for schistosomiasis treatment.

Other drugs that have shown promise against schistosomes include synthetic artemisinin analogs, other antimalarials (mefloquine), and oxadiazoles. Clearly, a larger portfolio of drugs is necessary to preserve the few effective drugs we have and also to combat inevitable resistant parasites.

3.8 Control

The control of schistosomiasis is a monumentally difficult undertaking. To show how much goes into organizing and carrying out region-wide control measures, one can study the large *S. mansoni* control project on the Caribbean island of St. Lucia in the 1970s–1980s [41]. The research team that was assembled for this project examined the feasibility and cost of three control measures: snail control, chemotherapy, and provision of fresh water on the effect on transmission. The island geography lent itself well to these separate points of attack, since the studied areas were not contiguous with one another. A vast amount of data was compiled over the 15-year period of the project, and a detailed cost analysis was made for the reduction of transmission to a certain level. What became apparent was that the most cost-effective measure for reducing transmission was the use of chemotherapy. The provision of fresh water supplies, with all the engineering and labor-intensive efforts

that they required, was less cost-effective, but brought benefits to the local population above that of only reducing transmission. Snail control was difficult to achieve and used alone, it was not as cost-effective as chemotherapy.

These and other field studies showed that probably the most efficient mode of attacking the problem was to rely mostly on mass drug administration, in the hopes of reducing morbidity in the population. Here, control programs, using mass chemotherapy, usually target entire communities or selected subgroups, such as school-aged children in places where the prevalence of schistosomiasis is 50 % or higher. Such efforts would have a good chance for reducing infection intensity, and if repeated on a periodic basis, gradually reduce transmission and the frequency of severe disease. However, reinfection (especially in children) is a severe problem for effective mass drug administration programs. Therefore, along with mass treatment campaigns, there is a need for parallel operational research, to better define the best approach for control.

In the ideal world an integration of control measures would be the most productive and effective means of combating the disease. The WHO currently recommends a two-pronged strategy for control that includes: (1) morbidity control in high transmission areas using PZQ, and (2) integrated control in low transmission areas where elimination may be possible [42]. Scientists have long realized that eradication of schistosomiasis, especially in highly endemic regions, is not likely to occur without huge monetary outlays and substantial country and local government cooperation. Elimination of the disease has as much to do with raising the standard of living of those at risk as to establishing and constantly monitoring a series of control measures. The integration of a variety of control measures, such as chemotherapy, snail control, education, access to uncontaminated water sources, and improved hygiene takes significant manpower and financial resources. The level of resources necessary is often beyond what the communities most affected by the disease can afford. Nevertheless, at the time of this writing, eradication is the goal of at least one large field project in Zanzibar, a multi-year effort spearheaded by the SCORE program (Schistosomiasis Consortium for Operational Research and Evaluation) based at the University of Georgia (USA).

In recent years, large control programs have been undertaken under the auspices of the Schistosomiasis Control Initiative (SCI, http://www3.imperial.ac.uk/schisto), a program established in 2002 through a number of major funding organizations, in particular the Bill and Melinda Gates Foundation. The mission of the SCI is to use anti-helminthic drugs to eliminate schistosomiasis and other NTDs such as soil transmitted helminths, lymphatic filariasis, and onchocerciasis from sub-Saharan Africa. These diseases not only cause significant mortality, but they often manifest in extreme morbidity that affects nutrition levels, learning, the ability to work, and ultimately serve to exacerbate poverty. The SCI estimates that 500 million people in sub-Saharan Africa are infected by two or more NTDs, so treating people with combination therapies will hopefully affect control of multiple diseases. From the period of 2002–2007, the SCI facilitated delivery of greater than 40 million treatments of PZQ and albendazole (a deworming drug effective against multiple species). They have also assisted countries in the development of sustainable NTD treatment

programs. SCI also works with other organizations such as the Global Network for NTDs and SCORE to launch new schistosomiasis control programs and maintain effectiveness of existing programs.

Historically, many of the control approaches have incorporated methods for snail control as well, and most investigators think this should be an integral part of effective long-term control efforts. The WHO has advocated snail control in association with chemotherapy since 1993 [43]. When used in sync with preventative chemotherapy, this integrated approach has led to success in many schistosomiasis control and elimination programs [42]. Before effective drugs for treating schistosomiasis were available, removal of snail populations (molluscicide) was the principal method of schistosomiasis control. Synthetic molluscicides such as sodium pentachlorophenate and niclosamide (Bayluscide) emerged in the 1950s and 1960s to combat snail populations. Niclosamide is now the only commercially available molluscicide and it has been used in many schistosomiasis control campaigns. Because of its expense, niclosamide cannot be used long-term and hence, it is not used in eradicating snail populations but to manage them and suppress transmission. A large number of other molluscicides have been used, including extracts of natural products such as Endod, from the African soapberry plant, *Phytolacca dodencandra*. Considerable research has also been devoted to biological control measures, using (among others) such natural predators as fish, birds, turtles, crayfish, and competitor snails. Although intriguing, these alternative molluscicides have not proven nearly as effective as Niclosamide.

Furthermore, schistosomes that infect humans may be capable of infecting other indigenous animals (e.g., water buffaloes with *S. japonicum*) and this potential zoonotic transmission must be taken into account when designing control programs.

3.9 Immune Responses and Pathology

No other area of schistosomiasis research has been as vigorously pursued over the past 30 years as the study of the immunology of the disease. This has led to some major advances, not only in our understanding of the basic disease, but in the broad area of inflammation and fibrogenesis of many types of chronic disease states.

In experimental settings, immune responses to the invading cercariae can be detected as early as 1–2 weeks, depending on the method used for detection. In mice, antigen-specific lymphocyte proliferation can be detected early in lymph nodes draining the site of cercarial penetration [44], followed by cytokine production, typically involving Th1 pathways.

Once eggs are produced and deposited by the female worms in the tissues, there are dramatic changes in both the magnitude and character of the immune response. In mice exposed to *S. mansoni*, this occurs around 4–5 weeks after cercarial penetration. The host launches a bewildering array of immunological checks and balances to limit or suppress an otherwise overwhelming infection [45]. What follows is a strongly polarized Th2 response [46], and immunoregulation occurs to freshly

deposited eggs in the tissues, so that the immune responses to new eggs later in the infection are not as florid as those in the earlier stages of the infection. This immunological modulation is a CD+ cell dependent phenomenon.

With so much antigenic insult to the host brought about by a schistosome infection, surprisingly little reactivity is seen to the live adult worm in the bloodstream. Specific humoral and cellular responses can be detected to adult stage antigens, although the adult worms do not seem to be affected much by them. Considerable research has been conducted on the reasons why this "immune evasion" of the adult stage may occur. It may be a combination of numerous pathways that the schistosomes have evolved over millennia to adapt to the otherwise hostile blood environment. Some research suggests that antioxidants, in particular glutathione peroxidase (GPX) and glutathione-S-transferase (GST), may have some role in protecting the worm surface from free radical-induced tegumental damage [47]. The schistosome may also have the ability to actively shed and replace the outer tegument [48]. Numerous host molecules have been shown to be associated with the adult tegument, and there is speculation that, somehow, these may cover and protect the tegumental surface from immune attack.

About one-half to two-thirds of the eggs produced by the mature schistosomes are never excreted, but remain in the tissues, and are eventually destroyed by the host's immune system. As mentioned earlier, the egg-induced granulomatous formation phase (Fig. 3.7a) sets the stage for the pathology we later see in the infection, and a greater understanding of this process is absolutely essential if we are to make progress in limiting the damage that tissue fibrosis causes.

The major pathology in schistosomiasis is associated with the development of tissue fibrosis. It is this accumulating fibrosis, or internal "scarring," that can result in irreversible, space-filling lesions, directly affecting tissue architecture and blood flow through the affected organs. In the case of *S. mansoni* and *S. japonicum* infections, this can lead to complications associated with high portal blood pressure, such as esophageal varices, and death when these vessels rupture. For *S. haematobium* infections, complications arising from fibrosis in the urogenital tract include numerous serious clinical illnesses, as mentioned previously.

In humans and some other primates, an interesting pathologic condition in the liver can develop called Symmer's clay pipestem fibrosis, wherein obvious and distinct fibrotic lesions have developed, as if someone had sectioned a liver after a series of clay pipestems had been thrust through it (Fig. 3.7b). Figure 3.7c shows a section of a liver showing the magnitude of the fibrosis around the portal vessels. The physiologic complications of such invasive liver involvement include portal hypertension and the related blood flow problems that accompany it. Liver function seems not to be much affected due to the infection. Liver pathology caused by a schistosome infection is not to be confused with cirrhosis, which has a different etiology entirely.

Since fibrosis plays such a large role in the clinical picture of schistosomiasis, many studies have investigated the underlying control of this process. In fact, much of the progress in the broad field of fibrosis research has been made possible by studying schistosomiasis in the mouse. The strong Th2 responses driven by the eggs

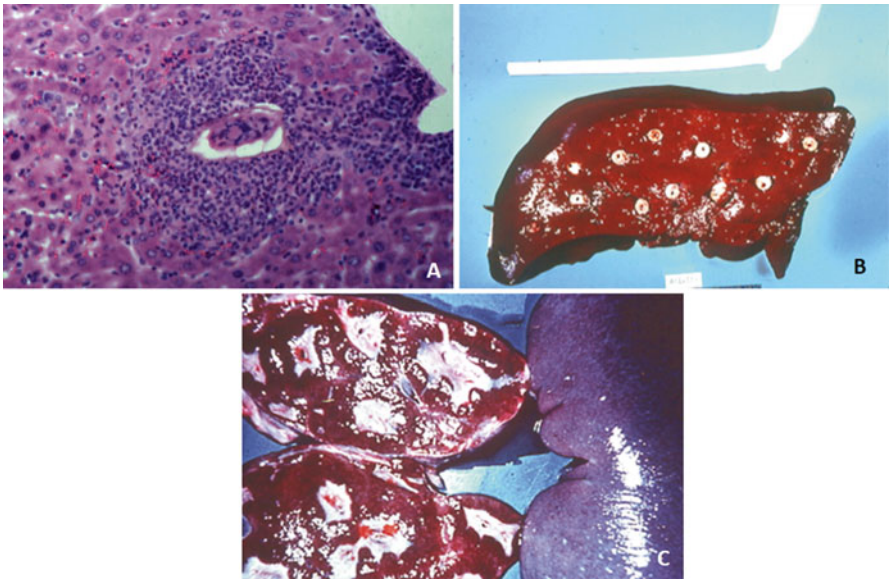

Fig. 3.7 Photomicrographs representing pathology of schistosomiasis. (**a**) Section of a mouse liver exhibiting an acute inflammatory cell (granulomatous) reaction surrounding a live *S. mansoni* egg (center of photograph). (**b**) Human liver in which clay pipestems had been embedded in it, prior to sectioning. This gives an approximation of the pathology leading to the term "clay pipestem fibrosis" in cases of schistosomiasis mansoni. (**c**) Sections of a liver (*left side* of photo) from a *S. mansoni*-infected human (chronic infection). Periportal fibrosis is clearly evident as whitish regions. Shown on the *right* is a portion of the spleen

have been a good model to decipher early events in inflammation and later fibrotic responses. Studying immune responses during the natural infection though is complicated by the continuous egg production by the parasite, and the associated overwhelming and complicated reactions to the various stages. However, one elegant experimental model uses the intravascular injection of isolated eggs that lodge in the lungs of mice, allowing synchronized measurements of inflammatory responses, cytokine involvement, and other processes involved in egg-induced inflammation [49]. Similarly, the recently developed procedure of injecting *S. haematobium* eggs into the bladder walls of mice is being made use of for exploring the development of bladder cancer, and other fundamental inflammatory processes caused by this parasite [50].

The regulation of fibrosis is a subject that is beyond the scope of this chapter, but a detailed summary by Wynn and Ramalingam describes the latest information on the regulatory processes involved and the potential pathways that may be targeted as potential therapies [51].

Investigators have taken experimental advantage of the fact that the schistosome egg is a potent driver of Th2 responses. In large part, the responses to the eggs have been studied most extensively in the context as the responses to the soluble egg antigens (SEA)—a crude PBS-soluble extract obtained from purified and

homogenized eggs [52]. It is a complex mixture of different glycoproteins, proteins, polysaccharides, and glycolipids, and efforts have been made to determine more precisely the components that drive the Th2 response to such levels [53].

3.10 Vaccines

A substantial amount of effort has been spent on trying to develop a vaccine for schistosomiasis [54–56]. It has always been thought that an effective vaccine would reduce the need for repeated drug administration, since an effect of the vaccine might be reduction or prevention of re-infection. A vaccine would be another tool in the arsenal of control.

When experimental models were first developed for schistosomiasis, in particular in the mouse model, it became apparent that there existed some "immunity" to a cercarial challenge in mice that had an existing adult infection. This so-called "concomitant immunity" seemed to be directed against the earlier stages, leaving the adult worm population unaffected by any immune responses that may have been stimulated. It gradually became accepted however that (at least in the mouse) this type of immunity was not based on antibodies or specific cellular responses directed to the parasite, but rather reflected altered blood flow patterns (as a consequence of the pathology of the disease) that diverted the normal migration of the immature worms—shunting them to sites where they could not develop [57]. However, some evidence in humans does suggest some anti-schistosome specific immunity can develop after repeated reinfection and drug treatment [23]. Numerous studies are being conducted in an attempt to determine the mode of action of this protective immunity, and if there are genetic factors involved in this protection.

In experimental animals, the highest and most consistent levels of protection have been achieved after exposure to irradiated cercariae. The most "resistant" mouse strains, such as the C57Bl/6, can develop protection as high as 70 % (i.e., 70 % of the challenge infection, compared to controls, does not mature). Most evidence suggests also that the target of this type of immunity is the lung stage schistosomule [58]. Since it would be enormously expensive to develop a standardized attenuated "live" cercarial vaccine, much effort has been devoted to try and develop a nonliving antigenic extract or recombinant vaccine in the hopes of developing one with at least the potency of the live attenuated vaccine. Of the several current candidates, two have reached the level of clinical trials: (1) Sm14, a fatty-acid binding protein, and (2) the Sh28GST, a GST derived from *S. haematobium*. Another experimental vaccine showing promise for future development is an *S. mansoni* tetraspanin (Sm-TSP-2). This antigen has also been shown to be recognized by IgG1 and IgG3 from parasite resistant individuals from *S. mansoni* endemic areas in Brazil [59].

As with any of these experimental vaccines, it may be one thing to develop an effective one in mice, or other lab animals, but quite another when used in field trials. Recent work in Brazil showed that preexisting IgE to a helminth vaccine (in this case for hookworm) led to urticaria in some individuals [60].

3.11 Genomics and Proteomics

Within the last few years, scientists have gathered a wealth of information about the genomes of *S. mansoni*, *S. japonicum*, and *S. haematobium*. The sequences for both *S. mansoni* and *S. japonicum* were published in 2009 [61, 62], followed by that for *S. haematobium* in 2012 [63]. By mining these datasets scientists are hoping to develop new approaches for drug discovery, identify vaccine candidates, and discover other tools in the arsenal for controlling schistosomiasis. This new information may allow us also to gain a better understanding of the mechanisms whereby these organisms are able to live for extended periods of time in tissues of both vertebrate and invertebrate hosts, as well as spending a shorter period of time, as different stages entirely (cercariae and miracidia), as free-living organisms.

We have known for some time that schistosomes have eight chromosome pairs (seven autosomal pairs and one pair of sex chromosomes) [64]. The females are of heterogametic sex (ZW), and the males homogametic (ZZ). Early characterization of schistosome DNA showed that it contains both moderate and highly repeated components [65]. From the recent sequence data, repetitive elements comprise about 40 % of each of their genomes. About one-fourth of the genome is composed of retrotransposon mobile genetic elements.

The recently published sequence data show obvious similarities in the overall genomic profiles for all three species. For example, draft genome estimates are 397 Mb for *S. japonicum*, 363 Mb for *S. mansoni*, and 385 Mb for *S. haematobium*. All three species possess a GC content of about 34 %. The numbers of coding genes for all three are also roughly comparable; 13,469 for *S. japonicum*, 13,184 for *S. mansoni*, and 13,073 for *S. haematobium*. Some differences are noted, however, indicating what previous studies have inferred—*S. mansoni* and *S. haematobium* are more closely related to each other than they are to *S. japonicum*. This is perhaps not surprising, considering the geographic distribution of each species, and by the vastly different families of snail hosts used by them (planorbids for *S. mansoni* and *S. haematobium*, and prosobranchs for *S. japonicum*).

A broad range of genomic and proteomic studies are now emerging taking advantage of the information gained from the sequence information that is now in the public domain. Among them are studies investigating potential drug targets, vaccine candidates, genes important in reproductive biology [66] and basic knowledge that render schistosomes such successful parasites, in both mammalian and molluscan hosts [67, 68]. These should complement also new approaches for drug screening, such as that being developed *in vitro* that may enable a more logical and cost-effective approach to the design of antischistosomal drugs [69].

3.12 Concluding Remarks

Schistosomiasis has plagued humanity for thousands of years. However, the last two decades have shown that progress is being made to reduce it in large-scale efforts. While funding for schistosomiasis research is still negligible in comparison to the

worldwide problem, it is encouraging that funding is becoming available from a larger number of sources than in the past, and various research consortia have been established with the common goal of reducing the impact schistosomiasis has on those in endemic areas. A large part of reducing morbidity has been conducted through several mass-drug administration campaigns. Effort is also being made to fine tune the control approach so that they deliver the "biggest bang for the buck," while incorporating new scientific knowledge to the problems at hand. PZQ remains the most effective weapon for schistosomiasis control and its use will only increase in the foreseeable future. However, the over-reliance on PZQ in mass drug administration programs brings the potential for drug resistance to the forefront. Losing the effectiveness of this primary drug would be devastating to schistosomiasis control, along the lines of losing artemisinin effectiveness for treating malaria (which is emerging in Southeast Asia). Because schistosomiasis vaccines may not be formidable weapons to combat the disease in the foreseeable future, there is an urgent need for new effective drugs. Until we see new methods of control or other drugs emerge for treatment, schistosomiasis prevention and control programs must incorporate surveillance and monitoring of mass drug administration programs to detect any sign of emerging resistance.

In the meantime, it is encouraging to see more research being conducted on the basic biology of the parasite, and comparative studies now being conducted with available genomic information to address issues that have long confronted us. There are many, but the more compelling among them are as follows: (1) how does the parasite adapt so quickly to the environment between a molluscan and mammalian host, (2) what triggers stimulate the adults to migrate to their organ-specific egg-laying sites in venules, and (3) how can adults live so long in the bloodstream, an obviously hostile place for so many other pathogens?

As we have demonstrated, the research area of schistosomiasis is vast and varied. We hope this chapter gives the reader a starting point to explore the various avenues of this research in more depth.

We would like to draw the reader's attention to several online resources to gain a better understanding of schistosomiasis and the direction research and control measures are taking. The CDC DPDx Web site is an excellent resource for general information on schistosomiasis (http://dpd.cdc.gov/dpdx/HTML/Schistosomiasis.htm). A good source of information on drug treatment programs is given in the Web site for the Schistosomiasis Control Initiative (SCI, http://www3.imperial.ac.uk/schisto), as well as the one for Schistosomiasis Consortium for Operational Research and Evaluation (SCORE) at http://score.uga.edu. SchistoDB (http://schistoDB.net/) is a genomic database for *Schistosoma mansoni*, containing sequences and annotation for *S. mansoni* in a user-friendly database. For lab applications, there are several standard operating procedures available on the NIAID Schistosomiasis Resource Center Web site at http://www.schisto-resource.org.

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Chapter 4
Fascioliasis

Santiago Mas-Coma, M. Adela Valero, and M. Dolores Bargues

4.1 Introduction

Considered a well-known veterinary problem of worldwide distribution, fascioliasis is the vector-borne parasitic disease presenting the widest latitudinal, longitudinal and altitudinal distribution known at present [1, 2]. In the last two decades, many surveys have shown it to be an important public health problem as well [3–6], including estimations of 2.4 million, up to 17 million people, or even higher depending from the hitherto unknown situations mainly in several regions of Asia and Africa [7].

The increasing number of human case reports in many countries of the five continents and the results of studies on pathogenicity and immunity, mainly regarding the chronic period of the disease, are the reasons why it has been decided to no longer consider fascioliasis merely a secondary zoonotic disease, but an important human parasitic disease [8] and include it as a food-borne trematode disease priority within the agenda of the World Health Organization (WHO) [9].

4.2 Systematics and Morphology of Casual Agents

Fascioliasis is caused by two species which belong to the subfamily Fasciolinae: *Fasciola hepatica* and *F. gigantica*. This subfamily includes digeneans which infect the liver and more rarely duodenum and lungs of their mammal hosts, are morphologically characterized by branched caeca and dendritic testes, and are transmitted by snails of the family Lymnaeidae [6].

S. Mas-Coma (✉) • M.A. Valero • M.D. Bargues
Departamento de Biología Celular y Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicent Andrés Estellés s/n Burjassot, 46100 Valencia, Spain
e-mail: S.Mas.Coma@uv.es; Madela.Valero@uv.es; M.D.Bargues@uv.es

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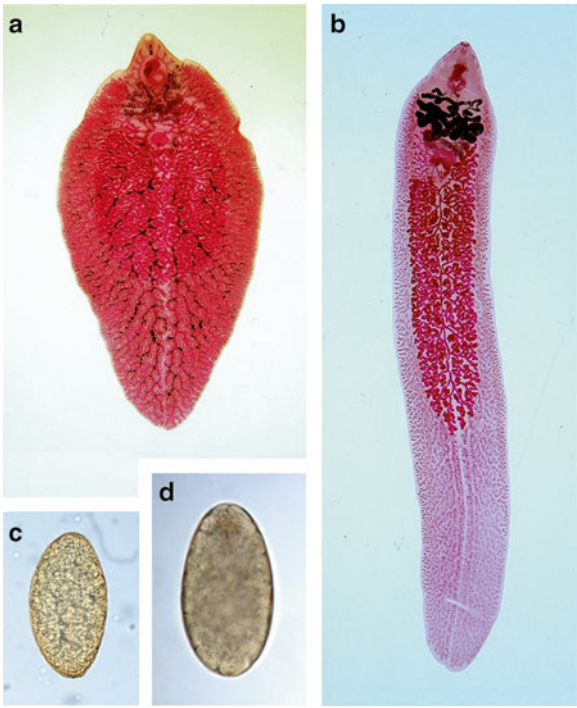

Fig. 4.1 Adults and eggs of fasciolid species: (**a**) adult stage of *Fasciola hepatica* from Bolivia; (**b**) adult stage of *F. gigantica* from Burkina Faso; (**c**) egg of *F. hepatica* found in stools of a human patient from the Bolivian Altiplano endemic area; (**d**) egg of *F. gigantica* found in a faecal sample of a bovine from Bobo Dioulasso, in Burkina Faso. Note almost absence of shoulders and parallel lateral body borders in the adult of *F. gigantica* (**b**). (**a**, **b**) at the same scale; (**c**, **d**) at the same scale. For measurements of adult stages and eggs of both fasciolids see text and Table 4.1 (Orig. S. Mas-Coma)

The adult stage of both fasciolid species has a leaf-shaped body, with a broadly pointed posterior end. The two suckers are relatively small and located close one another in a cone-like anterior extension of the body. The pharynx is well visible. The intestinal caeca are long, reaching the posterior end of the body and presenting a large number of lateral branches. The two branched testes are located in a longitudinal tandem, within the second and third fourth of the body. The cirrus pouch, containing a protrusible spined cirrus, is prominent, preacetabular and opens in a postbifurcal genital pore. The branched ovary is pretesticular and dextral. The vitellaria extend bilaterally up to the hindbody. The short uterus is located between the ovary and the caecal bifurcation. The eggs are operculated, ovoid, yellow and non-embryonated when laid (Fig. 4.1).

The two species differ in size. The adult stage of *F. hepatica* has a maximum length of 29.0 mm and a maximum width of 14.1 mm (Fig. 4.1a), whereas in *F. gigantica* it shows a maximum size reaching 52.3 mm and 11.8 mm (Fig. 4.1b), respectively. Thus, *F. gigantica* is more elongate and narrower, with lateral walls tending to be parallel, and with non-existent or less marked shoulders of the cephalic cone. Moreover, in *F. gigantica* caeca are more branched, mainly those toward the midline of the body, and the branches of the ovary are more numerous and longer. Morphometrically, all the measurements overlap in specimens of "pure" *F. hepatica* and "pure" *F. gigantica*, except the maximum body length, maximum body width,

body length–body width ratio, body roundness, and the distance between the ventral sucker and the posterior end of the body [10]. These features allow for the phenotypical differentiation between the two species.

However, hybrid specimens may give rise to intermediate forms in those endemic areas where the two species overlap [6]. The presence of such phenotypically intermediate adult and egg forms has been proved in Egypt [11], Iran [12] and Pakistan [13]. Additionally, comparisons of adults and eggs of liver fluke populations from different host species, and adults and eggs experimentally obtained in laboratory rats infected with isolates from different natural hosts revealed that the definitive host species decisively influences the size of adult worms and eggs, and that this influence does not persist in a heterologous host [14]. Thus, morphometric comparisons of fasciolid populations should always be made inside the same definitive host species.

4.3 Life Cycle

The adult stage of *F. hepatica* and *F. gigantica* parasitizes the large biliary passages and the gallbladder of ruminants, mainly sheep, goats and cattle, and many other herbivorous domestic and wild animals, including horses, donkeys, mules, and also Old and New World camelids. Buffalo, deer, wild boar, various marsupials, rabbit, hare and nutria are also susceptible hosts. Grazing domestic pigs may also be infected, but this host usually shows a higher natural resistance against the liver fluke [15]. Several African wild animals and many rodent species have been found naturally infected, and other species are usually used for experimental purposes [16–18]. Humans are susceptible hosts for the infection by both *Fasciola* species [6].

The life cycle of the two fasciolids takes around 14–23 weeks and follows a similar pattern [1, 15].

Fasciolid adults produce eggs inside the mammal host. These eggs reach the external milieu by way of bile and intestine. The transit between the definitive mammal host and the intermediate snail host includes the long resistance phase of the egg and the short active phase of miracidium. Eggs shed with the mammal faeces will only continue their development if they reach freshwater of appropriate physico-chemical characteristics. If the climatic conditions are suitable (15–25 °C), the miracidia develop and hatch in about 9–21 days. However, when conditions are unfavourable, they may not mature but may remain viable for several months.

The miracidium hatches under light stimulation and swims rapidly until it contacts an appropriate aquatic or amphibious snail host. The development takes place inside the intermediate snail host and includes miracidium penetration into the snail, sporocyst, redial generations, production of cercariae and shedding of the latter into water. A maximum of four redial generations have been found, although 3 generations are usually produced after monomiracidial infection. The redial generations follow the same developmental pattern in different lymnaeid species. Redial generations follow a complex development [19]. The stage of cercaria develops within

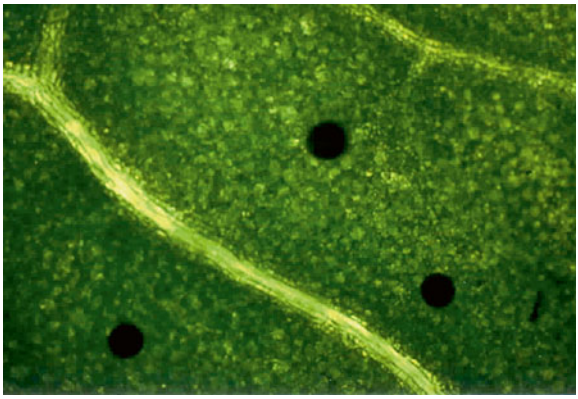

Fig. 4.2 Encysted metacercariae of *Fasciola hepatica* attached to a leaf of a freshwater plant (Orig. S. Mas-Coma)

6–7 weeks at 20–25 °C, its development being delayed at lower temperatures. Thus, the prepatent period is dependent on temperature, higher temperatures reducing it (15 °C: 56–86 days; 25 °C: 38 days).

A short swimming phase of cercaria and a long resistance phase of metacercaria allow for the transit between snail host and mammal host. The shedding process takes place between 9 and 26 °C, independently of light or darkness. Cercariae swim for a short time (1 h) until contacting a solid support, mostly leaves of water plants above or below the water line. They then lose their tails, quickly encyst and become infective within 24 h.

The definitive host is infected by ingestion of metacercariae (Fig. 4.2). Metacercariae excyst in the small intestine within an hour after ingestion, penetrate the host's intestine wall, and appear in the abdominal cavity by about 2 h after ingestion. Most migrating juveniles reach the liver within 6 days after excystment. In the liver they migrate for 5–6 weeks, preferentially feeding directly on liver tissue. They finally penetrate into the bile ducts where they become sexually mature.

The prepatent period (from the ingestion of metacercariae to the first appearance of the first eggs in the faeces) is about 2 months (6–13 weeks) in sheep and cattle, varies according to the host, and also depends on the number of the adult flukes in the liver [20]. In humans, a period of at least 3–4 months is necessary for the flukes to attain sexual maturity. The lifespan of the parasite in sheep can be as long as 11 years and 9–12 months in cattle. In humans, estimations from several long-term case reports suggest a lifespan of the adult fluke between 9 and 13.5 years.

4.4 Lymnaeid Snail Vectors

The development of fasciolid larval stages is very dependent of the environmental characteristics according to the nature of the free living phases which take place in the external freshwater milieu, and the parasite phase which develops inside the

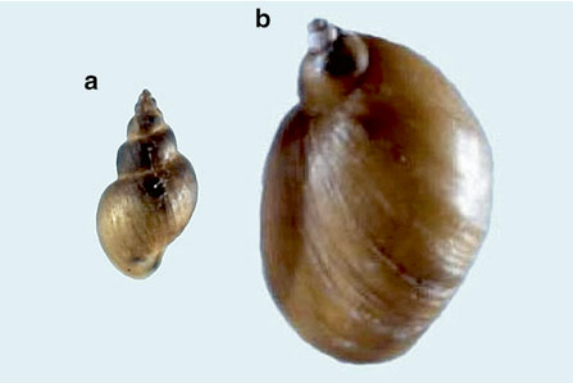

Fig. 4.3 Main species of lymnaeid vectors of fascioliasis in dorsal view: (**a**) specimen of *Galba truncatula* from Europe; (**b**) specimen of *Radix natalensis* from Africa. Note larger size of the latter (photographs at the same scale) (Orig. S. Mas-Coma)

freshwater snail, in its turn also very dependent from the environment. That is why this disease is pronouncedly influenced by climate change [21]. The similarity in the relationships between snails and climate/environment resembles the one known in arthropods participating in the transmission of many infectious diseases and underlies the recent trend of using the term vector also for the intermediate lymnaeid snail hosts transmitting fascioliasis.

Vectors of *Fasciola* are freshwater gastropod snails of the family Lymnaeidae (Fig. 4.3). Different lymnaeid species transmit the two fasciolids, which show a marked and different specificity. There are species of Lymnaeidae which cannot transmit fasciolids, other lymnaeid species which transmit *F. hepatica*, other lymnaeid species which transmit *F. gigantica* and a very few which are able to transmit the two fasciolid species. However, recent molecular studies on lymnaeids have shown that lymnaeid species misclassifications have been usual [22] and additionally hybridization phenomena between the two *Fasciola* species were unknown in the past, so that results of many of the old fasciolid–lymnaeid specificity experiments should be re-assessed [6].

Molecular studies indicate that *F. hepatica* is mainly transmitted by species of small size belonging to the so-called *Galba*/*Fossaria* group [23, 24], including *Galba truncatula* as the main vector and the only one in Europe, but also present in Africa, Asia and South America (Fig. 4.3a); *Lymnaea humilis*, *L. bulimoides* and *L. cubensis* in North America, *L. cubensis* in the Caribbean; *L. neotropica*, *L. cousini* and *L. viator* in South America; and *L. tomentosa* in Australia. The recent discovery of *L. schirazensis*, another species of the same *Galba*/*Fossaria* group which appears to have been always confused with *G. truncatula* and other similar vector species, in Asia, Europe, Africa, the Caribbean, North America and South America, has highlighted potential specimen classification problems distorting fasciolid–snail specificity/susceptibility and fascioliasis geographical distribution data. This unexpected finding now recommends the need to review a large body of literature on *G. truncatula* [24].

The species *Fasciola gigantica* is transmitted by species of the genus *Radix*, mainly *R. natalensis* in Africa (Fig. 4.3b) and varieties of *R. auricularia* and *R. viridis* in

Asia [22]. *Pseudosuccinea* is a monospecific genus including the species *P. columella* which has colonized all continents and appears to be able to transmit both *Fasciola* species [25].

A few species among the lymnaeid group of the stagnicolines have proved their capacity to transmit *F. hepatica* under exceptional or local natural conditions in a few areas, such as *L.* (*Stagnicola*) *palustris* and *L.* (*S.*) *fuscus*, and closely related species such as *Omphiscola glabra* [26].

Lymnaeid vectors, with their geographical distribution, define not only the distribution of fascioliasis, but may also explain the distribution of human infection within a country, as has been recently observed in Venezuela [27] and Chile [28], and, within an endemic area, its seasonality or permanent transmission [29].

4.5 Epidemiology

Despite of the restrictions imposed by the necessary climate/environment thresholds, *F. hepatica* has succeeded in expanding from the Near East original geographical area up to actually colonize the five continents. In its turn, *F. gigantica* appears restricted to areas of Africa and Asia where *Radix* vectors allow for their transmission [6]. It should be emphasized, however, that a global analysis of the geographical distribution of human infection shows that the expected correlation between animal and human fascioliasis only appears at a basic level. High prevalences in humans do not seem to be necessarily related to high prevalences in livestock.

Similarly to other water-borne parasitic diseases such as schistosomiasis, within a human endemic area it has been seen that human and animal infection appears irregularly distributed. The transmission foci are patchily distributed and linked to the presence of appropriate water collections, and human prevalences in school children appear to be related to the distance to water bodies presenting lymnaeids [29].

4.5.1 Distribution of Human Fascioliasis

In Europe, France is the endemic area where a higher number of human cases have been reported [30]. The first large modern epidemic of human fascioliasis occurred in that country in 1956 [31]. Between 1950 and 1983, a total of 3,297 cases from published reports were catalogued [32]. Most cases were reported from the areas of Lyon, Bretagne Nord—Pas de Calais and Sud-Ouest. More recent reports on Sud-Ouest France refer to more than 300 cases [33, 34]. Reports on 5,863 human cases were recorded from only nine hospitals between 1970 and 1982 [35], demonstrating that published data were largely underestimating the real situation. The disease is also important in Portugal, with the northern part of the country as a marked endemic area, including 1,011 cases diagnosed in Porto between 1970 and 1992 [36].

Fig. 4.4 Transmission focus of human fascioliasis in the Nile Delta region, in Egypt, with *Galba truncatula*, *Radix natalensis caillaudi* and *Pseudosuccinea columella* as vector species transmitting both *Fasciola hepatica* and *F. gigantica* infecting children (Orig. S. Mas-Coma)

In Spain, human fascioliasis appears to be underestimated and mainly distributed in the northern part [37], with imported cases recently added to authochthonous ones [38]. In other parts of Europe, human infection appears to be sporadic, although reported from almost all countries [39].

In Asia, the Near East appears as an important focus of human infection, concerning mainly Iran and Turkey. In Iran, human cases appear above all concentrated in the province of Gilan, at the Caspian Sea, where several large epidemics, including thousands of human cases, were reported from the end of the 1980s and during the 1990s [40–42]. In Mazandaran, fascioliasis has recently shown to be a human health problem too [43], and many reports have very recently been published on human cases diagnosed in other provinces. In Turkey, human infection does not seem to be rare. The detection of a 1.8 % human prevalence in a village in Eastern Turkey [44] suggests that this endemic area may be largely widespread throughout the eastern part of the country.

In the Far East, cases in Japan and Korea are sporadic, but recent information on Vietnam becomes bothering [45]. Only occasional cases of human fascioliasis were reported in Vietnam until the 1990s, but over 500 human cases have been diagnosed between 1997 and 2000 [46] and with non-stop increasing numbers thereafter [47, 48]. A recent report of a 13.8 % human prevalence in a village of Laos [49] may be interpreted as an epidemiological situation with a broader spread throughout southeastern Asia.

In Africa, numerous human cases have been detected in many governorates of Egypt, mainly children (Fig. 4.4) [50–54]. Initial estimations of 830,000 subjects

Fig. 4.5 Typical focus of transmission of animal fascioliasis in Cuba, with *Lymnaea cubensis* as vector species (Orig. S. Mas-Coma)

affected in the Nile Delta region [4] probably underestimate the real situation if the high prevalences reaching 18–19 % in total population in concrete villages [54] are considered.

In Latin America, human infection appears mainly in altitude areas of the Andean region. In the Bolivian Altiplano, human prevalences were of up to 72 % and 100 % in coprological and serological surveys, respectively [29, 55–59], and intensities reached up to more than 8,000 eggs per gram (epg) in children [6]. Similar situations, although with lower intensities, have been described in other altitude areas of Peru, such as in Puno [60], Mantaro valley [61] and Cajamarca [62]. Human infection has also been described in altitude areas of Ecuador, Colombia, Venezuela and recently also in Argentina [25, 63, 64]. A few human endemic areas have also been described in lowland areas in countries of the Southern Cone, such as Argentina [65] and Chile [28, 66].

Very recently, a human fascioliasis endemic area has been described for the first time in North America. Children proved to be infected in the state of Puebla, at a mean altitude of 1,840 m. Fascioliasis prevalences indicate this area to be mesoendemic, with isolated hyperendemic foci, a situation which adds concern about possible human fascioliasis underestimation in other areas of Mexico [67].

In the Caribbean region, human fascioliasis mainly poses problems in Cuba, where the first human case was already diagnosed in the first half of last century [68], many outbreaks have been reported [39] since the first one [69], losses in livestock husbandry due to fascioliasis are very high [70], and patients are continuously diagnosed [71, 72], even in high numbers [73]. In that island, the disease transmission is assured by two lymnaeid vectors, *L. cubensis* and *Pseudosuccinea columella* (Fig. 4.5). Unfortunately, appropriate field surveys are still lacking [74] and hence

the real situation in the different parts of the island remains unknown. Puerto Rico may still be considered a human infection risky area after the epidemiological situation in the past [75], and Haiti has recently proved to be also affected by this disease at human level nowadays [76], although human infection was already detected in Haiti time ago [77].

4.5.2 The Present Epidemiological Baseline

The present baseline on human fascioliasis pronouncedly differs from the knowledge available on human infection two decades ago. Many new concepts have been reached on human fascioliasis from the 1990s up to the present. A list of key aspects may be enumerated [6]:

1. In many areas, there are true human fascioliasis endemic situations, from hypo- to hyperendemics, which is very different of the old concept of humans only becoming infected sporadically in animal endemic areas.
2. In those endemic areas, high prevalences in humans (up to more than 70 % by coprology and even reaching 100 % by serology) do not appear to be necessarily related to high prevalences in domestic animals.
3. In human endemic areas, fascioliasis mainly affects children and females, with flukes infecting even at very precocious age (1–2-year-old children), usually showing a peak around 9–11 years and declining thereafter, although it may keep high prevalences in adults too (up to 40 % in given communities).
4. Worldwide estimations raised from the 2,500 reports of 1990 to 2.4 million, 17 million people and may even be higher at present if the almost total lack of knowledge about the situation of this disease in humans in many African and Asian countries is taken into account.
5. Human infection has been reported in 51 different countries from the five continents, showing how geographically expanded the problem might be.
6. The analysis of the distribution of the disease has shown that fascioliasis is the vector-borne parasitic disease showing the widest latitudinal, longitudinal and altitudinal distribution known.
7. Such a broad distribution including from under sea level (as in the Caspian area) up to the very high altitude (4,200 m at the Paso del Condor in Venezuela) is the consequence of the great capacity of both liver flukes and lymnaeid vectors to colonize new areas and their great capacity for adaptation to very different environments, habitats and climates, even of extreme conditions as the very high altitude regions in Andean areas, where mathematical models well-known for fascioliasis in lowlands of the Northern Hemisphere indicated that the disease could not exist.
8. In human endemic areas, intensities, estimated from amounts of epg of faeces, may reach up to more than 8,000 and amounts higher than 400 epg may be frequent in given communities, which markedly differs from the very low burdens (usually from less than 1 to 1–2 epg) reported before the 1990s.

9. Domestic animal species other than the usual sheep and cattle may also play an important role as reservoirs for humans in many different endemic areas, as mainly pigs, donkeys and buffaloes, depending from the regions.
10. The snail family of lymnaeids shows a systematic-taxonomic chaos which even impedes correct classification of snail specimens by malacology experts, as demonstrated by DNA sequencing methods; classification errors underlie a concept of fasciolid–lymnaeid specificity which must be revisited.
11. Lymnaeid species linked to the disease transmission in many human endemic areas were erroneously classified as local lymnaeid species, whereas in fact lymnaeid vector species imported from other continents were involved. This, together with importation/exportation of fasciolid-infected livestock, has given an international dimension to the public health problem in many areas where the disease was previously given local repercussion only.

The above-mentioned issues have given rise to a new platform for the analysis and interpretation of the human disease which is very different from a simple extrapolation from the traditional knowledge of fascioliasis in livestock. Unfortunately, sometimes not sufficient importance is given to this new base or it is not considered at all and consequently incorrect interpretations and erroneous conclusions are increasingly appearing in the recent literature.

4.5.3 Epidemiological Heterogeneity of Human Fascioliasis

After many years of studies on different areas presenting human infection by fasciolid liver flukes in South and Central America, Europe, Africa and Asia, the classification of epidemiological situations proposed by Mas-Coma et al. [6] still appears to be fully valid and useful. This classification includes the following human infection situations:

- *Authochthonous, isolated*, *non-constant cases*: humans acquire the infection in an area where they live and where animal fascioliasis is also present; these human cases appear sporadically, without any constancy.
- *Imported cases*: human cases diagnosed in a zone lacking the parasite, even in animals, who were infected in an area where transmission occurs.
- *Endemic*: three types of endemic situations can be distinguished according to human prevalences in the total population obtained by coprological diagnosis (data from serological tests may be somewhat higher).
  - *Hypoendemic*: prevalence less than 1 %; arithmetic mean intensity less than 50 epg; high epg numbers only in sporadic cases; human participation in transmission through egg shedding may be neglected; hygiene–sanitation characteristics usually including latrines and waste or sewage disposal facilities; outdoor defaecation is not commonly practised.
  - *Mesoendemic*: prevalence between 1 and 10 %; 5–15-year-old children may present higher prevalences (holoendemic); arithmetic mean intensity in human communities usually between 50 and 300 epg; individual high epg numbers can be found, although intensities over 1,000 epg are rare; human

subjects may participate in transmission through egg shedding; hygiene–sanitation characteristics may or may not include latrines and waste or sewage disposal facilities; outdoor defaecation may be practised.
  - *Hyperendemic*: prevalence more than 10 %; 5–15 year-old children usually present higher prevalences (holoendemic); arithmetic mean intensity in human communities usually more than 300 epg; individual very high epg numbers are encountered, intensities over 1,000 epg being relatively frequent; human subjects significantly participate in transmission through egg shedding; hygiene–sanitation characteristics not including the use of latrines; no proper waste or sewage disposal facilities; indiscriminate defaecation is commonly practised.

- *Epidemic*: there are different types of outbreaks according to the endemic/non-endemic situation of the zone.
  - *Epidemics in non-human endemic but animal endemic areas*: outbreaks appearing in zones where previous human reports have always been isolated and sporadic; such outbreaks usually concern a very few subjects infected from the same contamination source (family or small group reports; contaminated wild, home-grown or commercially grown watercress or other metacercariae-carrying vegetables).
  - *Epidemics in human endemic areas*: outbreaks appearing in zones presenting human endemics; a more important number of subjects may be concerned; usually related to previous climatic conditions having favoured both the parasite and the snail life cycles; epidemics can take place in hypoendemic, mesoendemic and hyperendemic areas.

Fascioliasis presents a very wide spectrum of transmission and epidemiological patterns in human hypoendemic to hyperendemic areas. These are related to the large diversity of environments, including different human endemic/epidemic situations, different human demographies, races, diets, habits, traditions and religions, different domestic and wild mammal reservoir species, different lymnaeid transmitting species, zones in both the Northern and Southern hemispheres, altitudes from –27 m up to 4,200 m, hot and cold weathers, seasonal and yearly constant temperatures, scarce to pronounced annual rainfall, low and high mean annual potential evapotranspiration, and from lack of dry period to lack of wet period through different dryness/humidity rates. From the landscape point of view, these areas include from altiplanos to valleys, from islands to mainlands, from natural to artificial irrigations, from lakes to lagoons, from large rivers to small streams, and from permanent to temporal water bodies [1].

4.5.4 Transmission Patterns in Human Fascioliasis Areas

A classification of transmission patterns has been proposed [78] and is progressively updated to offer a baseline for future research [6]:

1. A very high altitude pattern related to only *F. hepatica* transmitted by imported *G. truncatula* in Andean countries following transmission throughout the

year; within this category, two subpatterns may be distinguished according to physiographic and seasonal characteristics.

   (a) The altiplanic pattern, with transmission throughout the whole year, e.g. in the Northern Bolivian Altiplano and the Puno Altiplano.
   (b) The valley pattern, with seasonality and prevalences and intensities related to altitude, e.g. in the valleys of Cajamarca and Mantaro [79];

2. A Caribbean insular pattern, with reduced but repeated outbreaks in human hypoendemic areas and lymnaeid species other than the main vector species being involved in the transmission, e.g. the Pinar del Rio Province in Cuba.
3. A pattern related to Afro-Mediterranean lowlands, including overlapping *F. hepatica* and *F. gigantica* and several *Galba/Fossaria* and *Radix* lymnaeids together with secondary transmitting *Pseudosuccinea*, and where seasonality is typical, e.g. the Behera Governorate in Nile Delta region in Egypt.
4. A pattern related to Caspian surrounding areas, including human hypoendemic areas in which large epidemics occur, occasionally involving up to 10,000 people and with overlapping of *F. hepatica* and *F. gigantica* and several *Galba*/*Fossaria*, *Radix* and stagnicoline lymnaeids, e.g. the area of Rasht and Bandar-e Anzali in the Gilan province in Iran.
5. A pattern related to lowland areas in Vietnam, which may perhaps be extrapolated to other neighbouring South East Asian countries; this pattern is able to give rise to large human epidemics and is related to only/mainly *F. gigantica* and consequently *Radix* lymnaeids.

Human fascoliasis shows a marked heterogeneity of different epidemiological situations and transmission patterns throughout the world. Thus, well-known situations and patterns of fascioliasis may not always explain the disease characteristics in a given area. In other terms, when dealing with an endemic zone not previously studied, the aforementioned situations and patterns of human infection must always be taken into account merely as the starting base. Only once epidemiology and transmission characteristics of the new area are sufficiently assessed, may appropriate control measures be designed for the endemic area in question.

The lymnaeid vector species show a relationship with the transmission pattern. Lymnaeids present pronouncedly different ecological and ethological characteristics depending on the species. Factors such as type of water collection habitats, population dynamics, temperature thresholds, seasonality, or susceptibility regarding liver fluke infection, are crucial for fascioliasis. As in other well-known vector-borne parasitic diseases, lymnaeids constitute excellent markers of the disease characteristics useful for the differentiation between different human fascioliasis situations and patterns, and consequently their assessment is necessary before the appropriate control strategies may be designed.

4.5.5 Seasonality and Long-term Impacts of Climate and Global Changes

Climatic factors are decisive in the transmission of fascioliasis. The yearly definitive host infection incidence of fascioliasis has been related to air temperature, rainfall and/or potential evapotranspiration. These factors affect the intermediate snail host population dynamics and the parasite population at the level of both the free living larval stages of egg and metacercaria and the intramolluscan parasitic larval stages of sporocyst, rediae and cercariae.

Seasonal variation of mainly rainfall and temperature gives rise to different fascioliasis seasonality depending on the areas. In Europe, the transmission of the disease is typically bi-seasonal, due to the activity periods of the lymnaeid vectors in spring and autumn. In the Bolivian Altiplano, however, the transmission takes place throughout the year, lymnaeid vector populations being always present because of inhabiting permanent water bodies instead of temporary ones due to the high evapotranspiration rates at the very high altitude [29]. In other areas, the transmission appears mono-seasonal, due to the existence of only 1 year period with water availability and another period of dryness covering the rest of the year.

Man-made modifications of the environment may also modify the seasonality of fascioliasis in a given endemic area. Thus, artificial field irrigation appears to be sufficient by its own to allow for fascioliasis transmission in Cambodia [80, 81]. In the province of Punjab, in Pakistan, a complex transmission model has recently been described, including bi-seasonality with a peak related to rainfall and another peak related to man-made irrigation [82].

Unfortunately, climate change overlaps other anthropogenic and environmental modifications which are included in the broad term of "global change." Global change refers to many man-made environmental changes such as hydrological changes, e.g. construction of dams, irrigation canals, water reservoirs, that establish suitable new environments for the snail vectors that transmit the parasites [21]. Hence, global change factors are able to pronouncedly influence parasitic diseases by their own, so that establishing the causality of disease emergence by climate change is usually not an easy task. However, the aforementioned Pakistani province of Punjab is the first endemic area where the emergence of human infection has been correlated with an increase of fascioliasis transmission risk due to an impact of climate change throughout a 20-year period by means of an analysis of forecast indices and remote sensing data [82].

4.5.6 Sources of Human Infection

The ingestion of infective metacercariae by humans may occur by different ways. Several infection sources have been distinguished in studies performed in the last two decades [7]:

- Ingestion of freshwater wild plants: important in animal endemic areas
- Ingestion of freshwater cultivated plants, mainly watercress

Fig. 4.6 Freshwater plant usually included in human diet in a focus of fascioliasis transmitted by *Galba truncatula* in Talesh mountains, province of Gilan, in Iran (Orig. S. Mas-Coma)

- Ingestion of terrestrial wild plants: collected in dry habitats but which were submerged in water a few weeks or months before
- Ingestion of terrestrial cultivated plants needing frequent irrigation
- Drinking of contaminated water
- Ingestion of dishes and soups made with contaminated water
- Washing of kitchen utensils or other objects with contaminated water
- Ingestion of raw liver infected with migrating metacercariae which may keep the capacity to restart migration

Cultural traditions prove to be highly important in given endemic areas. Experimental studies performed with plant-made foods showed the role they may play in human contamination in the province of Gilan, Iran (Fig. 4.6) [83].

In Mexican children, an association between fascioliasis and the habit of eating raw vegetables was identified, including watercress and radish with pronouncedly higher relative risk than lettuce, corncob, spinach, alfalfa juice, and broccoli. The link of fascioliasis risk with consumption of raw vegetables other than watercress should be highlighted, as it suggests contamination when washing terrestrial vegetables with untreated water and/or in plant cultures using natural water for irrigation [67].

It shall be considered that metacercarial infectivity is dependent upon storage time, being lower when metacercariae are older: the maximum longevity was 31 and 48 weeks using doses of 20 and 150 metacercariae per rat, respectively, although in the latter case only a very low percentage was viable. Moreover, metacercarial viability

and infectivity did not show differences between isolates from different reservoir species, demonstrating that flukes from secondary reservoirs as pigs and donkeys involve the same potential risk as those from the main ones sheep and cattle [84].

4.6 Pathology and Symptomatology Clinical Manifestations

Four clinical periods may be distinguished in fascioliasis [3, 8, 15, 85]. The incubation period includes from the ingestion of metacercariae to the appearance of the first symptoms. In man, this period has not been accurately determined (only "a few" days, 6 weeks, 2–3 months or even more). The invasive or acute period comprises fluke migration up to the bile ducts. The latent period includes maturation of the parasites and starting of oviposition. This period can last for months or years and the proportion of asymptomatic subjects in this phase is unknown, being often discovered during family screening after a patient is diagnosed [86]. Patients may have prominent eosinophilia suggestive of infection, gastrointestinal complaints or one or more relapses of the acute symptoms. Finally, the biliary, chronic or obstructive period may develop after months to years of infection. Of these four periods, the second and fourth are the most important, because patients are in one or another of these two periods almost always when diagnosed.

4.6.1 Invasive or Acute Period

The symptomatology which appears during this period is due mainly to mechanical destruction of liver tissue and abdominal peritoneum by the migrating larvae causing localized or generalized toxic and allergic reactions lasting 2–4 months. The major symptoms of this phase include fever, abdominal pain usually in the right hypochondrium or below the xyphoid, gastrointestinal disturbances such as loss of appetite, abdominal flatulence, nausea and diarrhoea, respiratory symptoms such as cough, dyspnoea, hemoptysis and chest pain, and also urticaria.

4.6.2 Biliary, Chronic or Obstructive Period

Once in the bile ducts, adult flukes cause inflammation, hyperplasia of the epithelium, and thickening and dilatation of duct and gall bladder walls. The resulting cholangitis and cholecystitis, combined with the large body of the flukes, are sufficient to cause obstruction. This phase includes biliary colic, epigastric pain, fatty food intolerance, nausea, jaundice, pruritus and right upper-quadrant abdominal tenderness, among others. Lithiasis of the bile duct or the gall bladder is frequent, whereas

cirrhosis does not appear to be so [87]. The bile duct and the gall bladder may contain blood mixed with bile (haemobilia), blood clots and fibrinous plugs. Symptomatology in children from human endemic areas of Peru includes abdominal pain localized in the epigastrium, the Murphy symptom and jaundice as the most frequent clinical biliary characteristics, the rest of the symptoms being non-specific [88].

4.6.3 Clinical Highlights

In a developed country, blood eosinophilia and the ingestion of watercress or any other suggestive freshwater plant in anamnesis are extremely useful in guiding towards a fascioliasis diagnosis. Unfortunately, these two aspects are usually not helpful in human endemic areas of developing countries, where eosinophilia may be also caused by other helminth infections and local food traditions including the ingestion of many uncooked plants may mask liver fluke infection [89].

In human endemic zones, there is usually a decrease of the prevalence from children and young subjects to adult subjects. Despite of this, results demonstrate that adult subjects either maintain the parasites acquired when young or can be newly infected as the consequence of inhabiting a zone of high infection risk [59]. It must be considered here that the lifespan of the adult fluke in man is between 9 and 13.5 years [15]. Such a picture suggests that, in those areas, the majority of adult subjects should be in the biliary period, acute lesions by repetitive infections being superimposed on chronic disease with relative frequency. Thus, the acute period may be prolonged and overlap with both latent and biliary periods.

An association between anaemia and fluke burden (the most important), epg, fluke body area, presence of blood in faeces, IgG1 and eosinophil levels, and percentage of splenic weight was verified in a multivariate analysis. These results lead to the assumption that a high risk of anaemia in subjects with a heavy parasitic burden in human hyperendemic areas is to be expected [90]. These results are crucial, because although there were several reports listing anaemia in patients from endemic areas, results could only be considered with great caution because coinfections were never excluded in those papers and in fact it becomes very difficult, not to say almost impossible, to find subjects from endemic areas only infected by fascioliasis. And among those parasites coinfecting fascioliasis-affected subjects, many are also known to cause anaemia.

The duration of fasciolid infection, intensity of fasciolid infection, and liver damage have been experimentally verified to be associated with bacterobilia by *Escherichia coli* (45 % of cases), *Enterococcus faecalis* (45 %) and *Klebsiella pneumoniae* (10 %). This supports that the obstruction caused by advanced chronic fascioliasis may be related to biliary sepsis. These results lead to a reconsideration of treatment features in human disease, i.e. therapeutic strategies should also consider the possibility of bacterial co-infection [20].

The presence of gallstones was experimentally proved to increase with infection time. Therefore, the lithogenic induction by infection becomes manifest in

situations of advanced chronicity. Gallstone presence was strongly associated with the number of flukes located in the bile duct. The risk of pigment stones appears to depend mainly on factors that favour bile duct obstruction (cholangitis, fluke body development versus time, intensity of infection). Situations of undiagnosed cases, as in subjects presenting undistinguishable symptoms or in those keeping their infection for a long time because of non-treatment or of repetitive reinfections, usually in human endemic areas of developing countries, imply a higher lithiasis risk. Thus, a high gallstone risk may be expected in subjects inhabiting human hyperendemic areas where very high egg outputs detected in humans suggest that liver fluke burdens may also be very high [91].

Clinical pictures caused by fasciolids in locations of the human body different from the liver are known as ectopic fascioliasis. Flukes may deviate during migration, enter other organs and cause ectopic fascioliasis. In almost all patients, the causal agent is an immature juvenile, but a reduced number of ectopic cases caused by mature flukes shedding eggs have also been reported [89]. In humans, the most frequent ectopic lesions are in the gastrointestinal tract. Other such lesions are in abdominal wall, pancreas, spleen, subcutaneous tissue, heart, blood vessels, the lung and pleural cavity, skeletal muscle, appendix and epididymis [15]. Pathological effects of ectopic lesions are due to the migratory tracks causing tissue damage with inflammation and fibrosis.

Very recently, a wide analysis has shown that neurofascioliasis or intracranial infection by *Fasciola* and ophthalmofascioliasis or direct affection of the eye by migrating flukes may be rare, although not sporadic as previously believed. However, manifestations including a very wide range of neurological symptoms, signs and syndromes, together with meningeal, psychiatric or neuropsychic manifestations, and ocular disorders caused at distance by flukes infecting the liver may be frequent but underestimated due to misdiagnosis, mainly in low-income regions. The impressive clinical pictures should be highlighted. They include from hemiplegia and paraplegia to disturbances and difficulties of walking capacity, speech disorders, convulsions, epilepsia and coma, amnesia, or visual hallucinations and permanent blindness, only to mention a few, plus the clinical complexity of the puzzling polymorphisms, the disconcerting multifocality of the manifestations, and their changes along the evolution of the disease in a same patient, as well as differences between the clinical pictures shown by different patients. Moreover, these studies emphasize post-treatment sequelae and mortality in neurological patients and the need to consider neurological fascioliasis when estimating the global burden of this disease [90, 92].

4.7 Immunobiology and Coinfections

Fasciolid trematodes promote its own survival through several strategies to downregulate the host's immune response during the early phase of infection [93]. Another study proved that immune response modulation occurs in advanced chronic

fascioliasis too. The results indicated that during early chronic infection there was a predominance of a Th2 response, which decreased in the advanced chronic infection characterized by a persistent immune suppression [94]. Fascioliasis is a potent inducer of Th2 responses which impair the ability to mount any effective Th1 responses against bacteria and other pathogens [93, 95, 96].

The rapid and potent ability of fasciolids to suppress the protective arm of the immune response explains why infected hosts do not develop immune resistance. Within 24 h after oral infection, peritoneal macrophages express markers for the Th2-associated phenotype and display a reduced ability to respond to Th1 stimulants. This implies that by the time the newly excysted juveniles have penetrated the intestinal wall and entered the peritoneum, they have already initiated the immune events that will dominate throughout infection. So, these early-stage parasites secrete immunomodulatory molecules that influence the function of innate cells (dendritic cells, macrophages, neutrophils, mast cells, etc.) in the intestinal wall and peritoneal cavity. A systemic antigen-specific Th2 response is firmly established already at 7 days postinfection and is characterized by the secretion of IL-4, IL-5 and IL-13 from splenocytes. As the infection develops (3 weeks), regulatory macrophages (TGF-ß and IL-10 producing) and dendritic cells (IL-10 producing) are recruited to the peritoneum and dendritic cell maturation is inhibited. Mast cells recruited to the site of infection exhibit impaired Th1 promoting abilities. Most CD4* T cells in the peritoneum secrete IL-10 but not IL-4 or IFN-gamma. IL-10 secreting Tregs are induced which exert a suppression of both Th1 and Th2 cells that become non-responsive to parasite-specific antigens and mesenteric lymph nodes produce IL-10 and IL-5, but not IFN-gamma and IL-17, in response to stimulation by parasite antigens [97].

The chronic disease is also typified by Th2 responses and suppressed Th1 responses. Serologically, this polarity of immune response is strikingly displayed in the isotype of circulating antibodies. Fluke-infected animals secrete high titres of IgG1 antibodies and virtually no IgG2. Furthermore, blood macrophages are non-responsive to stimulation with endotoxin and exhibit elevated levels of arginase indicative of a phenotype that metabolize L-arginine and are important in promotingTh2 responses and facilitating tissue repair and fibrosis [97].

A consequence of liver fluke infection is the suppression of immune responses directed against concurrent or secondary pathogenic infections. The synergistic capacity of fasciolids in coinfection with other pathogenic agents is well known, immunological responses to pathogen antigens being markedly suppressed and concomitant infection being exacerbated following fascioliasis infection. The parasitological spectrum of protozoan and helminthic species found in the inhabitants of the human fascioliasis endemic areas, the multiparasitisms, and the associations between liver fluke infection and infection by other pathogenous parasites, all appear to be similar in the different human endemic zones [54, 57–60]. These synergistic associations of fascioliasis with other pathogens are believed to underlie the high morbidity and mortality rates of Aymara children inhabiting the Northern Bolivian Altiplano [7].

4.8 Genomics and Proteomics

Many different tools known to be useful for intraspecific variability analyses have been applied to fasciolids. Most studies on fasciolid proteins have concentrated on isoenzymes. Only a very few studies considered individual or population-level variation. The same isoenzymes of *F. hepatica* were detected regardless of the host species (cattle, sheep, goats), but densities of some isoenzyme bands did differ according to host [98]. Profiles of whole-body proteins and excretory/secretory products obtained with isoelectric focusing differ among worms from different hosts [99], and isoelectric focusing is therefore not a good technique. Random amplified polymorphic DNA (RAPD) markers applied to *F. hepatica* showed that the majority of genetic diversity occurred within, rather than between hosts and was also greater within than between populations. Individual cows were infected by numerous genetically different liver flukes, suggesting the influence of mainly migrations and transportation of definitive hosts [100]. Five among six microsatellite markers proved to be polymorphic in *F. hepatica* from Bolivia. No genetic differentiation between sampling sites or between definitive host species (sheep, cattle, pig) was found when applying these microsatellites [101].

Similarly, the restriction fragment length polymorphism (PCR-RFLP) technique has been applied repeatedly to fasciolids [102–104], but unfortunately these assays are only useful for the differentiation of pure species, but not for hybrid forms [6]. Indeed, this was already initially detected three decades ago. Restriction endonuclease maps of rRNA genes were distinct for *F. hepatica* and *F. gigantica*, Japanese *Fasciola* sp. being identical to *F. gigantica*. No intraspecific variations in the maps of *F. hepatica* or of *F. gigantica* were detected, but length heterogeneity was noted in the intergenic spacer, even within individual worms [105].

The aforementioned problems posed by isoenzymes, RAPD, microsatellites and RFLP techniques explain why these genetic tools have been abandoned or only sporadically used in *Fasciola*. That is why genetic studies on fasciolids mainly rely on DNA sequencing techniques at present.

In an initial approach, a total of six differences were detected between *F. hepatica* from Ipswich and *F. gigantica* from Malaysia in a 28S rRNA gene D1 domain fragment [106]. However, within the nuclear ribosomal DNA (rDNA) operon. Studies in invertebrates in general have shown that the ITS spacers are the most adequate markers for species differentiation [107]. The complete sequence of ITS spacers of fasciolids was obtained for the first time at the beginning of this century [108]. Nowadays, a large amount of literature is already available about the variability of ITS-1 and ITS-2 in *F. hepatica*, *F. gigantica*, and also hybrid or intermediate forms from throughout. This large information has recently been reviewed, corrected and a useful standardized nomenclature for DNA markers in fasciolids proposed [6].

In a large analysis of samples of "pure" *F. hepatica* from numerous countries and continents, only two haplotypes of the ITS-2 differing in only one mutation were found: the most spread FhITS2-H1 and the apparently more geographically

restricted FhITS2-H2. On the contrary, the sequence of the other spacer ITS-1 always proved to be identical in "pure" *F. hepatica*: FhITS1-HA. In "pure" *F. gigantica*, only one haplotype in ITS-2 (FgITS2-1) and similarly one in ITS-1 (FgITS1-A) were found. When comparing ITS-2 sequences, the two haplotypes of *F. hepatica* (FhITS2-H1 and H2) with the only one of *F. gigantica* (FgITS2-H1), five polymorphic sites enable the two species to be distinguished. When comparing ITS-1 sequences, the only haplotype of *F. hepatica* (FhITS1-HA) differs from the only haplotype of *F. gigantica* (FgITS1-HA) also in five polymorphic sites [6].

A comparative study with sequences obtained in countries not included in that countrywide study allowed to reach the conclusion that up to four haplotypes of ITS-2 could be distinguished in *F. hepatica* (FhITS2-1 to 4) and up to five in *F. gigantica* (FgITS2-1 to 5). On the contrary, the ITS-1 appeared to be fully uniform in both *F. hepatica* and *F. gigantica* everywhere (FhITS1-A and FgITS1-A) [6].

With regard to mitochondrial DNA (mtDNA), the complete genome of *F. hepatica* has been already sequenced, which will be suitable for studies of variation [109]. Unfortunately, only small fragments of the mtDNA codifying genes *cox*1 and *nad*1 have been used in the numerous local studies, and this becomes a problem due to the biased information gene fragments furnish [107].

Only in the aforementioned wide multicountry analysis of samples of "pure" *F. hepatica* and "pure" *F. gigantica* from different continents were these mitochondrial genes analyzed in its complete length [6]. "Pure" *F. hepatica* showed a mtDNA *cox*1 codifying gene providing a total of 69 different haplotypes (Fh*cox*1-1 to 69), including a total of 78 polymorphic sites. A total of 23 different haplotypes of the COX1 protein were found (FhCOX1-1 to 23). In its turn, the mtDNA *nad*1 codifying gene provided a total of 51 different haplotypes (Fh*nad*1-1 to 51). A total of 15 different haplotypes of the NAD1 protein were found (FhNAD1-1 to 15). "Pure" *F. gigantica* showed a *cox*1 gene providing a total of 11 different haplotypes (Fg*cox*1-1 to 11). A total of 5 different haplotypes of the COX1 protein were found (FgCOX1-1 to 5). In its turn, the *nad*1 gene provided a total of 15 different haplotypes (Fg*nad*1-1 to 15). A total of 10 different haplotypes of the NAD1 protein were found (FgNAD1-1 to 10) [6].

Proteomic tools have provided wide information about the profiles of soluble proteins secreted by fasciolids, mainly on cathepsin L and cathepsin B family of peptidases and their temporal expression as the parasites progresses from tissue to tissue [110], as well as about the expression and function of several antioxidant molecules glutathione S-transferases, fatty acid binding proteins and peroxiredoxin which, besides protecting the parasite from damaging reaction, may also have immunoprotective functions [111].

Given the intimate contact between the fluke and host tissues through its migration, antigens associated with the tegument of *Fasciola* also modulate host immune cell function. The tegument of *Fasciola* is a unique syncitial layer that plays the interface between host and parasite. In recent proteomic studies on the adult stage, extracted fractions of *F. hepatica* showed to contain 172–229 proteins, providing valuable insights into the complex protein composition within the tegumental layer

as a whole [97]. Regarding *Fasciola* juveniles, the enzymatic shaving of peptides from the surface of liver flukes and their subsequent identification, has allowed at least some of the tegumental surface proteome to be identified [112].

4.9 Diagnosis

Although some suggestive clinical presentation aspects may be useful, mainly in human endemic areas where physicians are aware about liver fluke infection risk in humans, verification needs the use of at least one among the direct parasitological techniques or indirect immunological tests. Other non-invasive diagnostic techniques presently available may be additionally helpful. Non-invasive diagnostic techniques which can be used for human diagnosis are radiology, radioisotope scanning, ultrasound, computed tomography and magnetic resonance (see reviews in [39, 113]).

4.9.1 Coprological and Other Direct Diagnostic Techniques

Analysis for the detection and identification of fasciolid eggs found in stool sample (Fig. 4.3), duodenal contents or bile continues to be the most appropriate diagnostic strategy for both detection of infection and estimation of intensity. This is even in spite of the recognized lower sensitivity of egg detection in faecal samples and its uselessness for the diagnosis of patients in the acute period, as well as the lack of an accurate relationship between egg counts per g of faeces and the fluke burden [114, 115]. Identifying fluke adults obtained during an endoscopy of after surgical intervention either by microscopic morphometry [10] or molecular tools [6, 102] may also be performed nowadays, although such occasions are evidently not frequent at all. Moreover, the infrastructure for endoscopy or surgery is in general not available in rural endemic areas.

Techniques ranging from a simple direct smear to different concentration methods may be used. Egg concentration has been achieved by flotation and sedimentation techniques. The sedimentation techniques appear to be more accurate and sensitive than flotation techniques [6, 39].

The size of the fluke eggs has always been used for human diagnosis. Basing on studies in livestock, the borderlines allowing differentiation between the two species were traditionally considered to be 150 μm in length and 90 μm in width, lower values representing *F. hepatica* and higher values *F. gigantica*. However, large variations were first observed in the size of *F. hepatica* eggs in livestock from different geographical locations [116]. Furthermore, it has been experimentally shown that the final host species (sheep, cattle, pig and donkey) decisively influences the size of the *F. hepatica* eggs even within the same endemic area [14]. Additionally, the existence of intermediate forms between the two fasciolid species and genetic

hybrids of both in overlapping areas increases the problem. The existence of these intermediate forms posed a question mark on whether egg characteristics are suitable as a tool for the differential diagnosis of fascioliasis caused by either species [115]. A concrete example of this problem was already emphasized in the diagnosis of fascioliasis in humans [117]. All in all, the aforementioned problems plus the use of serological tests, all of which unable to differentiate between the two fasciolids, or the lack of a calibrated microscope for measurements with an ocular micrometer explain why subjects diagnosed from areas where both species co-exist, such as in areas of Africa and Asia, are currently referred to as infected by *Fasciola* sp. or simply *Fasciola* [78].

A study of fasciolid eggs from different continents, using a computer image analysis system (CIAS), revealed that eggs shed by humans show morphological traits different from eggs shed by animals. In humans, *F. hepatica* eggs are bigger and *F. gigantica* eggs are smaller than reported to date from livestock, and their measurements overlap when compared. Measurements of *F. hepatica* and *F. gigantica* eggs originating from humans and animals from sympatric areas overlap, and, therefore, they do not allow differential diagnosis when within this overlapping range (Table 4.1) [89, 115]. These new results should aid clinicians since the application of the classic egg size range in human samples may lead to erroneous conclusions. Consequently, fasciolid egg size in human stool samples ought to be corrected in books and monographs as well as in guides of medical parasitology and tropical medicine.

Quantitative coprological analyses become important in epidemiological surveys as well as post-treatment monitoring. Egg burden is also crucial in the moment of deciding the appropriate treatment dose. The 400-epg threshold has been proposed for identifying high intensity infections. To avoid risk of colic, a repeated, timely spaciated mid-dose is recommended in patients shedding more than 400 eggs [118, 119]. The second half of the regimen is administered 24 h later, once the absence of secondary effects verified. The Kato–Katz technique appears to be appropriate, because of its simplicity, very low cost and reproducibility [8]. Its low sensitivity may be solved by repeated application.

Besides eggs in coprological analyses, adults and eggs may be also found elsewhere by means of other invasive techniques: obtaining duodenal fluid, duodenal and biliary aspirates; surgery (laparotomy, cholecystectomy, sphincterotomy); histological examination of liver and/or other organ biopsy materials [8].

4.9.2 Serological and Other Indirect Diagnostic Techniques

Numerous serological, intradermal and stool antigen detection tests have been developed. Immunological techniques present the advantages of being applicable during all periods of the disease, but fundamentally during the invasive or acute period, as well as to the other situations in which coprological techniques may present problems. However, immunological techniques offer other types of problems related mainly to sensitivity and specificity. Different serological tests have been

Table 4.1 Measurements of eggs of *Fasciola hepatica* and *F. gigantica* in different world regions according to the absence or existence of overlap of the two fasciolid species (intermediate hybrid forms have egg size ranges different from pure species)

Endemic areas	Geographical distribution	*Fasciola hepatica*		*Fasciola gigantica*	
		In humans	In animals	In humans	In animals
Areas where *F. gigantica* is absent	The Americas and Europe	100.6–162.2/65.9–104.6	73.8–156.8/58.1–98.1	–	–
Areas where both fasciolid species are present	Parts of Africa and Asia	106.5–171.5/63.9–95.4	120.6–163.9/69.2–93.8	150.9–182.2/85.1–106.2	130.3–182.8/74.0–123.6
Areas where *F. hepatica* is absent	Parts of Africa	–	–	137.2–191.1/73.5–120.0	129.6–204.5/61.6–112.5

Size given in length/width. All measures in μm
Data from Mas-Coma et al. [89] and Valero et al. [115]

used for human diagnosis. Almost all these techniques concern the detection of circulating antibodies and only a very few are designed to detect circulating antigens and immune complexes.

In recent years, efforts have been concentrated in obtaining purified excretory/secretory antigens and/or recombinant molecules to improve serological tests, owing to the problems of the parasitological diagnosis because of the delay in its usefulness in the acute period (coprological examination positive only after 3-4 months postinfection), intermittent egg output dynamics, very low or even absence of egg shedding in cases of only one or a few fluke adults and old, chronic infections, ectopic infections, "false" fascioliasis related to eggs in transit after ingestion of infected liver from domestic animals, or flukes unable to attain maturity in human subjects in non-human endemic areas [8, 39].

Several cysteine proteinases offer highly sensitive and specific markers for human fascioliasis serodiagnosis for *F. hepatica* [36, 120–128] as well as for *F. gigantica* infection [129–133]. *Fasciola hepatica* recombinant cysteine proteinases produced in yeast [123] or in *Escherichia coli* [134] have been used in ELISA methods for human infection diagnosis.

Very recent studies in two human hyperendemic areas of Bolivia and Peru have shown that the MM3 coproantigen-detection test allows for high sensitivity and specificity, fast large mass screening capacity, detection in the chronic period, early detection of treatment failure or reinfection in post-treated subjects, and usefulness for surveillance programs. However, this technique falls short when evaluating the fluke burden on its own [119]. The use of a new preservative/diluent CoproGuard™, developed for preservation of *Fasciola* coproantigens, proved to enhance coproantigen extraction without affecting the detection limit of the assay, and the antigenicity of *Fasciola* coproantigens in faecal samples stored at 37 ºC was retained throughout the entire observation period [135]. Thus, MM3-COPRO ELISA combined with the use of CoproGuard™ may be a very useful tool for the diagnosis of human fascioliasis.

Another study demonstrated that the commercialized DRG *Fasciola hepatica* IgG (human) ELISA is highly sensitive and specific, has a high negative predictive value but has a low positive predictive value. No correlation between egg output and the *F. hepatica* IgG ELISA test values was observed. It was concluded that this test could be used both as an individual serodiagnostic test for human fascioliasis when backed up by a compatible clinical history together with a second diagnostic technique for other cross-reactive helminth infections and in future large-scale epidemiological studies of human fascioliasis worldwide [136].

A new lateral flow test (SeroFluke) for human diagnosis appears to be a useful step forward [137]. This test was constructed with a recombinant cathepsin L1 from *F. hepatica*, and uses protein A and mAb MM3 as detector reagents in the test and control lines, respectively. In comparison with an ELISA test (MM3-SERO), the SeroFluke test showed maximal specificity and sensitivity and the advantage of being applicable to both serum and whole-blood samples. Its simplicity allows it to be used in major hospitals as well as in endemic/hyperendemic regions where point-of-care testing is required.

4.9.3 *Fasciolid Species Differentiation by Molecular Tools*

Infection by *F. hepatica* and *F. gigantica* cannot be differentiated by clinical, pathological, coprological or immunological methods. This is a problem in overlapping areas because this differential diagnosis is very important owing to the different pathological, transmission and epidemiological characteristics of the two fasciolids, as well as due to intermediate forms in which egg measurements may overlap.

To distinguish between *F. hepatica* and *F. gigantica*, a simple and rapid PCR-RFLP assay, using the common restriction enzymes *Ava*II and *Dra*II, has recently been described. It is based on a 618-bp-long sequence of the 28S rRNA gene recently obtained from populations of South America, Europe and Africa. This sequence showed no intraspecific variations within each species and a few nucleotide differences between both fasciolids. This assay provides unambiguous results and may be useful for both individual subject diagnosis and epidemiological surveys of humans and animals in endemic regions of sympatry in Africa and Asia [102]. A similar PCR-RFLP assay using restriction endonucleases *Hsp*92II and *Rca*I has been recently applied to differentiate between Chinese liver flukes [103]. Another such PCR-RFLP method was later developed [104].

Unfortunately, these three aforementioned PCR-RFLP assays are only useful for the differentiation of pure species, but not for hybrid forms [6]. A similar comment may be applied to the recently developed single step duplex PCR for simultaneous detection of both fasciolid species [138], as well as to the TaqMan real-time PCR-based assay [139], and other Specific PCR-based assays [140]. None of these methods proves to be able to detect the wide introgression capacity the two fasciolid species have [6].

Therefore, DNA marker sequencing still remains as the only appropriate method for both haplotyping of the two pure fasciolid species, as well as for the detection of hybridization in intermediate forms. For such a purpose, the complete sequences of the two rDNA spacers ITS-2 and ITS-1 together with those of the mtDNA genes *cox*1 and *nad*1 have so far proved to be the markers of choice, and a complete baseline and nomenclature for these four markers have already been provided [6].

4.10 Treatment

Many drugs have been used to treat human fascioliasis. Emetine derivatives, the classic drugs, were used widely and still continue to be used today, given intramuscularly or subcutaneously at doses of 1–10 mg/kg a day for 10 days. However, the use of emetine was progressively abandoned due to their toxic side effects involving heart, liver and digestive tract. The same occurred with dehydroemetine despite its better tolerability. Dehydroemetine, at a usual dose of 1 mg/kg daily for 10–14 days, was even considered the therapy of choice a few decades ago [89].

Chloroquine was also used to treat *F. hepatica* infection. Although no cidal effects on the flukes were shown, treatment by this aminoquinoline derivative improved the symptoms dramatically when applied in the acute phase. Among xylol derivatives, hexachloro-para-xylol was effectively used, mainly in the old Soviet Union and China. Bithionol, an halogenated phenol derivative, was proposed as the drug of choice for the treatment of *F. hepatica* infection during the last three decades of last century. It was usually applied at a dose of 30–50 mg/kg daily, divided into 3 oral doses on alternate days for 20–30 days. In cases of fascioliasis resistant to emetine and praziquantel treatment, bithionol achieved cure in dosages of 50 mg/kg daily for 10 alternate days or 40 mg/kg daily for 14–15 alternate days. Occasionally, the patients required a second course to obtain a complete cure. The side effects, including diarrhoea, anorexia, nausea, vomiting, pruritus, urticaria and abdominal pain, were usually mild [3, 39]. Another halogenated phenol derivative such as niclofolan was also assayed for liver fluke treatment in humans but rapidly abandoned due to its wide side effects.

Praziquantel is an isoquinoline-pyrazine derivative which was widely applied for the treatment of human fascioliasis during the 1980s and 1990s, basing on the fact that it is the drug of choice for human trematode infections. However, controversial results were found already from the beginning of its application to fascioliasis patients, including many reported praziquantel failures even at high doses. Today, it is generally accepted that *Fasciola* may be the only trematode genus that has practically no response to praziquantel.

Metronidazole and albendazole and sporadically also mebendazole are imidazole derivatives which have been also applied for human fascioliasis treatment with more or less success. But another imidazole derivative as triclabendazole (Egaten®) has become the drug of choice for human fascioliasis caused by both *F. hepatica* and *F. gigantica* at present [141]. This drug is better adsorbed if administered after meals [142]. The recommended dosage is two separate regimens of 10 mg/kg. A cure rate of 79.2 % when first used and 100 % after a second round of therapy was found in Chile [143], and 79.4 % and 93.9 %, respectively in Egypt [144]. Triclabendazole appears to keep its efficiency at standard regimes in human endemic areas after years [145], although the need for a third dose has been reported in Cuba [71].

The risk of appearance of resistance to triclabendazole can neither be forgotten taking into account the veterinary use of triclabendazole (Fasinex®) for livestock treatment in endemic areas since long ago, the tradition of human self-treatments with Fasinex® owing to the very general availability of this drug, and the appearance of triclabendazole resistance in animals in different countries. Triclabendazole resistance was first described in Australia, later in European countries such as Ireland, Scotland, the Netherlands, and Spain [146]. Very recently it has also been found in southern Brazil [147] and Argentina [148], and thus already in the New World. Up to that moment, triclabendazole resistance only concerned livestock in animal endemic areas, but unfortunately it has very recently been also described [149] in a human highly endemic area such as the Andean valley of Cajamarca, Peru [62]. The strategies to minimize the development of resistance include the use

of synergistic drug combinations [150], although this approach has the risk of building up multiple drug resistance [151]. Additionally, studies suggest that our understanding of the mechanism of resistance to triclabendazole remains far from complete [152–154], so that the spreading capacity of triclabendazole resistance remains unknown.

Nitazoxanide is a pyruvate ferredoxin oxidoreductase inhibitor with reported efficacy on a broad parasitological spectrum, such as intestinal protozoans and helminths. It may be considered a good alternative to triclabendazole, at least for the chronic stage of fascioliasis, mainly in those countries where Egaten® is still not registered but nitazoxanide is since several years. Nitazoxanide had demonstrated its efficacy against human fascioliasis in a few trials, in Egypt [155, 156] and Peru [157]. Its long 7-day treatment course may nevertheless become a problem. However, its usefulness for the treatment of human cases not responding to triclabendazole [158] is of important additional value. A good nitazoxanide efficacy has recently been reported when applied to liver fluke infected children in Mexico [67]. However, differences in fasciolid susceptibility to nitazoxanide may exist depending on geographical strains. Thus, no response to nitazoxanide treatment was reported in 24 cases of liver fluke infection in Esmeralda, Camagüey, Cuba [159], and a triclabendazole-resistant *F. hepatica* infected patient not responding to nitazoxanide treatment has recently been reported in the Netherlands [160].

Mirazid® is a drug prepared commercially from myrrh (Arabian or Somali) which is an oleo-gum resin obtained from the stem of thorny trees *Commiphora molmol* and other species of the family *Burseraceae*. Introduced to the local Egyptian market, it has been highlighted by its efficacy against human fascioliasis in many reports, although a recent evaluation proved that it showed only an insignificant activity against the liver fluke [161].

Artemisinin derivatives initially showed a high fasciolicidal activity in sheep infection, which was encouraging. Artesunate and artemether, given by the intramuscular route, yielded high egg and worm burden reductions. A study in Vietnam showed that the complete response rate at 3 months was lower than in triclabendazole, although those treated with artesunate were significantly more likely to be free of abdominal pain [162]. Unfortunately, a last study in Egypt demonstrated that artemether, administered at malaria treatment regimens, shows no or only little effect against fascioliasis, and hence does not represent an alternative [163].

4.11 Control

Prevention and control measures recommended for human fascioliasis were traditionally the same to be applied for veterinary fascioliasis, at the levels of domestic animals, snails and field [164–166]. However, studies on human endemic areas performed in the last two decades have shown that traditional epidemiological patterns of animal fascioliasis may not always explain the characteristics of human infection in a given area. Therefore, control measures for human fascioliasis should consider

Fig. 4.7 City market showing uncontrolled sale of vegetables involved in the transmission of human fascioliasis in Quy Nhon, Vietnam (Orig. S. Mas-Coma)

the results of the ecoepidemiological studies previously undertaken in the area concerned [6]. This is the reason why the WHO launched a worldwide initiative against this disease including different control strategies depending on the human endemic areas and countries.

With regard to individual measures, the prevention of human infection may be achieved by strict control of the human infection sources in each place, mainly with regard to watercress and other metacercariae-carrying aquatic plants for human consumption, especially in endemic zones. Unfortunately, potassium permanganate, which had been suggested to be the most effective preventive tool for killing metacercariae attached to leaves and vegetables used in salads, has been shown to have no effectivity on metacercarial viability, even at the very high doses [83].

Moreover, it should be considered that infection risks shall not be restricted to only ingestion of freshwater vegetables, as always mentioned. The different human infection sources may be taken into account, mainly in human endemic areas. Drinking of natural freshwater should be avoided in human endemic areas. In many human hyperendemic areas of the Americas, people do not have a history of eating watercress or other freshwater plants [60]. In the Nile Delta region, persons living in houses where piped water is present showed to have a higher infection risk [52].

The problem does not only concern rural areas, as usually believed. The possibility of human infection in urban areas should not be neglected. Thanks to transport of vegetables (both aquatic and terrestrial) from rural endemic zones to cities, plants carrying metacercariae can be sold in non-controlled city markets giving rise to urban infection (Fig. 4.7) [7].

Within general control measures to be applied in human fascioliasis endemic areas, education should always be included, mainly with regard to the need to let know inhabitants about the human infection sources. The community should be appropriately informed about the disease, its pathogenicity, its transmission, and where to go for diagnosis if suggestive symptoms appear.

The availability of a very effective drug against fascioliasis as triclabendazole prompted the WHO to launch a decisive step forward within its worldwide initiative against human fascioliasis [118, 167] in recent years. This initiative includes action in human fascioliasis endemic areas presenting different epidemiological situations and transmission patterns [6, 78]. Pilot schemes were designed to assess the best control strategies according to the different epidemiological situations and transmission patterns in the way to decrease morbidity, mainly in children. Selective patient treatments after passive detection in hospitals was the strategy applied in Vietnam, and infected subject treatment after active detection in surveys in the Nile Delta high human endemic region the one applied in Egypt. Bolivia and Peru were the other two countries selected for priority intervention due to the very large public health problem posed by this disease. The Northern Bolivian Altiplano was chosen as an example of the Altiplanic pattern, while the Cajamarca valley was chosen as an example of the valley pattern. The respective pilot interventions in the two Andean human endemic areas demonstrated the absence of serious side effects in triclabendazole treatments of schoolchildren [168], which subsequently allowed for the launching of mass treatments in these two Andean countries. Many other countries are nowadays receiving yearly triclabendazole donations through WHO for the treatment of their patients, in an expansion of the aforementioned WHO initiative.

In countries where watercress is included in food traditions, such as France, commercial growing of watercress should be carried out under completely controlled conditions, without access for ruminants and snail vectors.

In Egypt, the construction and utilization of the so-called "washing units," in which the water was appropriately filtered, gave rise to a marked decrease of human infection in a locality of the Nile Delta region where a high prevalence in humans was initially found [7].

Regarding veterinary control, previous epidemiological studies may provide for general recommendations on the appropriate time for treatment with effective drugs to achieve economic control, and better information from the livestock farming community. Forecasts of outbreaks may be made based on climatological data and epidemiological models. Recommendations for control measures should be made on a preventive rather than a curative basis, and all measures have to be considered from the point of view of the economy and assessment of local topographical and meteorological conditions. The efficiency of fascioliasis control depends on the correct and integrated application of several measures [15]: (1) reduction of the parasite load of the animal hosts and pasture contamination by regular strategic use of drugs (preventive treatment in appropriate year periods according to different regions); (2) reduction of the number of snails by physical, chemical and biological means; (3) reduction of the risks of infection through correct farm management

practices (rotational system through fluke-infected and fluke-free paddocks, combined with effective treatment).

Owing to the similarity of the life cycles of the two fasciolid species, prevention and control measures follow the same patterns for both *F. hepatica* and *F. gigantica*. However, the peculiarities of *F. gigantica* should be considered. Thus, in enzootic areas of *F. gigantica*, contraction of the infection by the animals and their contamination of the area with eggs shed with faeces take place when the animals go to drink, rather than when they are grazing in the pasture as is the case in *F. hepatica*. Accordingly, avoiding the watering of the animals from swampy banks of rivers and from bodies of water rich in vegetation would considerably reduce infection chances [15].

Lymnaeid vector control has unfortunately not received, by public health officers, the sufficient attention required to definitively eliminate transmission [3]. Intensive agricultural methods must be applied to reduce suitable snail habitats. Besides physical methods, there are available control strategies which consist of the use of chemical molluscicides, natural molluscicides of plant origin, biological control (including predators, competitors, the decoy effect and related phenomena, parasitic castration, interspecific trematode antagonism, and pathogens), genetic manipulation, and engineering control. However, the practical application of chemical methods in the control of snails is of doubtful value, requires labour and equipment, and regular yearly strategic molluscicide applications. Moreover, the application of molluscicides in the case of the small *Galba–Fossaria* vector species showing marked amphibious behaviour becomes almost impossible, due to the small size of the water bodies these vectors inhabit.

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Chapter 5
Paragonimiasis

David Blair

5.1 Introduction

Paragonimiasis is a zoonotic disease caused by lung flukes of the genus *Paragonimus*. Humans usually become infected by eating freshwater crabs or crayfish containing encysted metacercariae of these worms. However, an alternative route of infection exists: ingestion of raw meat from a mammalian paratenic host. Adult worms normally occur in pairs in cysts in the lungs from which they void their eggs via air passages. The pulmonary form is typical in cases of human infection due to *P. westermani*, *P. heterotremus* and a few other species (Table 5.1). Worms may occupy other sites in the body, notably the brain, but lung flukes have made their presence felt in almost every organ. Ectopic paragonimiasis is particularly common when infection is due to members of the *P. skrjabini* complex (Table 5.1). Human paragonimiasis occurs primarily in the tropics and subtropics of Asia, Africa, and the Americas, with different species being responsible in different areas (Table 5.1).

As agents of a "neglected tropical disease" [1], there is a tendency for people to regard lung flukes as unimportant and imposing a decreasing and trivial burden on human populations, having been eliminated in many formerly endemic areas. This is dangerous and misleading: recent estimates indicate that paragonimiasis is a major and continuing problem, with 292 million people at risk [1, 2], and about 23 million people in 48 countries (mostly in China) actually infected in the year 2005 [2]. Of this total, over five million likely had heavy (i.e., symptomatic) infections, and about 166,000 had cerebral involvement. Among the food-borne trematodes, *Paragonimus* species cost more disability-adjusted life years (DALYs) [3] than opisthorchiasis, fascioliasis, and intestinal fluke infections combined (Table 5.2). Only infections with *Clonorchis sinensis* imposed a higher burden in 2005 [2].

D. Blair (✉)
James Cook University, Townsville, QLD, Australia
e-mail: david.blair@jcu.edu.au

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Table 5.1 The major species of lung flukes responsible for human disease

Species	Geographical distribution	Type of paragonimiasis in humans	Natural definitive hosts[a]	Notes
P. westermani complex	East Asia (China and Taiwan, Korea, Japan, SE Siberia); SE Asia (Philippines, Malaysia, Thailand, Cambodia, Laos, Vietnam), South Asia (Sri Lanka, India), possibly also Nepal, Pakistan, and Papua New Guinea	Usually pulmonary; pleural disease not uncommon and other forms sometimes	Cercopithecids, canids, felids, herpestids, viverrids, mustelids, murids	A species complex exhibiting considerable geographical genetic variation. Apparently infective to humans only in East Asia and the Philippines
P. skrjabini and subspecies such as *P. s. miyazakii*	East Asia (China, Japan), Thailand; may extend westwards into NE India	Pulmonary forms rare; pleural and ectopic forms usual	Canids, felids, mustelids, viverrids, murids, hystricids	A species complex, but more restricted in distribution than *P. westermani*. Humans not usually suitable definitive host
P. heterotremus	South and SW China, Vietnam, Laos, Thailand, NE India	Usually pulmonary; ectopic cases reported [214]	Felids, sciurids, murids	Likely also a species complex: one human case reported of the recently described *P. pseudoheterotremus*
P. africanus	West Africa: Equatorial Guinea, Cameroon, Nigeria, possibly Ivory Coast	Usually pulmonary	Lorids, cercopithecids, herpestids, viverrids	
P. uterobilateralis	West Africa: Gabon, Cameroon, Nigeria, Liberia	Usually pulmonary	Canids, herpestids, mustelids, viverrids	
P. kellicotti	North America: Mississippi Basin and Atlantic coast of the USA; Ontario and Quebec in Canada	Usually pleural and pulmonary	Didelphids, canids, felids, mustelids, procyonids, suids, bovids, murids	Rare in humans, but humans are permissive hosts
P. mexicanus	Central and South America: Mexico, Costa Rica, Panama, Guatemala, Ecuador, Peru; probably other countries in the region	Usually pulmonary	Didelphids, cebids, canids, felids, mustelids, procyonids, suids	Possibly a species complex

Additional species may occasionally infect humans

[a]Omits experimental hosts, paratenic hosts, and humans

Table 5.2 Showing global estimates of the burden of food-borne trematodes in 2005 from Table 4 in [2]

	Total infected	Heavy infections	Cerebral infections	Deaths	YLD	YLL	DALYs
Clonorchiasis	15,313,219	1,131,982	N/A	5,591	37,083	238,287	275,370
Paragonimiasis	23,155,105	5,084,729	165,860	244	183,738	12,972	196,710
Intestinal flukes	6,723,551	926,137	N/A	0	83,699	0	83,699
Opisthorchiasis	8,398,230	329,987	N/A	1,323	11,300	63,067	74,367
Fascioliasis	2,646,515	299,510	N/A	0	35,206	0	35,206

Ranked by DALYs

DALY disability-adjusted life years, *YLD* years lived with disability, *YLL* years of life lost

A questionnaire administered to experts in human parasitology prior to a WHO meeting sought a ranking of 24 food-borne parasite taxa in order of importance. *Paragonimus* species were collectively ranked at 14 [4].

Even in the twenty-first century surveys of newly discovered endemic foci, or new examination of known foci, uncover prevalences of paragonimiasis that, for more fashionable diseases, would lead to great alarm. For example, paragonimiasis occupied fourth place on the list of causes of morbidity in the Municipality of Roxas, Philippines, in 2003 [5] and 27.2 % of over 900 registered tuberculosis patients had *Paragonimus* eggs in their sputum in 2002 [6]. A survey in NE India found local prevalences (based on serology) of 51.7 % in children under 15 years of age and 18.7 % in adults [7]. Of these, 20.9 % of children and 4.1 % of adults had eggs in their sputum. In SE Nigeria, 13.2 % of a population surveyed was egg-positive for paragonimiasis [8]. But it is important not to extrapolate too far from these focal data. They represent prevalences in particular communities or small areas and not national levels in any country.

In keeping with the persisting view that paragonimiasis is a rather quaint and unusual condition, medical journals sometimes feature quizzes and commentaries in which readers are invited to arrive at a diagnosis [9–11]. Veterinary journals in North America, where infections of domestic pets with *P. kellicotti* sometimes occur, also quiz their readers on this topic [12, 13]. It would be interesting to know how many reach unprompted the correct diagnosis of paragonimiasis.

Lung fluke disease is not a recent affliction of human societies. Evidence of paragonimiasis has been found in mummies and other archeological material in Japan, Korea, and pre-Columbian South America [14]. Eggs of *Paragonimus* species have been found at a Third Century AD archeological site in Japan [15]. In Korea [16], DNA sequences from eggs in tissues of a 400-year-old mummy confirmed the presence of *P. westermani*.

Despite its antiquity, paragonimiasis has spread its wings in the modern world. Cheap travel allows adventurous tourists the opportunity to try food that is new to them (and to take parasitic souvenirs home). However, even the stay-at-homes in non-endemic countries can sample parasites readily [17]. Crabs and crayfish, incidentally containing lung fluke cysts, are now regularly shipped around the world,

taking this and other diseases virtually anywhere. There are many papers reporting paragonimiasis cases in returned travelers, or in people who have eaten imported foods. Such papers almost always emphasize the difficulty in reaching a diagnosis: signs and symptoms of paragonimiasis are often vague and nonspecific. In most cases, they initially raise suspicions of other diseases such as tuberculosis or cancer [18, 19]. Indeed diagnostic tests for cancers may not initially change those suspicions [20, 21].

Recent publications focusing on the situation and trends in particular countries or regions are as follows: China [22, 23], Japan [24–27], Korea [28–32], Vietnam [33, 34], Laos [35], Thailand [36], the Philippines [5, 6], India [37], Brazil [38], Ecuador [39], Colombia [40], USA [17, 41, 42], Africa in general [43], South Africa [44], Cameroon [45], and Nigeria [8].

A few of the numerous previous general reviews and historical accounts are [46–52].

5.2 Life Cycle of *Paragonimus* Species

Like other trematodes, *Paragonimus* species have complex life cycles (Fig. 5.1). Hermaphroditic adult worms, resembling small coffee beans in size and shape in life, typically occur in the lung parenchyma of mammals, encapsulated in (usually) pairs in a fibrous cyst about 10–15 mm in diameter [41]. The cysts can communicate with bronchioles, allowing exit of eggs and cyst debris. Eggs, voided in sputum, or in feces if swallowed, hatch in fresh water to yield motile miracidia. These penetrate the tissues of particular species of freshwater snails, where a process of asexual multiplication yields many motile microcercous cercariae which leave the snail and enter the tissues of a crustacean, usually a crab or crayfish [53, 54]. Here, each cercaria develops and grows into a metacercaria (usually in the gills, heart, digestive gland, and other viscera, or the muscles), a "sit-and-wait" stage that is infective to mammals. Freshwater crabs and crayfish are prized food for many mammal species, including humans. Ingestion of inadequately prepared crabs or crayfish leads to infection, with metacercariae emerging from their cysts in the stomach or intestine and migrating through tissues towards the pleural spaces and lungs [49]. Worms can persist for many years in the lungs, thus obscuring their geographic origin if the patient has moved between countries. For example, a man was still expectorating eggs at least 20 years after leaving an endemic area [47].

If infected crustaceans are eaten by mammals in which worms are unable to mature, the juvenile worms may remain quiescent in the tissues [55]. If a suitable host eats such a paratenic host, worms from the latter may mature normally in their new home. Occurrence of paragonimiasis in top predators such as tigers, which are unlikely to eat small crabs directly, occurs via a range of paratenic hosts [55]. By such a route, human hunters and their hunting dogs in Japan can become infected by eating undercooked meat of wild *boars* [27, 56].

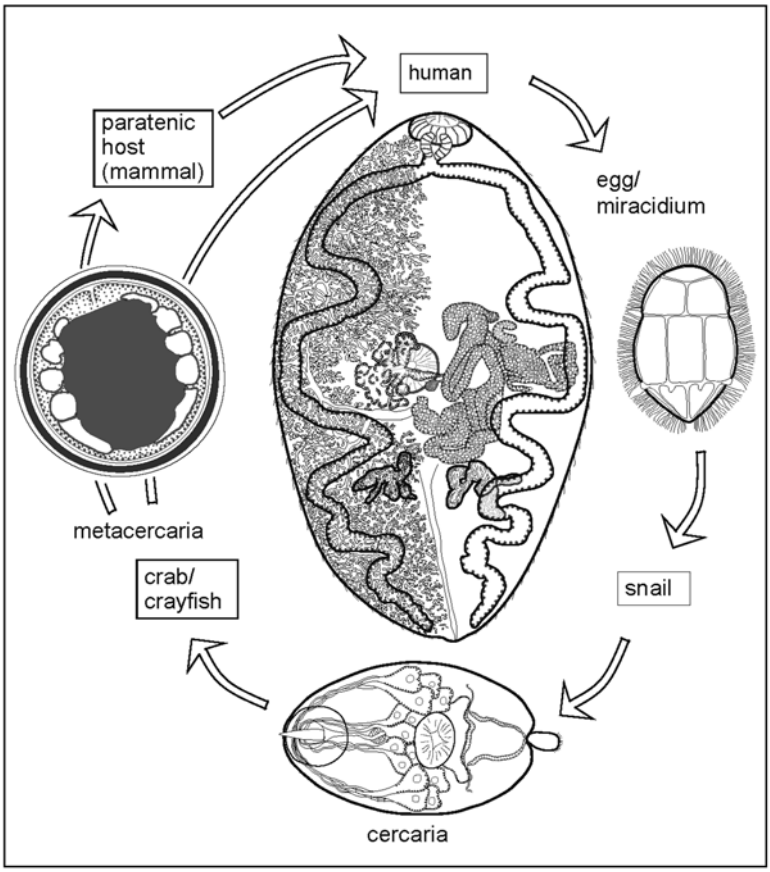

Fig. 5.1 Generalized life cycle of *Paragonimus* species. A miracidium hatching from the voided egg swims to find a suitable snail host. Within the hemocoel of the snail, cycles of asexual reproduction produce numerous cercariae which leave the snail and penetrate a crab or crayfish. Here, they grow and transform into metacercariae. If eaten by a suitable mammal definitive host, metacercariae migrate to the lungs, where they mature. In some unsuitable mammal species, metacercariae burrow through the gut wall and remain as small juvenile worms within the musculature. If such paratenic hosts are eaten by a suitable definitive host, the juvenile worms can mature in their new home. The drawing of the adult in the center of the figure is a dorsal view based on a photograph of a strongly compressed specimen of *P. westermani* from Vietnam, supplied by Dr. P.N. Doanh. Vitellaria are indicated on the left side of the body only

5.3 Taxonomy of *Paragonimus* Species

Paragonimus Braun 1899 is the sole genus in the family Paragonimidae [57]. There have been two recent major taxonomic treatments of the family [58, 59]. The earliest record of a lung fluke was the description by Diesing in 1850 of *Distomum rude*, based on specimens found in the lungs of a giant otter in Brazil by Natterer in 1828.

This was later placed in the genus *Paragonimus* [60]. Both the original description [61] and a redescription by Braun [62] failed to reveal some features vital for unambiguous identification. The type specimens are in poor condition [63] and no additional specimens have been found since, despite recent surveys of the original locality [63, 64]. The earliest described species that can be assigned to *Paragonimus* without doubt is *P. compactus* (Cobbold, 1859) from an Indian mongoose [65]. The first human cases reported were from Taiwan and date to 1879 [66]. Since then, >50 names have been applied to members of the genus, the majority of these in eastern and southern Asia [50]. Even within the last few years, new species have been proposed and described [67–69].

Paragonimus arguably presents more taxonomic problems than any other genus of food-borne trematodes. It will be important to resolve some of these, because species [49] and even different populations within a nominal species (see below) can differ in their biology and pathological effects. It is still not possible to say how many species of *Paragonimus* exist. Critical reviews of the literature and recent molecular work suggest that many names ought to fall as synonyms [50, 70].

Differentiation between species of lung flukes has long been based on some reasonably obvious morphological features [37, 49, 50]. For adults (see Fig. 5.1 for a typical example), these include arrangement of spines on the tegument, degree of branching of ovary and testes, body length–width ratios, and relative sizes of the suckers. Metacercarial cysts were long assumed to be morphologically conservative within a species, and a number of species have been proposed based only on the distinctive appearance of particular metacercariae. Characters of metacercariae that have been used [49, 50, 71] include details of cyst walls (presence or absence, number and thickness), relative diameters of suckers, the anterior extent of the excretory bladder, presence and length of a stylet on the oral sucker, presence of colored granules in the body, numbers of flame cells, body spination, and arrangement of papillae around suckers. In the case of eggs, there can be specific differences in size, shape, and shell sculpturing [50].

Until the application of molecular techniques to the taxonomy of the group, the stability and reliability of these morphological characters to distinguish among ~50 species could not be assessed. For more than 20 years, it has been possible to sequence DNA from adult worms, metacercariae, and even eggs. Such data (usually from portions of the mitochondrial genome and the nuclear ribosomal genes and spacers) have shown that many morphological features of metacercariae in particular are uninformative or misleading [70, 72, 73]. While tending to be morphologically rather conservative, features of adults can also be misleading. In particular, the arrangement of surface spines (singly, or in comb-like clusters) has long been regarded as a major taxonomic marker. However, very closely related species, that in some cases can actually hybridize, may differ with respect to this feature [74–76].

Molecular data have also indicated something else that was unexpected. Several "species" appear to represent species complexes. This is most apparent in the type species of the genus, *Paragonimus westermani* (Kerbert, 1878) Braun, 1899, described from the lungs of a Bengal tiger that had died in Amsterdam Zoo in 1877 [77]. It turns out that "*P. westermani*" is very widespread, occurring in eastern and southern Asia, from China (and adjacent parts of SE Russia [78]), Japan and Korea

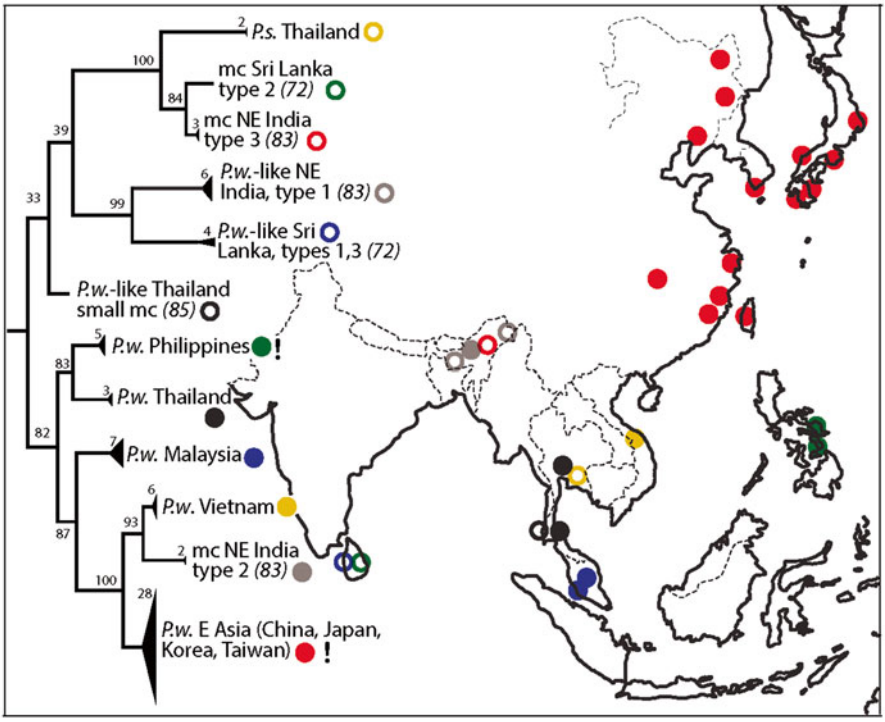

Fig. 5.2 Phylogenetic tree (based on partial mitochondrial *cox1* DNA sequences (382 bp)) of members of the *P. westermani* complex, with color symbols to show locations of populations on a map of eastern and southern Asia. The tree was inferred using Mr. Bayes and the substitution model GTR + I + G. Posterior support values are shown at nodes. The tree was rooted by outgroup (not shown). Most available sequences from GenBank were used. Subtrees containing multiple sequences have been collapsed to a triangle, the number of sequences represented is indicated to the top left of the triangle. In some cases, sequences were derived from metacercariae alone (abbreviated as "mc") and adults were not obtained. Some specific references are indicated (in italics) beside the relevant population. "!" indicates human pathogen. *P.w.—P. westermani. P.s.—P. siamensis*

west to India [79] and south to Sri Lanka [80], the Philippines [81], and possibly Papua New Guinea [82]. (Note that reports of endemic paragonimiasis caused by *Paragonimus westermani* in other parts of the world are likely to be erroneous, often based on the assumption that human cases are always due to this species. Note also that human cases due to *P. westermani* are likely only caused by populations of the complex from China, Japan, Korea, Taiwan, far SE Russia, and the Philippines.)

Morphologically, adults are very similar across this range, with spines spaced singly on the tegument, and testes and ovary possessing few, relatively simple lobes. Hence, the application of the name *P. westermani* has not been controversial until recently. Another nominal species, *P. siamensis*, has been shown by molecular tools to nest within the *P. westermani* complex [74, 83] (Fig. 5.2). Adults of the former species differ from those of *P. westermani* mainly in having spines arranged in comb-like clusters.

Molecular and morphological uniformity of adults and metacercariae is greatest in eastern Asia (China, especially the east, NE, and south, Taiwan, Japan, Korea, and adjacent parts of the Russian Federation). Such differences as exist in that region are primarily associated with ploidy differences (see below). This is also the region from which most human cases originate.

In the western part of the range (Thailand to India and Sri Lanka), there is more variation between populations. In the Indian subcontinent, Sri Lanka, and Thailand, various metacercarial morphologies have been noted that correspond with molecular differences yet remain within the *P. westermani* complex [79, 83, 84]. There are three morphologies in NE India (two close to *P. westermani sensu lato* and one close to *P. siamensis*) [83], two metacercarial types in Thailand that segregate according to molecular data in different parts of a phylogenetic tree, yet yield indistinguishable adults of the *P. westermani* type [85, 86] and at least two types in Sri Lanka [72, 84]. As far as is known, none of these variant forms is infective to humans, despite the reasonably close molecular correspondence of one of the Thai and one of the Indian forms with populations of *P. westermani* from East Asia that do infect humans (Fig. 5.2).

Individuals across most of the range of the *P. westermani* complex appear to be diploid, producing gametes by meiosis. However, in NE China, Japan, and Korea, triploid worms are common and often sympatric with diploids [87]. Triploids are unable to produce normal sperm [88]. However, they do produce eggs parthenogenetically. Thus, whereas diploid, sexually reproducing forms require partners for exchange of sperm and for cyst formation in the lungs of a mammal, each triploid worm is capable of cyst formation and egg production by itself (although more than one worm may occupy a cyst in heavy infections) [89, 90]. Triploid adult worms, their metacercariae and eggs are all larger than is the case for diploids. Tetraploid forms, which might produce viable gametes, have been found in China [91]. Research on relationships and origins of the polyploid forms is likely to continue for some time [92].

In addition to the genetic and ploidy differences within the *P. westermani* complex, there are marked biological differences in different places. Populations differ in their prepatent period [79]. Populations also differ in their preferred mammal hosts and in their snail hosts. For example, worm populations in the Philippines (and at least one member of the complex from India) mature readily in rats, whereas those in Malaysia and Japan can utilize rats only as paratenic hosts [55, 79]. Most strikingly, human cases attributable to members of the *P. westermani* complex are restricted to the eastern part of the range (with the possible inclusion of Papua New Guinea [82]. This is probably not due to differing dietary habits: *P. heterotremus* is the only species infecting humans in many areas where crabs containing metacercariae of *P. westermani* are also found (e.g., NE India, Laos), indicating unsafe consumption of crabs in those places. Knowledge of snail hosts in different regions is very limited and taxonomy of the relevant taxa is confused [93, 94]. However, different snail families are known to be used by members of the *P. westermani* complex in different countries [51, 95].

All of this genetic and biological diversity has been detected so far in a single nominal species! Genetic distances, based on DNA sequences, between some members of the *P. westermani* complex are as great as between very distinct species of *Paragonimus* [83]. If future taxonomists decide to recognize several different species within the *P. westermani* complex, there will be some difficult nomenclatural problems to deal with [51].

Other species complexes exist within the genus. The *P. skrjabini* complex [70, 96] of eastern and southern Asia is the most important of these in terms of public health. On the basis of both morphological and molecular data, it has been proposed that *P. miyazakii* from Japan and *P. skrjabini* from China are only distinct from one another at the level of subspecies [70]. Neither of these nominal taxa commonly matures in humans, but they are a frequent cause of severe ectopic paragonimiasis (Table 5.1). Also in Asia, *P. bangkokensis* and *P. harinasutai* belong to a distinct complex, the membership of which has yet to be fully established [75, 97]. These two species are able to hybridize and may co-occur in the same individual crab, yet have generally retained their separate identities [76]. In the Americas, it has recently been proposed that the human pathogen *P. mexicanus* includes cryptic species [98].

Paragonimus heterotremus is emerging as an important cause of paragonimiasis spanning the region from India to China. Molecular and other variation is now being discovered within this species and it may eventually be regarded as constituting another complex. In 2007, *P. pseudoheterotremus* was proposed as a sister species to *P. heterotremus* in Thailand, based on both morphological and molecular differences [68, 99]. Biological differences relating to mammalian hosts have also been reported. Rats act primarily as paratenic hosts for *P. heterotremus* in Thailand and NE India, but as normal definitive hosts for *P. pseudoheterotremus* [68, 100].

5.4 Evolution

Very distinct species of *Paragonimus* live in Asia, Africa, and the Americas. These lie far apart in molecular phylogenies and, where known, snail hosts are also distinct between continents. Given this, presence of *Paragonimus* species in the Americas likely long predates the arrival of humans. This is unlike the situation for *Schistosoma mansoni*, introduced to South America during the slave trade period [101]. Blair [102] proposed a Gondwanan origin for *Paragonimus*, with vicariance the explanation for its modern distribution, whereas Attwood [93] preferred an Asian origin with subsequent dispersal to other continents. Whichever is the case, it is clear that by far the greatest diversity in the genus is in eastern Asia. Attwood has speculated that this diversity in Asia, in contrast with the few endemic *Schistosoma* species there, could be due to transmission ecology: *Schistosoma* species have largely adopted very vagile final hosts (often humans), aiding dispersal over large areas and inhibiting local speciation. *Paragonimus* species, on the other hand, tend to utilize definitive hosts that are less vagile and more likely to establish local transmission cycles, facilitating speciation [93].

Below the level of genus, molecular trees have been used in support of biogeographic and evolutionary scenarios for the *P. westermani* complex. It used to be thought that the complex first appeared in southern Asia, perhaps in what is now Sri Lanka. From there, populations radiated towards the north and east, evolving as they went and adopting new intermediate and final hosts [84, 102]. However, the recent findings of pairs (or trios) of relatively distantly related populations in both NE India and S Thailand (see above) defy such simple interpretation. Worse, one of the NE Indian forms is phylogenetically moderately close to populations in East Asia (Japan, Korea, East China) that were regarded as the most derived under earlier scenarios. Much more work will be needed to reach an understanding of the evolution of the *P. westermani* complex. Identities of snail hosts, very poorly known outside East Asia, may be illuminating in this regard.

5.5 Epidemiology and Control Measures

5.5.1 Prevalence and Changes

It remains unclear to what extent the prevalence of paragonimiasis has changed globally in recent times. A large study comparing the global burden of disease in the years 1990 and 2010 indicated substantial global reductions (25–42 % depending on method of presenting the data) in years lived with disability due to paragonimiasis [103]. It should be noted that very high levels of uncertainty in the data were indicated. In many places, prevalences have dropped to very low levels. On the other hand, prevalences in some known endemic areas have proved to be higher than previously thought and new foci have been discovered in recent years (see Section 5.1). In Nigeria, paragonimiasis was a major problem during the Biafran War (1967–1970). It then largely disappeared from many areas, and was forgotten until recent surveys revealed startlingly high prevalences in the SE of the country [8, 104, 105]. New foci are regularly being found in NE India [37, 106, 107], Vietnam [33], and Laos [108, 109]. It seems highly likely that many more foci will be found, especially in Africa and the Americas where reported investigations are few.

As shown in the introduction, very large numbers of people remain at risk of paragonimiasis around the world, the great majority of these being in SE Asia. There is likely a downward trend in incidence of paragonimiasis world-wide, with some striking success stories. Japan, Taiwan, and South Korea, in particular, have been successful in reducing incidence to negligible levels. This has been done by mass-screening campaigns followed by chemotherapy and education as to the causes of the disease.

In S Korea, in excess of one million people were infected in the 1950s [110]. Infection rates were between 7.4 and 52.8 % in different parts of the country [111]. Huge reductions occurred from the 1970s to the 1990s [29] and the disease is nowadays regarded as rare, even a curiosity [112]. In Taiwan, prevalences of paragonimiasis

(based on findings of eggs in sputum samples) used to be as high as 24 % in some areas [113]. Nowadays, the disease has virtually disappeared and is regarded as very rare [114].

China has a well-developed public-health system that includes surveillance and control of parasitic diseases [115]. There have been targeted interventions to reduce prevalence of paragonimiasis [116, 117]. Despite this, the disease is still present in many areas, with an estimated overall seroprevalence in 2001–2004 of 1.7 % [118], implying ~22 million infected persons [2], and some suggestions that prevalences are increasing in certain areas [23, 119]. Little can be said about trends in many other countries of the eastern/SE Asian region. In Thailand, reduction in prevalence has been noted [120], whereas in endemic areas of the Philippines, prevalences remain high and likely unchanged [121].

In some hyperendemic areas, humans were probably the principal definitive hosts in the past [122]. However, crabs or crayfish infected with metacercariae can still be found in areas where human prevalence is nowadays negligible, indicating that the parasites are still maintained in reservoir hosts such as peri-domestic cats and dogs [23, 29, 119, 123, 124]. It is also certain that in many areas there is a true sylvatic cycle (wild mammals are the principal definitive hosts), a cycle that is very hard to break. *Paragonimus kellicotti* in parts of N America is maintained in a sylvatic cycle [125] and the same likely applies to *P. mexicanus* and to African species.

5.5.2 Environmental Change and Pollution

Although frequently alluded to in the literature, it is hard to find quantitative information on the reduction of paragonimiasis that is due to environmental change and degradation [29, 113, 126]. Low prevalence in one Chinese village in an otherwise highly endemic area was a consequence of death of local stream animals due to mercury and cyanide contamination from a gold mine [127]. Construction of the Three-Gorges Dam on the Yangtze River in China has raised concerns about the possible effects of this massive project on transmission of a number of parasite species [128]. However, it remains unclear what the effect of the dam will be on the upstream prevalence of *P. skrjabini*, and there seems to be diversity of opinion on this within China [129, 130].

5.5.3 Cultural and Dietary Habits Aiding Transmission

As a food-borne disease, host diet is obviously central in maintenance of paragonimiasis in a human population. Diet, in turn, is frequently culturally determined, making it hard to eliminate risky habits [34]. Freshwater crabs and crayfish are widely regarded as tasty snacks [29] or even as an important source of protein [121].

There are numerous accounts of appetizing local dishes that include undercooked crabs or crayfish [35, 42, 131–133]. Crabs and crayfish have also been used for medicinal purposes. Juice filtered from crushed crayfish was used in the past to treat measles in Korea [28, 29, 134], and a correlation was noted between prevalences of measles and paragonimiasis [135]. A similar use of crab juice persists in Laos [35]. In some parts of China and Africa, women eat crabs to enhance fertility [127, 136]. Infection also seems possible when simply handling infected crustaceans and utensils used in preparing them for the table [137].

Changing dietary patterns are having a profound effect on epidemiology of paragonimiasis. In Japan during the 1950s, highest prevalences were seen in children [138]. Today, paragonimiasis, although rare, is mainly seen in middle-aged males [27]. This is because these men like to eat raw meat of wild boars, a common paratenic host there and the route by which maybe 70 % of human cases in Japan are now infected [139]. Many of the dogs used for boar hunting are also infected [56, 139] and about 75 % of wild boars captured in Kyushu were seropositive for *P. westermani* [56]. In Korea, economic development, convenience food and technological toys have lured people indoors and changed their diets [29]. In Thailand and Nigeria, younger people are becoming less fond of traditional food, leaving older age-classes to carry the burden of paragonimiasis without them [8, 120].

Diet is also influenced by age, gender and role in society. Thus in some areas where paragonimiasis is (or was) largely uncontrolled, it is (or was) a disease of children, as mentioned above for Japan. More recently, studies in NE India [7], China [127, 129], and SE Nigeria [104, 140] have all found highest prevalences in children. In SW Cameroon, the number of paragonimiasis cases has declined among adults but prevalence is still high in children under 10 years of age [141]. However, in some cultures, prevalences are highest in adults [35, 142].

Gender biases have been noted in many reports, with male patients generally outnumbering females [143]. One meta-analysis [2] estimated a global male–female ratio of 1.267 in numbers of paragonimiasis cases, but did acknowledge that ratios vary a lot from place to place. In China, boys outnumbered girls three to one in a sample of 58 children infected with *P. westermani* [144], suggesting that boys are more adventurous in catching and eating raw crabs. The bias towards infection in adult male Koreans in the past might have been because they were more likely than females to drink alcohol, which was accompanied by ingestion of freshwater crabs [143]. In Laos, prevalences are higher among males, possibly because they were more likely to work outdoors where they could find and eat crabs [35]. Some reports from NE India noted that almost all patients were male [145], but this could be due to failure of females to seek medical attention for cultural reasons [42]. A survey in a different part of NE India did not detect a gender bias [7]. In one Nigerian study, women had a relatively high prevalence because they were more likely to eat crabs [8], perhaps while handling and preparing food, but prevalence was also high among fishermen working in local rivers [142]. Cases of paragonimiasis due to *P. kellicotti* in the USA are nowadays mostly seen in male campers and canoeists who eat raw crayfish to show off [146].

Nor are urban dwellers and tourists safe. Metacercariae are still found in crabs sold in markets in large Japanese cities such as Tokyo and Fukuoka [147]. A recent national survey for parasitic diseases in China found the highest prevalences of paragonimiasis in the municipalities of Shanghai and Chongqing [118, 119]. Presumably this is because urban dwellers have a relatively high disposable income and can afford to eat luxuries such as crabs imported from rural areas. Infected crabs may also cross oceans [148, 149], exported to Japanese restaurants in the USA where they have caused paragonimiasis in American patrons [150, 151]. Increased globalization of the food supply is likely to lead to many more cases confounding medical practitioners in non-endemic areas [4]. Not surprisingly, immigrants arriving in non-endemic areas often bring their diseases with them [17]. And of course, tourists might return from tropical holidays with more souvenirs than they bargained for [152].

5.5.4 Control Measures

In most documents dealing with the subject of neglected tropical diseases globally, paragonimiasis is included in the category "food-borne trematodes" [153]. The World Health Organization, in a list of milestones and targets, stated that by 2015, food-borne trematode infections should be included in mainstream preventive chemotherapy strategies [154] and morbidity controlled where feasible. By 2020, 75 % of the population at risk of food-borne trematode infections should be reached by preventive chemotherapy and morbidity due to these infections controlled in all endemic countries [154]. The usefulness of preventive chemotherapy for control of paragonimiasis alone remains to be evaluated [153].

Despite the obvious and sensible approaches outlined in WHO reports, exactly how control of paragonimiasis and other zoonotic trematodes is to be achieved at the national level is unclear. Comments from China [119] indicate a decrease in resources for this purpose in that country and a lack of coordination between veterinary and medical areas.

In the documents mentioned above, relatively little attention is paid to the low-cost provision of relevant education, an intervention which also has the benefit of lacking clinical side effects and ethical issues. Awareness of the disease and how it can be prevented, both among medical professionals and the general public, are key to efforts to reduce prevalence of paragonimiasis. Lack of such awareness is likely one of the factors predisposing towards recent apparent increases in numbers of cases in SE Nigeria [104, 105]. In China, a new program has been established in Chongqing to train medical workers about *P. skrjabini* [155]. Localized awareness campaigns have been mounted recently in the USA (targeting campers and canoeists who might be tempted to eat crayfish during their adventures) [156] and Colombia (targeting school children) [157].

The possibility of developing a vaccine against paragonimiasis has been mentioned a few times in the literature [158] and candidate molecules have been suggested [158, 159]. However, given the difficulties involved in producing

vaccines against any helminth species [160] and the logistical difficulties of distribution, storage, and mass administration, it does not seem practical to pursue the idea of a vaccine for paragonimiasis.

5.6 Genomics and Proteomics

Proteomic and genomic data should soon provide new insights into molecular mechanisms used by parasites in all areas of their physiology, but especially those involved with pathogenesis and immune evasion, and those that might be drug targets [161, 162]. Research on the "omics" of *Paragonimus* species has lagged behind that on the genus *Schistosoma*. Nevertheless, work to produce draft genomes of several *Paragonimus* species has now started (http://genome.wustl.edu/projects/detail/helminth-sequencing-project/). Transcriptome data for *P. kellicotti* produced as part of this sequencing project was used to show that genes encoding diagnostic antigens in *P. westermani* differ substantially from their orthologs in *P. kellicotti* [163].

The GenBank nucleotide database contained (on 8 August 2013) 1,334 sequences from *Paragonimus* species. Of these, 38 % were ESTs [164]; 29 and 26 % were short portions of the mitochondrial genome or portions of the nuclear ribosomal repeats respectively, mostly used in phylogenetic and taxonomic studies; 4 % were retrotransposons and 4 % coded for a variety of proteins. The database also contains the coding portions of two mitochondrial genomes.

Recently, microRNAs have been reported from *P. westermani* [165]. Small RNA molecules were sequenced using high-throughput technology on a Solexa machine.

The only relevant proteomics study to date using mass spectrometry identified many proteins in excretory–secretory products (ESPs) of adult *P. westermani* and established which of these elicited antibody responses [166]. The majority of proteins recognized by the host immune system were cysteine proteases (CPs).

5.7 Immunobiology

In their mammalian host, lung flukes need to be able to move through tissues, feed, and void wastes and eggs. They also need to evade and subvert, for months or years, the effects of the host immune system that might react against their bodies or products. All this they often do without causing overt disease in the host, and if disease becomes overt, it is likely to have a strong immunopathological component [167]. Compared to the range of immunological studies on *Schistosoma* species, relatively little work has been done specifically on *Paragonimus* species. However, all helminths elicit rather similar immune responses in their hosts [168], so it is likely that their methods of immune evasion have features in common. To quote Maizels et al. [168] directly: "Helminths do not simply ward off immune attack; rather, they influence and direct immune responses away from the modes most damaging to them,

regulating the host immune response to create niches that optimize successful feeding and reproduction". The main players most often mentioned on the host's team are the cells and molecules involved in the T_H2 response, including effector cells such as eosinophils [169]. Eosinophilia is a very frequent finding in paragonimiasis. Many functions and properties have been attributed to eosinophils, including exocytic attack on helminths via released substances, and pro-inflammatory effects as well as mechanisms to modulate immune processes [170]. Although in vitro destruction of parasites by eosinophils has been demonstrated, the situation in vivo is far from simple and will be an important focus for future research [171]. The literature on *Paragonimus* infections has tended to emphasize the interplay between eosinophils and the parasite. Lung fluke species field against the immune system a wide range of substances, notably proteases and other ESPs. Cysteine proteases (CPs), of which there are at least 15 different types [166], are heavily represented among ESPs and genes encoding them are among the most abundantly transcribed in the adult worm genome [164]. CPs are involved in metacercarial excystment [172], lysis of host tissue to allow migration of larvae [173] and interaction with the immune system in various ways [174]. In the last class of activities, CPs have been demonstrated to break down host IgG [175] thus having a suppressive effect on eosinophil function [176]. CPs may also stimulate eosinophils to degranulate and produce superoxides [174, 177].

There are many other ESP components which remain poorly characterized: over 140 protein spots could be seen after two-dimensional gel electrophoresis of ESP material [166]. As-yet unidentified components of ESP, in relatively high concentrations, increase the rate of apoptosis in eosinophils, likely favoring worm survival [178]. Substances in ESP can also regulate IL-8 production in human eosinophils [179]. Other molecules produced by *P. westermani* can act to detoxify reactive oxygen species produced by cells of the immune system [180–182].

5.8 Clinical Manifestations and Pathology

Many useful overviews of clinical aspects have appeared since the year 2000 [19, 37, 42, 183, 184].

5.8.1 Early Infection

After ingestion, metacercariae excyst in the small intestine and migrate through the wall of the gastrointestinal tract. From the abdominal cavity, juvenile worms generally migrate to the pleural cavity, sometimes via the liver. This is the acute stage of the disease that lasts for less than a month [42, 116]. Hepatic injury may occur in early infection, especially in children [185]. Signs and symptoms are often few during this phase except in heavy infections. However, if present, these may include fever,

abdominal discomfort and diarrhea, which can appear in as little as 2–4 days after ingestion of metacercariae [116]. Eosinophilia and elevated IgE levels are typical laboratory findings in the early stages [186].

5.8.2 Pleural Manifestations

Pleural manifestations may appear prior to parenchymal/pulmonary ones [42] and may persist for long periods, even after apparent cure [187]. Activities of worms in the pleural spaces can cause pleural effusion, pneumothorax and thickening of the pleura. Such effects may be particularly common in light infections of diploid *P. westermani* in which an individual, unable to find a mate, continues to wander in the pleural spaces eliciting a response from the host. Such unmated individuals can produce unviable eggs [89]. Light infections, and hence pleural symptoms, are typically seen in Japan nowadays [188], but such symptoms appear to be less common in Korea [19] and Laos [189]. Eggs of North American *P. kellicotti* seem to elicit a particularly strong inflammatory response and pleural symptoms and effusion are usual [42]. Since cysts containing adult worms tend to be peripheral in the lungs, often close to pleural surfaces [190], it is not surprising that some cysts discharge eggs into pleural spaces [42].

Symptoms related to pleural manifestations include chest pains and those related to presence of pleural effusion. Laboratory findings might include peripheral blood eosinophilia and elevated IgE levels. Pleural effusion can be variable in appearance [26] but is frequently eosinophilic, with low pH, low glucose, and high lactate dehydrogenase and protein levels [19, 191–193] and is often a good source of anti-*Paragonimus* antibodies [186]. It may also contain fluke eggs [151, 194], or even adult flukes [187, 195]. Pleural manifestations are common and pulmonary symptoms rare in infections due to *P. skrjabini* subspecies [26, 129].

5.8.3 Pulmonary Manifestations

Although they are hermaphroditic, adults of *Paragonimus* species do not self-fertilize [89]. Diploid worms need to exchange sperm with another individual and move into a location from which they can void their eggs to the outside. Worms encounter others in the pleural spaces then move into the lung parenchyma where pairs become encapsulated in a cyst that becomes increasingly fibrotic and typically up to 2 cm in diameter [28, 196]. Within this, worms exchange sperm and produce eggs, sometimes for many years. This stage is reached not less than 1 month after initial infection, but can take much longer [50], and signals the starts of the chronic phase. The cysts rupture into bronchi or bronchioles, permitting escape of eggs and other cyst contents. Eggs may be expectorated in sputum, or swallowed and voided via feces. Presence of worms encapsulated in the lungs produces classic pulmonary

paragonimiasis, of which symptoms are cough (which may be worsened by exertion), hemoptysis, dyspnea, chest pains, fatigue, fever, and loss of appetite [185, 197]. The first four symptoms in this list are the most commonly seen. "Rusty" sputum, containing parasite eggs, blood, necrotic material, and Charcot–Leyden crystals, is reported in a proportion of patients and is the classic sign of paragonimiasis. Symptoms may be more pronounced in heavy infections. A proportion of individuals, even those with heavy pulmonary infections, remain symptom-free [190].

5.8.4 Ectopic Paragonimiasis

Although pulmonary paragonimiasis is the best known form of the disease in humans, ectopic paragonimiasis is quite common. Cerebral, cutaneous, abdominal [186, 198, 199], and hepatic [200–202] sites are the most commonly mentioned in the literature, but lesions due to the activities of worms have been found in almost every part of the body, including a fingertip [203] and eyelids [204]. Not surprisingly, cerebral paragonimiasis has received much attention, not least because it can have lethal consequences (causing an estimated 244 deaths in 2005 [2] and is the most commonly reported form of ectopic paragonimiasis [48].

Cerebral paragonimiasis due to *P. westermani* was regarded as the commonest form of "brain tumor" in Korea in the 1960s, with an estimated 5,000 cases living in Korea in January 1966 [111]. The condition was seen predominantly in adolescent and young adult males [111]. Today, its rarity in Korea is such that medical practitioners encountering it there are likely to misdiagnose it [112]. At least in the case of infection due to *P. westermani*, cerebral paragonimiasis is more likely in individuals with an established pulmonary infection, and about 0.8 % of those with active pulmonary paragonimiasis in Korea develop the cerebral form [111]. The cerebral form is much more prevalent among hospitalized paragonimiasis patients [111, 205]. A recent estimate is that 0.72 % of all paragonimiasis cases have cerebral involvement [2]. The proportion of people infected with members of the *P. skrjabini* complex who develop cerebral symptoms is likely to be relatively high [205] and lung lesions are much less common [129, 206]. Sixteen percent of 213 cases of paragonimiasis due to *P. skrjabini* in China had symptoms suggestive of neurological involvement (severe headache and vomiting) [129]. Reports of cerebral paragonimiasis caused by other species are few [18, 42].

Worms possibly reach the brain by travelling via tissue through the jugular foramen into the interior of the skull [207], penetrating the meninges and burrowing into the brain [208]. This is the "active" phase, during which worms are alive and moving in tissues, secreting a range of biologically active compounds, and frequently eggs. Worms and eggs become surrounded by granulomatous lesions that can be cystic or solid, the former type containing caseous necrotic material [208]. These lesions eventually become calcified. Adult worms may not persist for long periods, but numerous eggs may be found in the lesions they have produced [137], even when many years have probably elapsed since the death of the adult worms [114].

Many neurological signs and symptoms of cerebral paragonimiasis have been reported [207]. The most common of them are seizures, headache, vomiting, nausea, and fever and localized weakness [190, 207]. Decline in cognitive function and visual disturbances are also frequent [205]. Many of these are most pronounced in the early stages while worms are actively moving around, and most deaths occur during this stage. Epileptic seizures are often associated with cerebral paragonimiasis [209].

Subcutaneous nodules, often migratory, are a common manifestation of ectopic paragonimiasis. These most frequently appear on the abdomen and chest [116]. On surgical removal, such nodules sometimes contain worms [210] or eggs [198, 211]. This form of ectopic infection is relatively uncommon when *P. westermani* is the causative species, but cases are reported from time to time [144, 185, 198, 211–213]. The first reported case of paragonimiasis due to *P. heterotremus* presented in Thailand with subcutaneous nodules [210] and cases from India [214] are likely also to be due to this species. Such nodules are particularly common in paragonimiasis caused by members of the *P. skrjabini* complex that rarely mature in humans [117]. In China, two different studies reported that 56 and 42 % of patients exhibited subcutaneous nodules [129, 215], which were often migratory. A single nodule was typical, but a few patients had multiple nodules [129].

5.9 Diagnosis

As is the case in infections due to many species of food-borne trematodes, symptoms of paragonimiasis are often rather vague and nonspecific, and hence creating diagnostic difficulties. In many cases, there are no overt signs or symptoms at all [188]. Diagnostic confusion with pulmonary tuberculosis and lung cancer remains very common: indeed such confusion is possibly more common than not [7, 216–219]. Hence, unless paragonimiasis is actually suspected, correct diagnosis is delayed and the patient might be subjected to expensive and ineffective treatments. Paragonimiasis appears to be more common than pulmonary tuberculosis in many endemic areas such as NE India [7, 217], parts of Laos [109], and parts of SE Nigeria [104]. Very often, reports note that failure of the patient to respond to treatment for tuberculosis, or the unexpected finding of lung fluke eggs [112] or worms [21] during surgery, prompt clinicians to enquire about past eating habits of their patients, and the way to a diagnosis of paragonimiasis is clear.

The four classes of diagnostic approaches are serology/immunology, parasitology, radiology/medical imaging and molecular (DNA-based approaches). Demonstration of lung fluke eggs in sputum, feces, bronchial washings, or surgical specimens provides a definitive diagnosis. However, eggs can be hard to find, even in some active pulmonary cases, and especially in cases of ectopic paragonimiasis. Consequently, immunologically based tests are regarded as more sensitive for diagnosis [7, 34, 45, 185] and are discussed first.

5.9.1 Serology/Immunology

A number of recent reviews include immunological methods for diagnosis of paragonimiasis [42, 51, 52, 71, 184, 220]. Here, only tests that are most often mentioned in recent literature, or are of particular historic interest, will be discussed. These include the intradermal test, ELISA, multiple-dot assay, immunoblotting, and the dot immune-gold filtration assay. Of these, ELISA and immunoblotting are the main methods in current use. Both are useful in a laboratory setting, but less easy to apply in field situations.

5.9.1.1 Commonly Used Immunological Methods

The earliest serological/immunological method to be used widely was the intradermal test [47]. A small amount of diluted worm antigen is injected into the skin of the forearm and the size of the resulting wheal compared after a few minutes with that elicited by a control injection [47]. The test is simple, inexpensive, and quite sensitive. But there are some drawbacks, mainly (1) a high proportion of false positives [221]; (2) cross-reactions with other trematodiases, especially if the antigen used is not purified [47, 222]; and (3) a positive reaction can persist long after the infection has been cleared [47]. The intradermal test has often been used as the first step in mass screening programs in Japan [48], China [23], and Korea [28, 122], generally supplemented by more specific serological tests and/or examination of sputum for eggs. Huge numbers of people have been screened in this way [26]. Its use continues to the present day in a number of countries including Colombia [40], Laos [35], India [133], and China [129].

Many variants of the enzyme-linked immunosorbent assay (ELISA) have been described in the literature [51], and these are among the most widely used of tests today [223]. In the commonest formats, antigens from the parasite are bound to a surface, typically in a microtiter plate, for ease of handling. Antibodies in host sera are allowed to react with these. The quantity of complexed antigen/antibody is determined using a secondary antibody, which is generally commercially purchased, specific for particular classes or subclasses of host antibody, and conjugated with a reporter enzyme that produces a color change in a substrate. Other variants have been successfully tried. For example, immunoglobulin-binding proteins of bacterial origin, such as Protein A, can be used instead of parasite antigen [224] and peroxidase-conjugated Protein G can be used instead of secondary antibody [45].

Lung fluke antigens that have been used include crude antigen extracts, ESPs [225], recombinant peptides [226] and various purified or partially purified antigens including cysteine proteases [227]. A comparison of somatic and excretory–secretory antigens from local *P. heterotremus* in NE India, done during development of an ELISA test for paragonimiasis, found that both preparations of antigens had 100 % sensitivity but that the latter was more specific (100 % as opposed to 91.3 %) [225].

Multiple dot-ELISA is used in Japan and elsewhere to screen for several parasites simultaneously. Antigens from a range of parasite species are dotted onto a nitrocellulose membrane, and the membrane is dried to bind the antigens and then flooded with patient serum to permit detection of antibodies against all the parasites assayed at the same time, following essentially the same steps used in microplate ELISA [34, 52, 228–230]. In a conceptually similar approach, also aimed at simultaneous screening for multiple parasites, antigens from parasites can be partially purified using SDS-PAGE and antigen bands recovered from the gel, spotted onto microarray plates, and screened as for ELISA [231]. In this case, a 35-kDa band was the target antigen for paragonimiasis.

The DIGFA (dot immunogold filtration assay) method is said to be better than ELISA because it is easier, faster, and cheaper, but exhibits comparable sensitivity and specificity. Reagents required are known to be stable at 4 °C for at least a year [232]. Results can be read within a few minutes and no special equipment is required [52, 233]. Antigens from one or more parasite species are dotted onto a nitrocellulose membrane. A dot containing diluted human serum or similar is used as a positive control. Serum from a patient is added to the nitrocellulose membrane. Next is added anti-human antibody or Protein A conjugated with colloidal gold, which forms a colored spot where it reacts with antigen–antibody complexes on the membrane. The Chinese-language literature contains some recent examples [234–237].

There may be cross-reactions when sera from patients with other parasites (schistosomes, liver flukes etc.) are tested for paragonimiasis, especially when crude extracts of parasite antigen are used [163, 228]. In immunoblotting, parasite antigens are separated electrophoretically before western blotting and probing with sera. In this way, immune reactions with diagnostic antigens of particular molecular masses can be identified [238]. This approach has been used for diagnosis of paragonimiasis by the Centers for Disease Control, Atlanta, Georgia in the USA, since 1988, the antigen generally used being a crude extract from *P. westermani*. However, extracts from *P. kellicotti* are better for diagnosis of that species [163].

It remains difficult to distinguish between infections due to different species of *Paragonimus* using ELISA or immunoblot [45, 163]. ELISA inhibition tests can go some way to dealing with this problem [239] but their use is not widespread. The multiple dot-ELISA (above) was developed partly for this reason, and can provide a stronger reaction with the homologous species when antigens from *P. westermani* and *P. s. miyazakii* are both included [228, 240]. Ouchterlony's double diffusion test [239] and the DIGFA method [233] can also help in distinguishing between *Paragonimus* species.

5.9.1.2 Immunological Indication of Cure after Treatment

It is well known that intradermal tests can be positive long after cure of paragonimiasis (see above). Similarly, IgG ELISA tests may yield positive results for long periods after treatment, and antibody levels may even exhibit a transient rise after chemotherapy, probably in response to material released from dead worms [241–243].

Hence, a positive immunological test does not always indicate that an active infection is present. IgG levels return to normal at from 4 to 18 months [243, 244] after cure, or even longer [245]. The variation is possibly related to intensity and duration of infection. The presence of worm eggs in tissues may partly explain the slow decline of specific antibody levels [243, 245].

5.9.2 Parasitological Diagnosis

Direct parasitological diagnosis, by finding eggs in sputum or feces, is likely to be the only option available in clinics in poorly equipped rural areas. If "rusty" colored sputum containing blood, parasite eggs, and Charcot–Leyden crystals is found, then a definitive diagnosis of pulmonary paragonimiasis can be reached easily. However, such diagnostic manifestations are not always seen, even when mature worms are present in the lungs, and never during prepatent early infections or ectopic paragonimiasis. Multiple sputum samples should be examined from each patient before a negative result can be declared with any confidence. The record seems to be 27 for the number of sputum specimens examined before eggs were found [143]. Eggs are rarely found in feces, but this most commonly in children, who tend to swallow rather than spitting [216]. Eggs may also be found in bronchial washings and brushings [246], pleural effusion and surgical specimens such as lung biopsies. Of course, rural clinics are not likely to have facilities to perform relevant procedures.

Paragonimiasis and tuberculosis co-occur in many places. The Ziehl–Neelsen staining (ZNS) technique, used to stain mycobacteria in sputum, had long been assumed to destroy lung fluke eggs [247]. Thus, diagnosticians trying to exclude infections with either of these pathogens have tested sputum samples separately for each. Handling and microscopic examination of fresh sputum for *Paragonimus* eggs carries some risk of tuberculosis transmission to laboratory staff. A recent reevaluation of the ZNS technique, and especially of modifications that avoid long periods of heating the slide, has shown that destruction of eggs can be avoided. The ZNS technique therefore provides a low-cost method for distinguishing between paragonimiasis and tuberculosis [247]. Furthermore, the method reduces the risk of tuberculosis transmission [247] and the ZNS slides can be kept for further evaluation and archiving.

5.9.3 Molecular Diagnosis by DNA Detection or Sequencing

Steps towards molecular diagnosis by detection and/or amplification of parasite DNA have been taken by a number of researchers. However, there has been very limited application of these to date in clinical practice. Eggs, either in sputum or feces, have generally been the targets of these efforts. It proved possible to PCR-amplify and sequence the nuclear ribosomal ITS2 region from as few as 3–5 eggs of

P. westermani from sputum [248], and from a single egg of *P. heterotremus* [34]. This same genomic region has been the target for other studies using PCR on eggs from patients [7, 45, 106, 125, 249–252]. Only one study has detected DNA from eggs in human fecal samples [45] and it was suggested that molecular detection of *Paragonimus* DNA in feces might be more efficient than attempting a physical search for eggs in feces. Others have detected DNA from feces of experimental hosts [253, 254].

In one interesting development, it proved possible to amplify and sequence ITS2 from a paraffin-embedded section of lung tissue from a suspected case of paragonimiasis due to *P. kellicotti* [125]. Such approaches might make it possible to obtain data from archived pathological specimens.

A loop-mediated isothermal amplification (LAMP) protocol has been established for *P. westermani* [255]. Using purified DNA from this species, the detection limit was 10^{-8} ng/μL of DNA [255]. LAMP is said to be far more sensitive than conventional PCR and can possibly be applied to for example pleural fluid. The approach uses four species-specific primers that recognize six regions in the target DNA. The reaction can be completed in an hour at a single temperature (60 °C) and a simple in-tube visualization of successful amplification is possible [256]. No complicated equipment is needed. This method may be suitable for field labs and small hospitals. It could also be applied to field identification of metacercariae and eggs, provided a species-specific set of primers is available.

Next-generation DNA sequencing methods are beginning to feature in work on *Paragonimus* species and may find practical application in the future [257].

5.9.4 Radiology/Medical Imaging

There have been several recent reviews including material on medical imaging as it relates to paragonimiasis [42, 258–261]. Imaging methods include conventional chest X-ray, X-ray and computed tomography, MRI and ultrasonography. Paragonimiasis is often not suspected when imaging procedures are requested by clinicians, and findings often lead to an initial diagnosis of other conditions, such as tuberculosis or cancer [20].

Chest X-rays have been used for very many years for diagnosis of paragonimiasis [47] and increasingly are coupled with computed tomography (CT) for more detailed anatomical observations of the chest [190, 258, 259, 262–264]. Abnormalities are not always apparent, even in cases of active pulmonary paragonimiasis [7, 145, 265]. Common observations in early paragonimiasis include pneumothorax, pleural effusion, airspace consolidation and linear opacities [19, 190]. The last of these probably represent migration tracks of worms from the pleural spaces into the lung parenchyma [190, 263] and might provide strong support for a diagnosis of paragonimiasis [259]. Distinct cystic lesions and nodules less than 3–4 cm in diameter and bronchiectasis tend to be seen in established infections [19, 190]. Cysts may appear as ring shadows, within which worms may be detected in some cases [190]. Pleural thickening may be present adjacent to worm nodules [263].

Radiographic abnormalities can be slow to resolve—months to years—after treatment [136, 187]. Occasionally, nodules may resolve as small calcifications in the lungs [190].

Ultrasonography, using reflection of sound waves from tissues to produce images, provides relatively little anatomical detail. However, it can supplement other imaging techniques [266]. Furthermore, it is capable of real-time imaging of moving structures and can be used to guide biopsy procedures to obtain diagnostic material (inflammatory material, eggs, fragments of worms) from nodules in abdominal and thoracic organs [266–268].

CT methods and MRI are useful for imaging cerebral paragonimiasis [190, 208]. In early "active" infection, characteristic ring-like granulomatous lesions are commonly seen using CT scans. These may be solitary or resemble clusters of grapes, each usually 1–3 cm in diameter and each surrounded by edematous areas [190]. Later these become calcified, resembling "soap-bubbles" or egg-shells with surrounding parenchymal damage [190].

Magnetic resonance imaging (MRI) methods do not involve the use of ionizing radiation such as X-rays and are good for visualizing lesions in soft tissues such as the brain. T1- and T2-weighted images differ in the ways they show fat and water in tissues. Images obtained are generally sequential "slices" through the body, similar to those produced by CT methods, and descriptions of brain lesions seen using MRI are generally similar to those based on CT images. In the earliest stages of cerebral paragonimiasis, especially in children, hemorrhage might be present, seen as high signal intensity in T1-weighted images or as high or low intensity on T2-weighted images [269]. As granulomatous lesions form, these may be seen as "soap bubbles" or a "grape cluster" of ring-like structures with surrounding edema, each ring being up to 3 cm in diameter [270]. The walls of these were described as "… usually isointense relative to brain parenchyma on T1 weighted images and isointense or hypointense on T2 weighted images" [208]. Following eventual calcification, the lesions may resemble egg shells with central content of varying intensities [270, 271]. A lesion thought to be the migration track of a worm has been reported [269]. MRI has also been used on a number of occasions to image lesions caused by spinal paragonimiasis [270, 272].

CT imaging results from series of hepatic paragonimiasis [200] and abdominal paragonimiasis cases [267] have recently been reported.

5.10 Treatment

5.10.1 Chemotherapy

In the past, bithionol was often the drug of choice, but is no longer commonly used. It requires a long course of treatment [273] and side effects can be a disincentive to patient compliance [185]. Two drugs are now generally recommended for treatment

of pulmonary paragonimiasis: triclabendazole (TCL) and praziquantel (PZQ) [153, 274, 275]. PZQ has a long history of use in paragonimiasis [274, 276]. The recommended course is 25 mg/kg body weight three times daily for 2–3 days. Cure rates are generally very high [273]. However, repeat rounds of treatment are sometimes necessary [185], especially when pleural effusion is present [186, 187, 277, 278]. PZQ has also been used for treatment of cerebral paragonimiasis, with good results reported [206, 279]. However, PZQ is only likely to alleviate symptoms in "active" cases, when worms are still present: mechanical insult and release of eggs and bioactive compounds will cease when the worms die. Of course, PZQ treatment can eliminate any pulmonary infection that might be a source for later cerebral invasion [205]. Co-administration of anti-inflammatories is sometimes recommended for cerebral paragonimiasis because of the risk of reaction against substances released from dying worms [207]. The extent to which this is a problem remains unclear [206].

Side effects of PZQ administration are rare and generally mild. Patients may report mild and transient insomnia, nausea, headache, dizziness, vomiting, and abdominal pain; less common effects include rash and hypotension [275]. A very few patients exhibit an allergic response after administration of PZQ [274, 280]. A strong inflammatory reaction was observed in a Lao patient after PZQ administration [281]. There is no indication yet of resistance to PZQ by *Paragonimus* species, although there is evidence of resistance in *Schistosoma mansoni* [282] and the possibility of resistance developing in other trematode species remains a cause for concern [273, 283], especially if the drug is used for mass administration. Caution is suggested in administering PZQ to pregnant or breastfeeding women, people with ectopic infections, and children under 4 years of age. The main contraindications are hypersensitivity and cysticercosis [115, 275].

Triclabendazole is also effective against paragonimiasis [153, 275] and might have some advantages, given that only one or two doses are required [284] and that dose rates are lower than for PZQ. The recommended regimen is 10 mg/kg body weight in a single dose (which may be repeated after 12–24 h in heavy infections [275] or 20 mg/kg of body weight, in two separate doses of 10 mg/kg, administered on the same day [153]. TCL might be better tolerated by patients than PZQ [5]. It is also the better option for mass drug administration, in part because the one or two doses required can be given under supervision, avoiding compliance problems that might arise if subjects are given tablets to take at home over several days. In the Philippines, a single dose of TCL (10 mg/kg body weight) was found to be as effective as the three-dose regimen of PZQ [5]. One case of suspected triclabendazole resistance has been reported [280].

Common side effects are similar to those seen after PZQ administration and include mild and transient abdominal and epigastric pain, sweating, and eosinophilia; less common effects include nausea, vomiting, headache, dizziness, cough, fever, urticaria, pruritus, and skin rash. Again, caution is suggested for treatment of pregnant or breastfeeding women, people with ectopic infections, and children <6 years [275].

Although newer drugs, and combinations of drugs, are being explored for treatment of food-borne diseases, including trematodes [273], no alternatives suitable for treatment of paragonimiasis are apparent as yet [285]. Some authors have suggested that inhibitors against some parasite-specific molecules might provide an excellent means of chemotherapy [286–288].

5.10.2 Surgical Intervention

Often surgeons discover underlying paragonimiasis when they were expecting some other condition [114, 198]. However, surgery is not normally appropriate for uncomplicated pulmonary paragonimiasis: treatment with praziquantel or triclabendazole should suffice. Invasive interventions are more common in pleural cases, especially when voluminous pleural effusions are present that have not resolved after drug treatment. Draining of effusion is a common procedure which may need to be repeated [187]. Surgical decortication of the pleura is also sometimes required to remove fibrous material [289, 290]. Surgery is also appropriate for uncomplicated ectopic cases, especially subcutaneous [203, 213].

Surgery was the only intervention available in cerebral paragonimiasis until the drug bithionol was introduced around 1961 [111]. Since then, PZQ and TCL have superseded bithionol [291]. Drug treatment is the treatment of choice in active early cerebral cases [111, 208], but is of little value in chronic "inactive" cases, except as a means of treating any concomitant pulmonary infection. In one study in China, 73 of 88 patients with cerebral paragonimiasis showed improvement when treated with PZQ [292]. Surgery was done only on those with relatively superficial lesions that were accessible and easy to remove [292].

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Chapter 6
Liver Flukes: *Clonorchis* and *Opisthorchis*

Weerachai Saijuntha, Paiboon Sithithaworn, Nadda Kaitsopit, Ross H. Andrews, and Trevor N. Petney

6.1 History and Taxonomy

Opisthorchiid flukes most commonly occur in the bile duct, gall bladder, and liver of their mammalian and avian hosts [1]. They are small to medium sized with 33 recognized genera in the family Opisthorchiidae. These are divided into 13 subfamilies [1, 2]. Both of the genera *Clonorchis* and *Opisthorchis* fall within the subfamily Opisthorchiinae. *Clonorchis sinensis* from East Asia and *Opisthorchis viverrini* from the Lower Mekong Basin are currently recognized as the most important human pathogens. Both are involved in the development of human cholangiocarcinoma (CCA) and have been classified as class one carcinogens by the International Agency for Research on Cancer, a part of the World Health Organization [3].

W. Saijuntha (✉)
Walai Rukhavej Botanical Research Institute, Mahasarakham University, Maha Sarakham, Thailand
e-mail: weerachai.s@msu.ac.th

P. Sithithaworn • N. Kaitsopit
Department of Parasitology and the Liver Fluke and Cholangiocarcinoma Research Centre, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
e-mail: paibsit@gmail.com; nd_tg_na@yahoo.com

R.H. Andrews
Department of Parasitology and the Liver Fluke and Cholangiocarcinoma Research Centre, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Faculty of Medicine, St Mary's Campus, Imperial College, London, UK
e-mail: rhandrews@gmail.com

T.N. Petney
Department of Ecology and Parasitology, Institute of Zoology, Karlsruhe Institute of Technology, Kornblumenstrasse 13, Karlsruhe, Germany
e-mail: trevor.petney@kit.edu

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The presence of *C. sinensis* and *O. viverrini* in East and continental Southeast Asia, respectively, is strongly correlated with the incidence of CCA particularly in northeast Thailand where there is the highest incidence worldwide [3]. Although *Opisthorchis felineus*, which occurs from Europe across to eastern Siberia, has not yet been recognized as a carcinogen, there is evidence suggesting that this is the case [4]. It is, nevertheless, a pathogen of considerable significance in its own right [5].

Opisthorchis lobatus, a new species recently found in freshwater fish in Lao PDR, may also cause zoonoses, but its role in humans is not known, and it will not be dealt with here [6].

Clonorchis sinensis was first described by J.F.P. McConnell in the August 21st issue of the Lancet in 1875 from postmortem specimens collected from the bile duct of a Chinese seaman who died in Calcutta, India. On September 18th of the same year T.S. Cobbold wrote a short letter, also published in the Lancet, indicating that from McConnell's description of the trematodes it was undoubtedly a new species which he then named *Distoma sinense*. This name was later revised by Looss [7], as *Clonorchis sinensis*, the type species for the genus. At the time of its discovery, McConnell noted "The morbid anatomy of the liver in this case seems unequivocally to point to the presence of the flukes in its biliary ducts as the exciting cause of the acute and extensive structural degeneration of the proper structure of that organ, and of that cholaemic condition induced by the obstruction of the biliary channels which appears to have been the immediate cause of death." clearly pointing out the pathological significance of this species.

The discovery of *Opisthorchis viverrini* initially followed a quite different path. Poirier [8] was the first to discover and describe this species which had been retrieved from the biliary ducts of a fishing cat (*Prionailurus viverrinus*) which had been kept in the Zoological Gardens attached to the Paris Natural History Museum. It was only over 20 years later that Leiper [9] described the first specimens from humans supplied by W.F.J. Kerr from Chiang Mai in the north of Thailand. In 1916 Kerr published a parasitologically more detailed paper listing 17 % of the 230 adult male prisoners examined to be infected. Interestingly, 74 % of those examined were Laotians who had lived in the Chiang Mai area for their whole lives—there were very few Thais in the sample group. In this paper Kerr originally identified the species as *Opisthorchis felineus*, but in a postscript indicates that they were identified by Leiper as *O. viverrini*. About a decade after Kerr's paper, Prommas [10] reported the first case of infection (again as *O. felineus*) from the northeast of Thailand at Roi-et while Bedier and Chesneau [11] reported high prevalences of 25 and 15 % from Thakhek and Vientiane, respectively, in Lao PDR.

Gurlt was the first to describe and illustrate *O. felineus* (subfamily Opisthorchiinae), although he confused it with *Distomum conus* Creplin 1825 [4]. It was only in 1885 that *O. felineus* which had been isolated from cats were described as a valid species by Rivolta as *Distomum felineum* [12]. It was later moved from the genus *Distomum* to *Opisthorchis*, created by Blanchard in 1895. This is the type genus for the family Opisthorchiidae Looss, 1899. Opisthorchiasis in humans caused by *O. felineus* was first described by Vinogradoff [13] from Siberia.

6.2 Current Status and Geographical Distribution

Human populations show high levels of infection with all three liver fluke species within each of their distributional ranges. Up to 680 million people worldwide are at risk of infection [14]. Recent estimates indicate that 45 million people living in Asia and Europe are infected, with approximately 35 million *C. sinensis* cases, 10 million *O. viverrini* cases, and 1.2 million *O. felineus* cases [15, 16].

Clonorchis sinensis is the most frequent human parasite of the three with 600 million people at risk of infection [14] in East Asia from mid-Vietnam through much of China, including Taiwan, into Korea and the far east of Russia [17, 18]. Although *C. sinensis* was previously endemic in Japan, the last human case was in 1991 and no autochthonous case has been reported since [19]. The second most common species is *O. viverrini* which is found along the Lower Mekong and its tributaries in the north and northeast of Thailand, Lao PDR, Cambodia, and southern Vietnam. Data for the last two countries are sparse and for *O. viverrini* most of our information comes from the northeast of Thailand with an increasing list of publications from Lao PDR [20–22]. The information which is available suggests that as many as 67 million people may be at risk of infection [14].

Although *O. felineus*, the European liver fluke, is the most poorly studied of the three species it has been reported from continental European countries except Finland, Norway, and Sweden. It does not occur in the UK. Animal hosts are wild and domestic carnivores [4] but humans probably play a significant role in parasite transmission [21]. Human cases have been reported to occur in Belarus, Germany, Greece, Italy, Poland, Romania, Russia, Spain, the Ukraine, the Baltic countries, Moldova, and Kazakhstan, but records from some of these countries are over 50 years old [4, 5, 23]. *O. felineus* represents a significant health problem in certain areas of Siberia where evidence indicates that its prevalence in both humans and in animals increases from west to east [23]. In the Ob-Irtysh basin, where the prevalence of infection peaks, it is of particular medical significance [23].

Although clonorchiasis is endemic to East Asia and opisthorchiasis to the Mekong area of Southeast Asia and parts of eastern Russia all can occasionally be found in non-endemic areas having been introduced by infected tourists, refugees or workers who have moved from endemic to non-endemic areas [16, 24–27]. The flukes have, however, not currently become endemic in these areas due to the lack of suitable intermediate hosts.

6.3 Biology and Life Cycle

Liver flukes are hermaphroditic trematodes which are dorsoventrally flattened. The body has an oral sucker situated anteriorly and a ventral sucker at mid-body. The differentiation of the species is based on morphology. The adult worms differ in the shape and position of their testes and the arrangement of the vitelline glands.

Fig. 6.1 Adult worms of the liver flukes. (**a**) *Opisthorchis viverrini* (**b**) *Clonorchis sinensis*

C. sinensis can be separated from the other two species by the presence of branched testes in a tandem position and the continuously distributed vitelline glands (Fig. 6.1). Although *O. viverrini* is similar to *O. felineus* in having lobed testes and a cluster vitelline gland, it differs by having deeper lobulation of and greater extremity of the testes and also lacks transversely compressed patterns of vitelline follicles. The size of the adult flukes depends on the species involved. *O. viverrini* is the smallest, measuring 5.5–10×0.77–1.65 mm. *O. felineus* is somewhat larger measuring 7–12×2–3 mm [5, 28] while *C. sinensis* is the largest measuring 10–25×3–5 mm. Variation in the size of the adults is density dependent, with individuals being smaller the higher the infestation, and also dependent on the diameter of the bile duct they inhabit [29].

The eggs of *C. sinensis*, *O. felineus*, and *O. viverrini* are morphologically similar making them difficult to distinguish from one another. The operculum of each species has a distinct shoulder while a small knob or comma shape appendage is found at the abopercular end [28]. The surface of the egg shell is rough and irregular, having been described as having a "musk-melon pattern" by scanning electron microscopy [30].

The transmission cycle of all three liver fluke species goes through three phases: (1) the infection of the snail first intermediate hosts via host feces, (2) cercarial release and finding fish second intermediate host for development of the infective metacardariae stage, and (3) ingestion of metacercariae in raw or partially cooked fish by humans. Petney et al. [21] argue that the three species

differ in terms of the relative significance of the zoonotic and anthroponotic components of the epidemiological cycle with *O. viverrini* having mainly human final hosts, *O. felineus* in Europe mainly wild carnivore hosts and *C. sinensis* and *O. felineus* in its Asian range a mixture of the two. This has considerable epidemiological significance, particularly in control and prevention programs.

The life cycles of all three species are very similar with a snail first intermediate host with usually low prevalences of infection, a fish second intermediate host with substantially higher levels of infection, and usually a carnivorous mammal as final host [31, 32]. The low prevalences of infection in the snail first intermediate hosts are, at least in part, compensated for by the often very high prevalences in fish, the infective phase for humans.

The distribution of each species is closely related to that of their hosts. This is particularly true of the snail first intermediate hosts which tend to be more restricted in the number of species used [17, 33, 34]. For *O. viverrini* the presence of snails of *Bithynia siamensis goniomphalos* in nearby freshwater sources is a prerequisite for the presence of the parasite in the human population [34, 35].

The importance of fecal contamination of fresh water sources inhabited by intermediate snail hosts by wild and domestic animal hosts (i.e., the zoonotic cycle) varies greatly between the three fluke species. In its European range fecal matter containing *O. felineus* eggs is almost exclusively found in wild and domestic carnivores. In areas with widespread human infection, fecal contamination of freshwater by infected animals is certainly reduced and in the case of *O. viverrini* probably only plays a minor role in infecting snails.

Clonorchis sinensis is a species with a relatively broad range of hosts sharing anthroponotic and zoonotic components in its epidemiological cycle [21]. *C. sinensis* is known to use eight main snail species as intermediate hosts. These come from five different families (Assimineidae, Bithyniidae, Hydrobiidae, Melaniidae, and Thiaridae) [19]. There is some geographic differentiation in the distribution and prevalence of *C. sinensis* in the different snail species. Prevalences vary locally and may be as high as 27 % for *Alocinma longicornis* in parts of Guangdong and 8 % for *Bithynia fuchsianus* in parts of Guangxi, although most values are substantially lower [19].

Once the snail has ingested the embryonated eggs, which are passed in the feces of the final host, the eggs hatch to release miracidia which then undergo development to sporocysts then rediae and finally cercariae in the snail. The tailed cercarial stage escapes from the snail host and actively swims to find a suitable fish second intermediate host which it then penetrates, losing its tail and encysting to become a metacercaria embedded either in the muscles of the fish or under the scales.

C. sinensis utilizes predominantly fish in 11 families, with 46 genera and 132 species, of which 32 genera and 71 species belong to the family Cyprinidae, but also several crustacean second intermediate hosts Lun et al. [19]. The fish include a number of species which are commonly used in aquaculture fish, including the common carp (*Cyprinis carpio*), the grass carp (*Ctenopharyngodon idellus*), the silver carp (*Hypophthalmichthys molitrix*), as well as tilapia (*Oreochromis mossambicus*) [19, 36–38].

The mammalian final hosts are infected when they eat raw fish containing the metacercariae of *C. sinensis*. This fluke has an unusually broad final host spectrum which includes human associated species such as cats and dogs, stock animals such as pigs and the brown rat (*Rattus norvegicus*), all of which are effective reservoir hosts, as well as a wide variety of wild fish eating carnivores and occasionally birds [19, 39]. Nevertheless, humans are considered to be the most important final host [19].

After being eaten, the metacercariae excyst in the host's duodenum and then move through the hepatopancreatic ampulla into the biliary ducts and towards the liver. Here, they attach to the mucosal lining and develop into hermaphrodite adults. These begin reproduction after about 3–4 weeks and may remain viable for years [18]. Attwood and Chou [40] reported that parasites survive up to 26 years in an infected human. In the case of humans, infection occurs through the deliberate ingestion of raw, partially cooked or fermented fish containing the infective metacercariae as part of tradition a food culture [41].

Although there are varying estimates of the number of humans infected with *C. sinensis* it appears that this number is increasing [16]. This is particularly the case in China where prevalence was estimated to be 4.7 million in the early 1990s to almost 13 million today [16, 42]. In the Republic of Korea the picture is variable with estimates of 4.6 % in 1971, 1.4 % in 1997, and 2.4 % in 2004 [43]. Unfortunately, few long-term records are available from Vietnam.

In addition to its direct influence on human health, *Clonorchis* infection in the second intermediate host can also reach substantial prevalences in aquaculture fish potentially limiting profitability in the aquaculture industry [14]. Chen et al. [44] found that freshwater fish in aquaculture had a 37.09 % prevalence of infection with a mean number of 10.7 cercariae/fish, while 3.07 % of shrimps from freshwater ponds carried on average a single metacercaria.

Molecular methods will probably extend our knowledge of the distribution of opisthorchid parasites. Traub et al. [45], using PCR-based technology, detected *C. sinensis* eggs in 23 % (5/22) of human feces which tested positive by microscopy as well as PCR for *Opisthorchis* like eggs. The samples came from a rural community in Eastern Thailand (Chachoengsao Province) extending the known range of *C. sinensis* substantially to the south and east of its recognized distribution in an area where it is sympatric with *O. viverrini*. Whether *C. sinensis* overlaps elsewhere with *O. viverrini* or *O. felineus* is currently unknown. *C. sinensis* is known to infect a variety of mammalian hosts including domestic dogs, cats, and pigs [18]. Dogs and cats can have high prevalences of infection (0.8–48.5 % in dogs, 0–64.1 in cats) which, however, varies considerably between endemic areas in China [17]. The higher prevalence in cats, as for *O. viverrini*, is probably due to their preference for eating fish. Pigs, which are omnivorous, had a prevalence of infection of 27 % in southern China [46].

O. viverrini is known only from three currently recognized taxa within a single snail genus, *Bithynia funiculata*, *B. siamensis goniomphalos*, and *B. s. siamensis*, from Southeast Asia [34]. In Thailand, all three taxa of *Bithynia* are found, namely, *B. funiculata* in the north, *B. s. siamensis* in the center, and *B. s. goniomphalos* in the northeast [47]. No regional separation of *Bithynia* snails has been reported in other parts of Southeast Asia, probably due to insufficient surveys [48].

Table 6.1 List of first intermediate hosts in Southeast Asia infected by cercariae of *O. viverrini*

Country	Snail	Sample size	% Prevalence	References
Thailand	*Bithynia funiculata*	352	0.30	[270]
	B. siamensis siamensis	2,800	1.60	[271]
	B. s. goniomphalos	5,729	0.45	[272]
	B. s. goniomphalos	1,382	3.04	[33]
	B. s. goniomphalos	537	0.37	[273]
	Bithynia snails	18,078	0.13	[51]
	B. s. goniomphalos	4,874	0.61–1.30	[274]
	B. s. goniomphalos	N/A	0.14	[275]
	B. s. goniomphalos	6,150	0.05	[276]
	B. s. goniomphalos	48,327	0.07	[50]
Cambodia	*B. s. siamensis*	406	0.25	[277]
Lao PDR	*B. s. goniomphalos*	3,142	2.01	[33]
	B. s. goniomphalos	81	2.47	[278]
	B. s. goniomphalos	3,913	0.60	[239]
	B. s. goniomphalos	2,000	0.95	[279]

The prevalence of *O. viverrini* infection in *Bithynia* snails is variable, with numerous collections being parasite free. If the parasite is present, cercarial release commonly occurs from about 0.1 to 2 % of individuals, but some collections have infection rates of 6–9 % (Table 6.1) [33, 34]. Snail population density is strongly seasonal, being highly abundant later in the rainy season, when reproduction occurs. At this time the *Bithynia* are extensively distributed in shallow water and rice fields. They can be found at a depth of at least 3 m, albeit in a much lower density [49]. During the dry season the population density crashes and the snails which survive are often found buried in the mud for seasonal aestivation [34, 50].

The snails are infected by ingesting the embryonated eggs of the parasite which are excreted with fecal matter. Indeed, human fecal bacterial contamination of fresh water bodies can act as an indicator for the seasonal transmission of *O. viverrini* eggs to snail intermediate hosts [51]. Once ingested, the eggs hatch to a miracidium which in turn develops to a sporocyst [28]. Once the sporocyst has developed within its snail host, it gives rise to numerous rediae which in turn produce numerous pleurolophocerous cercariae. After being released, they actively seek an appropriate fish second intermediate host, in the case of *O. viverrini* these belong to the family Cyprinidae [23, 32, 47].

The process of host finding by cercariae is complex. Free-swimming cercariae are very efficient at actively locating the appropriate species of fish in a large volume of water [52]. The intensity of infection in fish varies by season, species, individuals, and types of water bodies. Most metacercariae are distributed throughout the body of fish with some being found in the head. For *O. viverrini*, metacercarial burdens peak in winter (October–February) and become low in the rainy season and summer; thus, transmission of the parasite from fish to humans is probably seasonal.

Many cyprinid fish species have been reported as potential hosts for *O. viverrini* [16]. In Thailand, Lao PDR and Cambodia, at least 40 species of fish from 18 genera have been reported to serve as intermediate hosts of *O. viverrini*. Of these, the genera *Cyclocheilichthys*, *Puntius*, and *Hampala* are considered to be the most important [53]. For *O. viverrini*, the prevalence of infection in the fish second intermediate host is very much higher than in the *Bithynia* snail first intermediate hosts (Table 6.2). This is, however, fish species and locality dependent and ranges from 2.1 to 100 % [54]. For example, 30.4–97.1 % prevalence was found in *C. apagon* [55], 43.1–100 % in *C. armatus* [56, 57], 69.9–93.7 % in *P. leiacanthus* [58], and 33.3–74.1 % in *H. dispar* [56, 57]. The average number of metacercariae infecting fish varies from one to thousands with the highest intensity (average 1,989.8/fish) in *C. armatus* from Savannakhet, Lao PDR [57].

Humans are the dominant hosts for *O. viverrini*, while other domestic mammals, for instance dogs and cats, can act as reservoir hosts [47, 59]. Domestic cats have been found to have a relatively high prevalence of 35.5 % in the northeast of Thailand, making them potentially significant zoonotic sources of the disease during human based control programs [59]. Dogs have a much lower prevalence of 0.37 % [59]. Hamsters, rabbits, and guinea pigs are experimentally highly susceptible to infection [16]. There is no current information for native mammals and other fish-eating animals which may be also infected.

For *O. viverrini*, when fecal contamination from humans is reduced or eliminated by mass treatment and/or improved sanitation the prevalence of infection can be substantially reduced [60, 61]. However, complete elimination of infection may not be possible if domestic cat and dog reservoir hosts maintain the source of parasite and hence play a critical role in maintaining the life cycle. Based on the pattern of age-intensity profiles, *O. viverrini* may survive for 10–20 years [22].

In the case of *C. sinensis* or *O. viverrini*, both are known to cause significant health problems [47]. For these two species considerable epidemiological information is available: e.g., prevalence and intensity of infection increase with age and males tend to have a higher prevalence of infection than females [47]. Thus, hepatobiliary morbidity is more frequent in older individuals and males than in younger people and females. In addition, as is usual with many parasite species, the distribution in the host population is neither random nor uniform, but overdispersed with a few individuals harboring most of the worms [62]. Interestingly, some individuals appear to be predisposed to a heavy infection, with the intensity of infection returning to pretreatment levels after treatment [63]. Such epidemiological characteristics must be incorporated into the models on which control and eradication programs are based.

The infective metacercariae of all species discussed here are found only in their specific (mostly cyprinid) fish second intermediate hosts. These fish, when eaten raw, fermented or undercooked, act as the source of infection to humans and animal hosts [41]. Traditional dishes based on raw fish are the main sources of infection of *C. sinensis* throughout its range, for example, goi ca mai (raw fish salad) and slices of raw silver carp in Vietnam; yusheng, a raw fish salad in China; and sushi, sliced raw fish, in Korea and Japan [18]. The length of time which the metacercariae remain viable depends of the method of preparing the food.

Table 6.2 List of second intermediate hosts (Family Cyprinidae) in Southeast Asia infected by metacercariae of *O. viverrini*

Fish	% Prevalence	Country	References
Labiobarbus siamensis	51.3–100	Thailand, Cambodia	[277, 280, 281]
Cyclocheilichthys armatus	19.16–100	Thailand, Cambodia, Lao PDR	[53, 56, 57, 252, 280–286]
Hampala macrolepidota	2.6–100	Thailand, Cambodia, Lao PDR	[55, 239, 279, 283, 287–289]
Amblyrhynchichthys truncatus	100	Cambodia	[281]
Neolissochilus stracheyi	100	Lao PDR	[286]
Lobocheilus melanotaenia	100	Lao PDR	[288]
Puntius partipentazona	100	Thailand	[58]
Cyclocheilichthys apogon	25.0–97.1	Thailand, Cambodia, Lao PDR	[55, 58, 281, 286, 290]
Hampala dispar	6.49–94.80	Thailand, Cambodia, Lao PDR	[55–58, 239, 252, 277, 279, 281–283, 285, 286, 289–291]
Puntius brevis (*Puntius leiacanthus*)	14.0–93.7	Thailand, Cambodia, Lao PDR, Vietnam	[55, 57, 58, 239, 277, 281, 285, 286, 288, 292]
Poropuntius laoensis	90.0	Lao PDR	[288]
Puntius orphoides	31.0–90.0	Thailand, Cambodia, Lao PDR	[55, 252, 281, 282, 290, 291, 293]
Cyclocheilichthys repasson	10.0–80.0	Thailand, Cambodia, Lao PDR	[56, 57, 239, 277, 279, 283, 289]
Cyclocheilichthys enoplos	2.1–80.0	Cambodia, Lao PDR	[55, 56, 286, 288]

(continued)

Table 6.2 (continued)

Fish	% Prevalence	Country	References
Esomus metallicus	10.0–75.0	Thailand, Lao PDR	[57, 252]
Labiobarbus lineatus	3.0–69.6	Thailand, Lao PDR	[56, 252]
Barbonymus altus	30.0–66.7	Cambodia	[55, 281, 291]
Barbonymus schwanenfeldii	66.0	Cambodia	[281]
Puntioplites proctozysron	2.0–60.0	Thailand, Cambodia, Lao PDR	[55, 56, 252, 281–283, 286, 291, 293]
Thynnichthys thynnoides	3.7–59.7	Thailand, Cambodia	[55, 281, 287]
Cyclocheilichthys lagleri	58.2	Cambodia	[281]
Hypsibarbus lagleri	50.0	Lao PDR	[57]
Mystacoleucus marginatus	50.0	Lao PDR	[57]
Onychostoma elongatum	44.4	Lao PDR	[57]
Henicorhynchus lineatus	42.9	Lao PDR	[56]
Labeo chrysophekadion	40.0	Cambodia	[277, 293]
Oreichthys parvus	40.0	Lao PDR	[288]
Henicorhynchus lobatus	33.3	Cambodia	[281]
Hypsibarbus pierrei	33.3	Lao PDR	[286]
Hypsibarbus wetmorei	33.3	Lao PDR	[286]
Poropuntius deauratus	33.3	Lao PDR	[286]
Puntioplites falcifer	33.3	Lao PDR	[57]

Rasbora ourotacniatiran	33.3	Lao PDR	[288]
Osteochilus waandersii	30.5	Lao PDR	[56]
Carassius auratus	28.1	Vietnam	[292]
Cyclocheilichthys furcatus	25.0	Cambodia, Lao PDR	[286, 291]
Puntius viehoever	22.0	Thailand	[252]
Osteochilus hasselti	6.1–20.0	Thailand, Cambodia, Lao PDR	[57, 277, 281, 282, 291]
Puntius stoliczkanus	16.67	Thailand	[294]
Barbonymus gonionotus	2.0–16.1	Thailand, Cambodia, Lao PDR	[53, 239, 252, 279, 281, 287, 289, 291]
Onychostoma fusiforme	14.3	Lao PDR	[286]
Henicorhynchus siamensis	4.3–10.9	Thailand, Cambodia	[55, 281, 282]
Paralaubuca barroni	10.0	Lao PDR	[286]
Crossocheilus reticulatus	5.6	Cambodia	[281]
Rasbora spp.	4.3	Vietnam	[292]
Osteochilus sp.	4.0	Thailand	[252]

In Southeast Asia, particularly in Thailand and Lao PDR, the raw or partially cooked fish dishes which act as the source of the liver fluke infection can be grouped into three categories. Fresh raw fish dishes without heating are called "koi pla." These pose a high risk of infection. A moderate risk is presented by quickly fermented dish (1–2 days) known as "pla som." The last dish is fermented fish known as "pla ra" which normally requires long-term fermentation, but short-term and variable ingredients may provide favorable environments for metacercarial survival. Pla ra is a common ingredient for cooking in Southeast Asia, for example in papaya salad (som tum) [41]. In Lao PDR, the fermented fish is known as "pla dak." In other countries such as Cambodia raw fish are prepared as "pla hoc" which is similar to "pla som" and this may serve as a source of infection. The usual sources of *O. felineus* infection in Russia are dried or salted fish. Other dishes include sliced raw fish (stroganina), which is popular among native Siberians, and fish pickled in vinegar.

The number of *Bithynia* species present in the Eurasian area has changed from a single species, *Bithynia leachi*, to four morphologically similar species, *B. inflata*, *B. leachi*, *B. troscheli*, and *B. sibirica* [64] of which the first three of these can act as intermediate hosts for *O. felineus* [23]. As with *O. viverrini*, cyprinid fish also act as the exclusive second intermediate hosts for *O. felineus* [4]. These include the ide (*Leuciscus idus*), roach (*Rutilus rutilus*), European dace (*Leuciscus leuciscus*), tench (*Tinca tinca*), verhovka (*Leuciscus delineatus*), and silver crucian carp (*Carassius auratus gibelio*) [23]. After an outbreak of *O. felineus* infection in Italy, 83.1 % of tench from Lake Bolsena were found to be infected [65]. Apart from this outbreak humans are seldom infected in Europe probably because raw fish is not common in the human diet in this area [5]. However, prevalences can be very high in humans in the Asian distributional area of this species where raw fish are more commonly consumed [66].

In Europe, domestic cats and dogs can act as hosts [4, 67] in addition to a wide range of wild carnivores. These include several fox species, the raccoon dog and wolves, as well as *Martes* and *Mustela* spp., badger, otter and wolverine [4, 68]. Unlike *C. sinensis* and *O. viverrini*, *O. felineus* has also been reported from the Pinnipedia: the Caspian seal (*Pusa caspica*) from the brackish Caspian Sea, the bearded seal (*Erignathus barbatus*) from the Arctic, and the grey seal (*Halichoerus grypus*) [4, 23]. Non-carnivore and therefore presumably accidental hosts include chipmunks (*Eutamias sibiricus*) [23], beaver (*Castor fiber*), European water vole (*Arvicola terrestris*) and the brown rat (*Rattus norvegicus*), rabbit (*Oryctolagus cuniculus*), and wild and domestic pig (*Sus scrofa*) [4, 23].

6.4 Molecular Biology, Genetics, and Evolution

Advances in molecular biology have provided opportunities for increasing our understanding of the developmental and reproductive biology, as well as genetic diversity and evolution, of medically important trematodes including *Opisthorchis* and *Clonorchis*. They have also helped us to understand host–parasite interactions

and the pathogenesis of the diseases caused by these flukes. This has aided in the improvement of diagnostic methods, new drug treatments and potentially vaccine development. In addition, it is likely that new molecular data will help in examining theories on the molecular basis of chronic clonorchiasis and opisthorchiasis induced CCA. Below we will also discuss the cytogenetics, genomics, transcriptomics, and proteomics of these species.

6.4.1 Cytogenetic Analysis

The cytogenetic study of the liver flukes focuses on the study of the structure and function of the chromosomes. It includes the analysis of G-banded chromosomes, other cytogenetic banding techniques, as well as molecular cytogenetics, such as fluorescent in situ hybridization (FISH), and comparative genomic hybridization (CGH). The karyotypes of *C. sinensis* are reported to be either $2n=14$ (samples from the far east of Russia) [69] or $2n=56$ (samples from Korea and China) [70]. The $2n=56$ karyotype was described according to the number and morphology of chromosomes, probably being the octaploid form of typical opisthorchiid karyotype with $2n=14$ [69]. The karyotypes from Russia consisted of pairs of large metacentric and submetacentric chromosomes and five pairs of small chromosomes [69]. Those from Korea and China could be divided into two groups based on their sizes, with 8 pairs of large and 20 pairs of small chromosomes. They have the same number of 16 submetacentric and 8 subtelomeric pairs, but the Korean isolates have 3 metacentric and 1 metacentric/submetacentric pairs, whereas the Chinese isolates have two and two pairs. In addition, the mean total length of the diploid complements of the specimens collected in China is slightly longer than that of those collected in the Korea [70]. The question of the phylogenetic relationship between *C. sinensis* from Russia, China, and Korea therefore remains open. The liver fluke described in China and Korea could be an octaploid form of normal *C. sinensis* or possibly distinct species [69].

The karyotype of *O. felineus* collected from West Siberia was $2n=14$ and consisted of two pairs of large submetacentrics and five pairs of small chromosomes. There are three pairs of metacentric and four pairs of submetacentric chromosomes [71]. A comparison of the relative length and centromere indices of the chromosomes of these *O. felineus* did not reveal significant differences [71].

In contrast, the karyotype of *O. viverrini* is $2n=12$ and includes two pairs of large-submetacentrics, one pair of medium-sized submetacentrics, one pair of small-sized subtelocentrics or acrocentrics, and one pair of small-sized acrocentric chromosomes [72]. The medium-sized submetacentric chromosome of *O. viverrini* is probably the result of the fusion of two chromosomes from ancestral karyotypes [69]. However, the comparative analysis of mitotic and meiotic chromosomes by heterologous FISH revealed six pairs of chromosomes in the karyotype of *O. viverrini*, indicating a relatively recent chromosomal fusion event which took place in the formation of the modern karyotype [69]. However, none of the *O. viverrini*

chromosomes have shown any interstitial telomere sequences (ITSs) after FISH by telomeric DNA probe or PNA telomere probe [73]. More recently, the chromosome number, length and nomenclature of each chromosome were determined by scanning electron microscopy. The six chromosomes consist of one large metacentric, one medium-sized metacentric, two small-sized metacentric, one small-sized submetacentric, and one small-sized acrocentric chromosomes [74]. Moreover, the repetitive sequences show that despite the small size of *Opisthorchis* genomes, a large amount of interspersed repetitive DNA sequence is distributed along the euchromatic regions [75].

6.4.2 Genome

There are some reports on the genomic features characterizing *C. sinensis*, but very few on *O. viverrini* and *O. felineus*. The assembled genome of *C. sinensis* has a total size of 516 Mb with approximately 16,000 reliable protein-coding gene models. Genes for the complete pathways for glycolysis, the Krebs cycle and fatty acid metabolism were found, but key genes involved in fatty acid biosynthesis are missing from the genome, reflecting the fact that the liver fluke receives lipids from the bile of its host. Moreover, genes encoding proteases, kinases, and phosphatase enzymes, tegument and excretory–secretory products, host-binding proteins and receptors were also discovered. In addition, 53 genes related to sex determination, sex differentiation, and sexual reproduction were identified [76]. The genome of *C. sinensis* contained more than 100 copies of a long terminal-repeat retrotransposon (*CsRn1*) which belongs to the Ty3/gypsy-like long-terminal-repeat transposon family. The functional domains of Gag, proteinase, reverse transcriptase, RNase H, and subdomains of integrase are strongly conserved in *CsRn1*, which has been predicted to be mobile element based on structural considerations and from the presence of mRNA transcripts [77]. Insertions of *CsRn1* appear preferentially at repetitive and agenic chromosomal regions. Furthermore, *CsRn1* was reported to induce variations in the genome that may influence the evolution of *C. sinensis* [78]. The finding of such genomic characters of *C. sinensis* reveal the evolutionary interplay between parasite and host, which may be valuable for understanding host and parasite interactions [76]. In the case of *O. viverrini*, the estimated genomes size reported by real-time PCR was 75.95 Mb [79]. However, whole-genome sequencing of these liver flukes has not been reported.

The complete mitochondrial genomes of all three liver flukes have been successfully characterized. The mtDNA sequences of *O. viverrini*, *O. felineus*, and *C. sinensis* were variable and ranged between 13,510 and 14,277 bp and comprised 36 genes [80, 81]. Of these, 12 genes encoded for proteins, i.e., cytochrome c oxidase subunit 1 (CO1), CO2, CO3, NADH dehydrogenase subunit 1 (ND1), ND2, ND3, ND4, ND4L, ND5, ND6, cytochrome b, and ATPase subunit 6. Two genes encoded for ribosomal RNA, i.e., small subunit rRNA (*rrn*S) and large subunit rRNA (*rrn*L). The number of tRNA encoding genes varied between the different species, i.e., 20 genes for

O. viverrini and 22 genes for *O. felineus* and *C. sinensis* [80, 81]. The gene content and arrangement were almost identical between species. There were two noncoding regions, the long noncoding region (LNR) and the short noncoding region (SNR). For the *C. sinensis*, there is a lack of tandem repeat [80], whereas there was tandem repeat, interrupted in LNR region, in *O. felineus*. Moreover, when comparing the length of noncoding region of the mtDNA of *C. sinensis* from Russia and Vietnam there were significant differences between species [81].

6.4.3 Transcriptome

Expressed sequence tags (ESTs) for adult *C. sinensis* and *O. viverrini* have been reported with at least 3,000 and 4,194 ESTs, respectively, and have been registered in public dbEST databases [82, 83]. The most abundant genes in adult *C. sinensis* include cysteine proteases and mitochondrial genes, which may support biliary epithelia destruction by adult liver flukes to evade host immune attack [84, 85]. The second most abundant gene transcripts were proteins constituting muscular tissues, which enable adult flukes to abrade and feed on the biliary epithelium [86]. Vitelline precursor protein was the third most abundantly expressed gene product. It is responsible for hardening the eggshell encasing the germ cell and surrounding yolk cells [87].

The ESTs of the metacercarial stage of *C. sinensis* could be assembled into 322 genes. Those expressed most abundantly were for proteases and metabolic, transcription, and translation housekeeping proteins [82]. To obtain an insight in the developmental gene expression and regulation of *C. sinensis*, the adult and metacercariae ESTs of *C. sinensis* were compared. It was found that genes encoding structural and cytoskeletal proteins, transcription and translation machinery proteins, and energy metabolism-related proteins were highly expressed in *C. sinensis* metacercariae, while the other genes were highly expressed in the adult stage. These data may explain, to some extent at least, that *C. sinensis* metacercariae in fish hosts have a quite different physiology and metabolism compared with adult *C. sinensis* in mammals [82].

The most abundant genes in adult *O. viverrini* encoded for myoglobin, vitelline precursors, egg shell proteins, and glutathione-*S*-transferase. The other abundantly expressed genes encoded proteins involved in host–parasite relationships and included proteases, saposin-like proteins, and dynein light chains [88]. Homologues of some of the most abundantly represented proteins in *C. sinensis* and *O. viverrini* ESTs are cysteine protease, myoglobin, and vitelline B precursors, whereas others were overexpressed in each species [88]. The open reading frame (ORF) region in ESTs was also used to predict the expressed proteins in proteomic analysis. Such ORFs were generated from 4,194 available EST sequences of *O. viverrini* and subsequently analyzed for secretory signal sequence and the transmembrane domain. A total of 897 potential ORFs were identified, of which 78 were predicted to contain a secretory signal sequence and 42 to contained two or more transmembrane domains [83]. Proteases were highly presented in the *O. viverrini* transcripts

encoding secreted proteins, with five different cathepsins, a legumain, and an S1 type serine protease all predicted to contain a signal sequence [83]. In addition, more than 50 % of the predicted protein sequences of *C. sinensis* and *O. viverrini* were inferred to be homologues, reflecting their relatively close biological and physiological relationships [89]. Comparison of the predicted proteins of liver flukes and other trematodes, *S. japonicum*, *S. mansoni*, *F. hepatica* found that 29–31 % protein sequences were homologous [89]. However, this prediction of expressed proteins may not be satisfactory as some ORF encoded sequences might not express or express a low protein level. Thus, additional proteomic analyses are needed to provide more informative data on the proteins expressed in these liver flukes.

6.4.4 Proteome

Proteomic analyses should provide information on potential new and specific targets for treatment the infection. Moreover, the identification of parasitic-specific proteins could clearly facilitate the design of new tools for rapid and cheap diagnosis, which in turn could help breaking the transmission cycle of the parasite, as well as help in the identification of potential targets for vaccination, one of the best ways to control these parasite infections [90].

The adult stage of all three flukes dwells in the bile duct which provides an anaerobic environment where a large amount of exogenous glucose is used as a carbon source for energy metabolism. Thus, the most important endogenous proteins examined in the liver flukes are glycolytic enzymes which play an important role in the glycolysis pathway. Phosphoglycerate kinase (PGK), a glycolytic enzyme, was found extensively, localized in the muscular tissues of the oral and ventral suckers, ovary, testes, tegument, and intrauterine eggs of *C. sinensis* [91, 92]. The inhibitors of several glycolytic enzymes of *C. sinensis* have also been reported, e.g., vanadate can inhibit phosphoglycerate mutase (PGM) [93], whereas lactase dehydrogenase (LDH) was inhibited by Cu^{+2}, Fe^{+2}, and Zn^{+2} [94]. The cytosolic and mitochondrial malate dehydrogenases (cMDH and mMDH) of *C. sinensis* share low amino acid sequence homology (22 %) and these enzymes are differentially inhibited by 4.4′-bisdimethylamino diphenylcarbol. cMDH is more stable against heat and acidity than mMDH. Moreover, cMDH plays a pivotal role on the cytosolic side of the malate–aspartate shuttle. mMDH is a key enzyme in the tricarboxylic acid cycle and in the malate–aspartate shuttle. Thus, these glycolytic enzymes are required for the survival and pathogenesis of these liver flukes [95–97].

The mechanism of pathogenesis due to liver fluke infection mainly involves the interaction between parasite antigens and the host immune response [98]. Therefore, the excretory–secretory (ES) proteins and tegumental proteins of the liver fluke play crucial roles in host–parasite interactions, pathogenesis, and disease outcomes. Myoglobin is an abundant protein in the ES products of *C. sinensis*. It may play an oxygen-capturing role and then slowly release this oxygen to metabolic pathways in bile duct. Recombinant myoglobin reacted with the sera of *C. sinensis*-infected

rabbits and clonorchiasis patients. Lysophosphatidic acid phosphatase (LPAP), belonging to the acid phosphatase family, has been identified as an ES-antigen in adult *C. sinensis*. It shows high sensitivity and specificity in the serodiagnosis of human clonorchiasis [99]. The proteomic analyses by the 2D proteome mapping of *C. sinensis* ES products identified 62 protein spots including thioredoxin peroxidase, myoglobin, and a number cysteine proteases that were expressed abundantly [100]. More recently, Zheng et al. [101] have reported a proteome analysis of ES products of *C. sinensis* using LC-MS/MS analysis and found 110 proteins including 39 known functional proteins and 71 unknown proteins. The enzyme fructose 1,6-bisphosphate (Cs-FPBase) within the ES product was a potential causative agent of hepatic fibrosis [101].

A comparative proteomic analysis of the developmental stages from juvenile to adults of *O. viverrini* was made by 2D gel electrophoresis [102]. The total number of protein spots ranged between 210 and 239 according to the age of the worm (1–4 weeks). Only small differences in the pattern of protein spot were found during parasite maturation [102]. The secreted and surface-exposed proteomes of *O. viverrini* has also been reported [83]. The secretory proteins were analyzed using peptide OFFGEL electrophoresis (OGE) and (multiple reaction monitoring) MRM. A total of 25 proteins, 13 from MS/MS analysis of OGE and 12 from MRM identifications, were positively identified as constituents of Day 1 ES (in vitro culture of worms), whereas the remaining 18 proteins identified in Day 17 ES are a likely consequence of the culturing process [83]. Proteases were abundant, but proteolytic enzymes were underrepresented in the ES of *O. viverrini*. However, the prediction of the secretory proteins from a signal sequence or based on a transmembrane domain in the ESTs found 26 known proteins and 39 unknown proteins to be secretory proteins. Of these, only five, i.e., cathepsin F-like cysteine protease, cathepsin D, venom allergen-like protein 8, cystatin, and granulin were detected by proteomic identification [83]. Granulin, which is a homologue of human granulin, is a potent growth factor involved in cell proliferation and wound healing. A granulin of *O. viverrini* (*Ov*-GRN-1) was examined and found to be expressed in most parasite tissue, particularly the gut and tegument. *Ov*-GRN-1 is probably the major growth factor protein in the ES products secreted by *O. viverrini* that can induce the proliferation of host cells which may ultimately manifest in CCA [103].

The tegumental syncytium, which is the outermost surface of liver flukes, is considered to be very important for host response and parasite survival. Thus, it is generally seen as the most susceptible target for vaccines and drugs. Several tegumental proteins of the liver flukes have been characterized and identified. The tegumental protein of *C. sinensis*, CsTP31.8, has been proven to be an antigenic protein [104]. CsTP20.8 is expressed in adult worms and metacercariae but not at the egg stage. However, CsTP20.8 protein is considered to have limited valuable for the serodiagnosis of clonorchiasis because it shows only moderate sensitivity and although it has high specificity [105]. Another tegumental protein CsTP21.1 was identified from adult *C. sinensis* by bioinformatics analysis. It is localized in the tegument of adult worms [106]. Interestingly, CsTP21.1 is considered a trematode–nematode pan-specific antigen that could be useful for the development of a universal immunodiagnostic kit for human infection with trematodes and nematodes [106].

Membrane-spanning proteins of *O. viverrini* are predicted to include 28 known proteins belonging to the transporters/channels, protease/hydrolytic enzymes, structural/membrane organization proteins, and other miscellaneous proteins, as well eight unknown proteins based on transmembrane domains. However, proteomic identification found only four membrane proteins, i.e., ATP–ADP antiporter, Sm-TSP-2, succinate dehydrogenase, and succinate dehydrogenase complex subunit C expressed in *O. viverrini* [83].

6.4.5 Vaccine Development

It is clear that a vaccine against any of the major human pathogens within the Opisthorchiidae would greatly aid control measures, although progress in research in this direction, which has been under way for some time has been limited [107]. The goal for the development of a vaccine against *O. viverrini* and/or *C. sinensis* is not only to limit the pathologic sequels due to acute and chronic infections, but, as is the case with other carcinogenic pathogens such as human papilloma virus, it could also protect against cancer [108]. Genomic studies on both *O. viverrini* and *C. sinensis* may well facilitate the development of vaccines with time. As indicated above, the tegument is generally viewed as the most susceptible target for vaccines and drugs in liver flukes because it is a dynamic host-interactive layer with roles in nutrition, immune evasion and modulation, pathogenesis, excretion and signal transduction [109, 110]. Large proteins such as multifunctional secreted proteases and tegumental proteins have been identified as potential targets for the development of drugs and vaccines [76].

DNA vaccines against *C. sinensis* were produced by encoding cysteine proteinase and a fatty acid-binding protein and tested in a rat model. They showed 31.5 and 40.9 % protection efficacy, respectively [111]. In addition, an oral vaccine using *C. sinensis* tegumental protein 22.3 kDa fused with the *Bacillus subtilis* spore coat CotC showed 44.7 % protection in the rat model [112]. An *O. viverrini*-crude somatic antigen (CSAg) administered with complete Freund's adjuvant or alum was used to stimulate immune responses in *O. viverrini*-primed hamsters. The greatest protection was 48.4 % and elevated TGF-β induced by *O. viverrini* may play an important role in parasite survival [113]. The reported protection rate of the vaccine candidate molecules against these liver flukes is not satisfactory and more studies are required.

6.4.6 Phylogenetics, Systematics, and Genetic Diversity

A variety of molecular markers/techniques has been used to examine the genetic diversity of *Clonorchis* and *Opisthorchis* species at the interspecies and intraspecies levels. Although there are considerable similarities between *C. sinensis*, *O. viverrini*, and *O. felineus*, there is also a great deal of regional variation both within and

between species. Adaptation to differences in the environment and host selection process, as well as limited gene flow between different water sheds can all play a considerable role in determining the genetic constitution of each isolated species and population [114]. Although all three species are closely related, their systematic position remains controversial. Some reports indicate that *O. viverrini* is more closely related to *C. sinensis* than *O. felineus* when examined using 12 mitochondrial protein-coding genes [115] and the ninth intron region of the paramyosin gene [80]. By contrast, it has also been suggested that *C. sinensis* is more closely related to *O. felineus* than to *O. viverrini* based on ITS2 and mitochondrial DNA [116–118], or even that *C. sinensis* and *O. viverrini* are more closely related to one another than to *O. felineus* when examined by ITS and CO1 sequences [119]. Thus, the situation is far from clear and more powerful genetic markers together with greater sample sizes and more geographical isolates need to be studied for a comprehensive phylogenetic analysis among these liver flukes.

Considerable genetic diversity has been observed in *C. sinensis*, based on its geographic distribution within China, Korea and the Russian Federation, as well as among different reservoir hosts including human. Isoenzyme markers can be used to differentiate *C. sinensis* into the two populations from two different geographical isolates from Korea and China [120, 121]. However, the DNA regions of ribosomal DNA and mitochondrial DNA sequences were strongly conserved and nearly identical between different isolates [120, 122, 123]. In another study based on ITS1 sequencing, two levels of intraspecific variation, i.e., interindividual and intraindividual, were observed and these showed a "northern" and a "southern" genetic group of *C. sinensis* according to their distribution in China, Korea, and Russian Federation [124]. Moreover, the eggs of *C. sinensis* collected from a well preserved Chinese body which had been buried in 167 BC revealed the differences in the ITS1 sequence at 15 nucleotide positions compared to the present samples, suggesting sequence divergence through time [125]. More recently, the genetic variation and phylogeography of *C. sinensis* have been studied from two geographic localities in the Russian southern far east and compared to the other geographic localities from China, Korea, Japan, and Vietnam by CO1 sequence. A total of 18 haplotypes were observed. Of these four were common to Russian and Chinese isolates, and the other two were common to Russian and the other isolates. The Russian samples differed from those of the other localities in haplotype frequencies [126].

The role which animal reservoir hosts play in genetic variation of *C. sinensis* is currently being investigated. Both RAPD and MGE-PCR was used to examine the genetic variation among individual adult *C. sinensis* collected from cats and dogs in two geographical areas, Guangdong province in the South and Heilongjiang province in the North of China [117, 127]. Both revealed genetic polymorphisms among *C. sinensis* individuals from these hosts in each location. In a recent study from different geographical localities in Korea, as well as in China, using mitochondrial genes sequences, the genetic variation present in *C. sinensis* from naturally infected cats, dogs, rabbits, and humans was examined. Intraspecific nucleotide variation of the Korean population ranged between 0 and 1.6 % [117], whereas 0–1.7 % was found in the Chinese population [128].

The genetic diversity of *O. viverrini* has been intensively investigated based on a variety factors, e.g., spatial, temporal, and host factors. Unlike *C. sinensis*, there is no report of genetic variation between specimens collected from different reservoir hosts. An initial report was published on different geographical isolates by Ando et al. [129] using rDNA and mitochondrial DNA sequences but with a restricted sample size. The next study involved the establishment of 32 enzyme (allozymes) loci using multilocus enzyme electrophoresis (MEE) to comprehensively examine the genetic variation among natural populations of *O. viverrini* from 11 different geographical localities in Thailand and four localities in Lao PDR [130–132]. Two major lineages of *O. viverrini* were found, which could be additionally subdivided into at least six distinct genetic groups which correlated with five different wetland systems [132]. RAPD and microsatellite analyses in *O. viverrini* also showed significant differences between the isolates from Thailand and Lao PDR [133]. Interestingly, the MEE data provided evidence of potential coevolution between *O. viverrini* and its snail host, *B. s. goniomphalos*, as there was a high concordance of lineages and specific genetic groups [48, 132]. An additional *O. viverrini* genetic isolate from Savannakhet, Lao PDR was analyzed using 20 allozyme markers and also found to be associated with a specific wetland system [134].

Microsatellite markers and MEE have been used to explore the population genetics and systematics of *O. viverrini* from different geographical isolates [132, 135, 136]. In addition, *O. viverrini* populations collected from different years (temporal), as well as from different fish host species, was carried out by MEE [118, 132]. The level of genetic differentiation between the populations from Thailand and Lao PDR was very high, whereas it was low for comparisons among populations from Thailand. The same pattern was found among different fish host species and temporal populations [118, 132]. Based on the MEE and microsatellite analyses, *O. viverrini* populations almost always deviated from Hardy–Weinberg equilibrium with varying levels of heterozygote deficiencies [132, 137]. In addition, microsatellite markers could be used to examine the genetic differences among *O. viverrini* populations over small scale geographical distances within Khon Kaen Province, Thailand [136]. MEE was also be used to explore the genetic structure of *O. viverrini* populations at Vientiane Province, Lao PDR (Kiatsopit et al., unpublished). The analyses based on microsatellites, together with allozyme data, revealed that the predominant mode of reproduction in *O. viverrini* is selfing (inbreeding) rather than cross-fertilization (outbreeding). The demonstration of significant genetic heterogeneity, as well as biological variation between the different geographical isolates of *O. viverrini* from Thailand and Lao PDR, provide independent evidence that *O. viverrini* is a species complex [132, 138].

Genetic variation within *O. felineus* from different geographical localities was investigated using three different polymorphic genetic markers, i.e., CO1, CO3, and ITS1 sequences [139]. All *O. felineus* populations were classified into three geographically isolated groups, namely, from eastern Europe (the drainage basins of the Volga, the Don, and the Ural rivers), northern Asia (Siberia, the Ob-Irtysh and the Yenisei river basins), and Central Asia (Kazakh, the Nura-Sarysu Basin, part of the endorheic Aral-Caspian basin). Only low genetic differentiation between these geographically distinct European and Asian *O. felineus* population was observed.

This homogenization of population structure could result from potentially high levels of gene flow between populations, accompanied by active migrations of definitive hosts, including humans, during the Holocene [139]. More recently, ISSR and allozyme analyses have been used to examine genetic variation of *O. felineus* from six rivers of Western Siberia. In addition, ISSR was also used to explore the genetic variability of metacercariae of *O. felineus* collected from different fish host species [140]. Again, only a low degree of genetic polymorphism and differentiation among *O. felineus* population was observed. Southern *O. felineus* samples from the Tobol and Tura rivers showed higher polymorphism levels than the samples from rivers in northern part [140]. However, the metacercariae of *O. felineus* collected from different fish species showed no genetic differences [140]. The results so far suggest that population genetic data based on other genetic markers such as microsatellite DNA are required.

6.5 Diagnosis

The most common diagnostic method for fish-borne trematode, which involves finding eggs in fecal samples, seems still to be far from ideal. In low egg output and low prevalence situations, sensitivity is also low, and using this method is a puzzle that challenges scientific efforts.

6.5.1 Parasitological Methods

Fecal examination is the routine method used for the diagnosis of liver fluke infection. It has the advantages of the simplicity of sample collection and of being non-invasive. Once a fecal sample is available, the modified formalin–ether (or ethyl acetate) concentration technique (FECT) [141], the modified thick Kato smear [142], or Stoll's dilution egg count technique can be used [143]. Although these techniques are highly specific there are limitations because of the prepatent period of infection before eggs are produced, poor sensitivity when infection intensities are low, or intermittent egg excretion associated with bile duct obstruction. Both sensitivity and specificity vary depending on the method used but also on the experience of the examiner. The diagnostic value of these methods lies in their ability to detect relatively light infections, which occur in the majority of infected individuals, and in individuals recently treated with praziquantel. As a single examination does not necessarily provide diagnostic certainty, repeated examinations are needed to improve diagnostic sensitivity. Thus, three consecutive Kato–Katz thick smears are more sensitive than a single examination by FECT [144]. However, even using such repeated stool examination there can be a discrepancy between egg count and worm detection so that a false negative diagnosis remains a real possibility.

In an autopsy study, adult *O. viverrini* were recovered directly from 139 livers. Examination of postmortem fecal samples from these individuals showed that only 67 % were positive for *O. viverrini* infection. The detection limit using normal fecal examination was estimated to be 20 worms or approximately 1,000 epg. Individuals with low infection intensities and limited egg output are likely to be underdiagnosed by as much as ~20 % [62]. Although there is some evidence of density-dependent fecundity, there is in general a linear relationship between fecal egg count and worm burden.

Diagnostic kits which reduce processing time (Parasep SF) have also become available; however, these show a lower sensitivity than FECT although they have a higher sensitivity than the simple smear method (Sithithaworn, unpublished). The performance of this kit is comparable with that of the widely used Kato–Katz method.

Eggs can also be detected during treatment of bile duct obstruction either in bile from nasobiliary or percutaneous transhepatic biliary drainage (PTBD) or in the duodenal fluid. Adult worms are ejected during expulsion chemotherapy [145–148]. Similar data to those available for *O. viverrini* are now also available for *C. sinensis*. These indicate that this species lays more eggs than *O. viverrini* (514 epg/worm), probably due larger size of adult worms [149]. Worm burdens determined by expulsion chemotherapy ranged from 1 to about 100 worms for *C. sinensis* [149, 150].

In some endemic areas for liver flukes, for example in Southeast Asia, intestinal flukes coexist leading to a potential diagnostic problem if conventional fecal examination is used. The eggs of *O. viverrini*, which are identified by their characteristic rough and thick egg shells are very similar to the eggs of several species of other food-borne trematodes belonging to the families Opisthorchiidae, Heterophyidae, and Lecithodendriidae. The latter two families are commonly referred to as minute intestinal flukes (MIF) due to their small size compared to the liver flukes [28, 151, 152]. These species are, like the liver flukes, fish-borne trematodes (FBT) or fish-borne zoonotic trematode (FZT) [153–155]. The similarity between the eggs of these other FBT species and those of *O. viverrini* and *C. sinensis* can substantially increase the likelihood of a false positive diagnosis, depending on the prevalence of these species. They thus reduce diagnostic specificity and the identification of adult worms and PCR confirmation may be necessary for correct species identification in areas where a number of species exist.

6.5.2 Immunological Methods

Several serological tests for clonorchiasis and opisthorchiasis have been developed for use in a diagnostic assay with greater sensitivity and specificity than fecal examination. These include the intradermal test (IDT), immunoelectrophoresis (IEP), indirect hemagglutination assay (IHA), indirect fluorescent antibody test (IFAT), and indirect enzyme-linked immunosorbent assay (indirect ELISA) [17, 156, 157]. Indirect ELISA is commonly preferred for the detection of antibodies although, due to the complexity of the antigen, neither sensitivity nor specificity is constant. Crude somatic extracts of adult worms of both *O. viverrini* and *C. sinensis* used for ELISA provide higher

sensitivities than fecal examination [17, 158, 159], while ES antigens show a superior or equivalent performance to the crude antigen [160, 161]. Interestingly, antigen extracted from the *Bithynia* intermediate host snails has also been used as an antibody detecting antigen for the diagnosis of human *Opisthorchis* infection [162, 163], although the value of such rests has yet to be evaluated.

Recombinant antigen for serum antibody detection by ELISA has been produced from eggs and egg shells [159, 164]. In addition, the propeptide of cathepsin L, glutathione *S*-transferases, adenylate kinase 3, phosphoglycerated kinases, PGM, LPAP, and cathepsin B, cathepsin F, cathepsin L-like, legumain, taurocyamine kinase have been characterized and show better diagnostic sensitivity and specificity over conventional fecal examination diagnostic methods [91, 106, 164–168]. Nevertheless, the increased specificity and reduced cross reactivity of these proteins need to be tested under field conditions before they can be judged good enough to replace the commonly used native crude antigen [17]. The detection by ELISA of antibodies in non-fecal clinical samples such as urine and saliva has been considered and saliva found to be of potential use for the serodiagnosis of opisthorchiasis [169].

One drawback of antibody-based detection is the inability of this method to differentiate between past and present infections because of the persistence of antibodies in the in the patient even after a cure has been effected [170–172]. One way of overcoming this problem is to use an antigen-based detection which indicates if current infection is present [173–175]. Monoclonal antibody (mAb)-based systems offer increased diagnostic sensitivity, as they are able to detect secretory products from only a few adult worms. This is effective in low-scale infections when eggs are not detectable in fecal samples [174]. This has been corroborated in an autopsy study [62]. Studies in animal models for *C. sinensis* [176] and *O. viverrini* (Duanngai unpublished) showed promising results. Recently it was suggested that coproantigen detection is useful for detecting positive cases, again especially when fecal examination negative, although the antigen level is also correlated with the intensity of infection [177]. This may be a useful approach for the detection of mild infections and for the evaluation of the effectiveness of pharmaceutical cure.

6.5.3 Molecular Methods

A number of target genes from both *C. sinensis* and *O. viverrini* have been tested for their diagnostic suitability including satellite DNA, ITS1, ITS2, and mitochondrial DNA. These were used for both conventional PCR and real time PCR diagnosis showing high specificity but variable sensitivity [17, 157, 178]. The detection of *O. viverrini* egg DNA in human stools using PCR and based on primers complementary to the repeat DNA element showed a specificity of 98 % and a sensitivity of 100 % for moderate-to heavy infections with more than 1,000 EPG. In light infections with less than 200 EPG the sensitivity was reduced to only 68 % [179, 180]. More recently, the retrotransposon of *O. viverrini* (*OV-RTE-1*) has been found to be the new alternative genetic marker of high sensitivity and specificity for the PCR diagnosis of opisthorchiasis [181]. Another PCR-based study using the same target

DNA showed low sensitivity (50 %) at high egg counts of more than 1,000 EPG in stool samples from Lao PDR [182]. However, if the quality of the DNA was improved by using cetyltrimethylammonium bromide during its preparation (CTAB) to remove PCR inhibitors the sensitivity was increased to 79 % [183]. PCR-positive tests occurred in a 29 % of cases which were parasite negative in this study using the conventional fecal examination method indicating its potential diagnostic value for light infections. Another *O. viverrini*-specific primer pair was established which was able to detect adult worms with 10–12 ng of DNA, and metacercariae when three or more occurred in a fish sample [184]. Loop-mediated isothermal amplification (LAMP) has been established for the detection of both *O. viverrini* and *C. sinensis* with a higher sensitivity than conventional PCR [185–187].

Species-specific PCRs are now also available to distinguish between the three species of liver fluke: *O. viverrini* [129, 179], *O. felineus* [188], and *C. sinensis* [189]. In addition, several genetic markers/approaches involving conventional PCR, PCR-RFLP, multiplex PCR, real-time PCR and multiplex ligation-depended probe amplification (MLPA) pyrosequencing can be used to differentiate between species involved [189–192].

The molecular methods discussed above will contribute significantly towards a more effective and accurate diagnosis of trematode infections, although further simplification of the tests and an understanding of cost-effectiveness under various socioeconomic scenarios is needed. In addition, the validation of DNA positive test results is required, although evidence from animal models is accumulating and supported human studies [193, 194].

Real-time PCR can also now be used to quantify the intensity of infection with *C. sinensis* [99]. In addition, molecular identification techniques can be used in cases of multiparasite infections in a single host [6, 191].

Such approaches can also be used in a food security setting to test for the presence of liver flukes in aquaculture or native fisheries products, particularly for export [184, 186, 195].

Due to their high specificity, such molecular diagnostic tests are likely to play an increasingly significant role in anthelminthic drug efficacy evaluations, the rigorous monitoring of reinfection patterns, and to investigate changes in the endemic range of the liver flukes [45, 55].

6.6 Consequence of Infection

6.6.1 Pathogenesis, Pathology, and Morbidity

Liver fluke infection causes significant pathological changes to the bile ducts which the worms inhabit. The pathology can also extend to affect both the liver and gall bladder [18, 47]. Syrian golden hamsters provide a suitable animal model to examine these changes [196, 197]. During the early phase of infection with *O. viverrini* there is an acute inflammatory reaction in the large intrahepatic bile ducts as well as portal connective tissue. Once the infection has become chronic (at about 30 days

post infection) hyperplasia and adenomatous formations of the bile duct epithelium occur [198]. Granulomatous responses to both the adult flukes as well as to the eggs which they produce lead to periductal fibrosis and scarring. This is the most prominent feature during the chronic stage of infection [196]. The extensive fibrosis is associated with a significant increase in the synthesis of and the hepatic content of collagen [199, 200]. With the onset of the chronic phase of infection the inflammatory responses become less severe suggesting that immune modulation may occur. Fibrotic tissue accumulates due to repair dysfunction and an imbalance in synthesis and degradation of the fibrotic tissue. These factors may lead to cell proliferation which, in the presence of cofactors, significantly contributes to cancer development [201]. In humans, periductal fibrosis is a significant cause of hepatobiliary disease and leads to an increased risk of CCA development [202, 203]. In *O. viverrini* patients with advanced periductal fibrosis there was an eight times higher level of IL-6 responses to *O. viverrini*-excretory/secretory products than in patients without fibrosis, indicating the role of IL-6 in the pathogenesis of advanced periductal fibrosis in opisthorchiasis [204].

Chronic infection by the liver flukes corroborated by a marked humoral immune response indicated by the presence of parasite-specific IgG, IgA, and IgE in the serum and bile of humans infected with *O. viverrini* [205, 206]. Although the IgG level against CSAg correlated with hepatobiliary abnormalities diagnosed by ultrasonography, there was weak correlation with the intensity of infection [207].

The bile ducts which harbor the adult worms show the most significant and potentially dangerous gross and microscopic pathological changes in both *O. viverrini* and *C. sinensis* infections but development is long-term taking up to 7–15 years for *O. viverrini* [208]. Immunomodulation during both the acute and chronic phases of infection is responsible for the pathological changes observed [18].

Light infections may be inapparent with no significant symptoms. Pathology depends on both the duration and the intensity of infection as well as to the susceptibility of the host [17, 47, 209, 210]. For heavy infections the peripheral bile ducts may become thickened beneath the fibrotic capsule of the liver. A recent outbreak of opisthorchiasis in Italy caused by *O. felineus* infection presented as a febrile syndrome with eosinophilia and cholestasis [211]. This outbreak is interesting as 37 (82 %) of the 45 infected individuals showed symptoms of the disease and 8 (17.7 %) were admitted to hospital for treatment.

As indicated above, inapparent infections are common with only about 5 % morbidity occurs among infected individuals [212]. Once the symptoms become apparent they are usually nonspecific, involving general abdominal discomfort. In such cases hepatobiliary abnormalities and/or CCA can usually be detected by ultrasonography [203, 213].

6.6.2 Liver Flukes and Cholangiocarcinoma

CCA is a cancer of the epithelial cells in the bile ducts arising along either the intrahepatic or extrahepatic biliary tree [214, 215], although studies on molecular pathogenesis are currently confined to the intrahepatic CCA type. CCA is responsible for

as much as 15 % of liver cancers worldwide, most of which are associated with trematode infection [216, 217]. Large-scale epidemiological studies of CCA indicate an increase in both the incidence and mortality rates. Currently CCA is the second most frequent primary liver cancer [218]. The highest incidence of CCA worldwide occurs in northeast Thailand [219]. In addition to CCA induced by either *C. sinensis* or *O. viverrini*, early observations indicate that around 400 cases of this disease currently occur every year in patients heavily infected with *Opithorchis felineus* [220].

The induction of cancer by these liver flukes appears to be dependent on a variety of factors including host genetic background, past exposure to infection as determined by elevated *O. viverrini* antibody levels, liver cirrhosis, chronic infection with hepatitis C virus and heavy alcohol consumption [221].

The association between *O. viverrini* and CCA was first determined in a hospital-based, case–control study conducted in Thailand in the late 1980s [222]. A total of 103 patients with CCA were compared with an equal number of age- and sex-matched controls and elevated *O. viverrini* antibody titers were positively correlated with an increased risk of CCA [222]. This was confirmed in a repeat study based on 129 cases the cancer. This study indicated that the population-attributable risk is as high as 88 % in endemic areas [223]. Another a case control study, this time on *C. sinensis* from Korea, compared 41 patients with CCA with 406 controls and found a similarly strong association between liver fluke eggs in fecal samples and CCA [224]. A recent meta-analysis including 912 cases and 4,909 controls confirmed this association [225]. The population-attributable risk was lower than that calculated for *O. viverrini* but was nevertheless 27.9 % for men and 16.2 % for women.

In patients with a *C. sinensis* infection the formation of calculi in the intrahepatic biliary passages is a characteristic pathological change. This may be associated with suppurative cholangitis, cholecystitis, and biliary abscess or so-called cholangiohepatitis. It can eventually lead to the development of primary liver cancer, especially CCA. The occurrence of calculi is probably caused by bile stagnation, which in turn causes mechanical obstruction by *C. sinensis* worms and eggs in the bile ducts. The calculi in the intrahepatic and extrahepatic bile ducts are made up of bilirubin and calcium salts. The formation of such pigment stones in clonorchiasis is thought to be due to bile stagnation leading to changes in the composition of bilirubin, cholesterol, phospholipid, bile acid and the activity of bacterial glucuronidase. The goblet cell metaplasia of the bile duct epithelium is responsible for the high content of mucous secretion in the bile. This mucin-rich bile in conjunction with the worms and eggs not only cause cholestasis but also provide a suitable environment for secondary bacterial infection. This is usually due to *Escherichia coli* which cause ascending cholangitis from the intestine [18]. Studies on *C. sinensis* indicate that this species also stimulates biliary epithelial hyperplasia [226], which is considered to play a significant role in carcinogenesis [197, 227]. Clonorchiasis-associated CCA involves substantial mucin secretion, usually accompanied by extensive fibrosis [228, 229]. Although the larger bile ducts are only slightly enlarged and fibrotic, they are commonly blocked by adult worms or calcium bilirubinate stones [230]. Clonorchiasis-associated CCA has develops in a discrete nodular or confluent mass in which smaller

ducts with adenomatous hyperplasia undergo malignant transformation occur [231]. Chronic inflammation is of particular significance for the induction of CCA due to oxidative and nitrative DNA damage [232].

Although most of these studies indicate that liver flukes cause tissue damage by mechanical and chemical irritation, some recent studies suggest that parasite-specific immune responses may also play a major role [232]. A genetic polymorphism in the detoxifying enzyme glutathione-*S*-transferase (GSTM1) in association with seropositivity for opisthorchiasis was found to modify the cancer risk factor for CCA [223]. Thus, gene–environment interactions (current or past infection of *O. viverrini* infection) can play a significant role in individual susceptibility to CCA.

Carcinogenesis of CCA is still not clearly understood; however, it appears to be a multistage process with a variety of factors being involved of which chronic infections and persistent inflammation are predominant [233]. It is also possible that nitric oxide (NO), which can generate DNA-reactive agents and N-nitrosamines, is involved [232]. Excess NO production plays an important role in a number of pathological processes, including the induction of cancer (see [232]). If a host becomes infected with a liver fluke, macrophages and other cell types (e.g., mast cells, eosinophils, and epithelial cells) are activated by parasite specific T cells and cytokines and synthesize NO from L-arginine via the induction of iNOS with the aim of eliminating the intruder. Nitric oxide is not only cytotoxic but also genotoxic by reacting with superoxide to form the highly reactive peroxynitrite which leads to oxidative and nitrative DNA damage via the formation of 8-oxodG and 8-nitroguanine [234]. These can be used to indicate DNA damage in the affected tissues. The overproduction of NO caused by *O. viverrini* infection can also lead to the endogenous nitrosation of amine precursors to form potentially carcinogenic N-nitrosamines such as *N*-dimethylnitrosamine (NDMA) [235]. NDMA, which is a carcinogenic product of the nitrosation reaction, has been detected in the urine of *O. viverrini*-infected subjects. It seems to be associated with a lymphoproliferative response to active liver fluke antigens which ceases after praziquantel treatment and the death of the parasites [235]. During an active *O. viverrini* infection of either hamsters (the animal model) or humans, an isoform of cytochrome P-450 (CYP) enzymes, CYP2A6 is formed [236, 237]. NDMA requires metabolic activation, mainly by CYP2E1 and CYP2A6, before becoming carcinogenic. It is hypothesized that this increase in CYP2A6-related enzyme activity in *O. viverrini*-infected individuals is an important link between inflammatory processes due to chronic liver fluke infection and a high risk for CCA.

6.7 Epidemiology

A somewhat dated national survey carried out by the Ministry of Public Health in Thailand in 2001 showed that helminth infections are common with a country wide total prevalence of 22.5 %. Of the species involved hookworms are the most common (11.4 %), while *O. viverrini* ranked second with an average prevalence of

9.6 %, although the central and southern areas of the country showed a very limited presence of the parasite [238]. In the northeast, there is substantial variation in the prevalence of opisthorchiasis among provinces, ranging from 4 to 33 % [238]. In Lao PDR, *O. viverrini* is common in the lowlands among people with close ethnic ties to the majority of the northeast Thai population probably due to high levels of partially enforced migration from Lao PDR into Thailand in the past [239]. The prevalence in certain areas is as high as 36–60 % [15]. This is much higher than previous records indicate [240, 241]. The presence of mixed infections including *O. viverrini* and heterophyid and lecithodendriid flukes found in communities along the Mekong River, potentially make conventional fecal diagnosis difficult [151].

Limited information on the incidence of infection in endemic communities in Thailand is available [61, 63, 242]. In a study of three villages in Khon Kaen Province, the incidence was 1.7–25 % over a 6 month period [61]. In a central Thai village containing a migrant population from the northeast of the country the incidence was 21.6 % per year [243]. The high levels of incidence in some villages correspond with the high prevalences in some areas. For example, with an incidence of 40 % per year, only 6 years are required for the prevalence of an originally uninfected cohort to exceed 95 % [244]. In northeast Thailand information on the rate of reinfection after treatment also show a high incidence of (re)infection. After a pretreatment prevalence of 55.1 %, it took 1 year for the prevalence to return to 54.8 % [242]. Upatham et al. [63] reported that in an area in Chonnabot, Khon Kaen Province, where 97.4 % of villagers were infected, the prevalence had reached 94 % 1 year post praziquantel treatment. It is significant that individuals with a high pretreatment intensity of infection tended to have a high intensity of reinfection. This may be a predisposition to heavy infection in some individuals. This hypothesis is supported by evidence from other parasites, such as *Ascaris lumbricoides* [245], *Necator americanus* [246], *Trichuris trichiura* [247], and *Schistosoma mansoni* [248]. Rapid reinfection after treatment shows little evidence for protective immunity, although this may occur in some individuals.

Although the rates of *O. viverrini* and *C. sinensis* infection vary considerably between villages, the pattern of infection is similar. Infection is age-dependent with the youngest age groups (0–5 years) having a low prevalence and intensity of infection. These increase through the preteen and early teenage years, often reaching a plateau in late teenagers (e.g., 15–19 years). In some areas, the intensity of eggs released increased with age [212], but the worm burden declined after the age of 50–60 [62, 249]. A number of possible reasons have been suggested for this decline including the late-development of an immune response, lower parasite survival in more heavily fibrosed bile ducts, death of parasite in heavily infected people, or reduced exposure to infection in older age groups. Infection in infants may be due to mothers feeding them raw fish which is often finely ground [212, 250, 251]. However, the reported intensities of infection under the age of four are invariably low and there is little evidence that young children experience frequent exposure to infection.

In general, the prevalence and average intensity of *O. viverrini* infection is either not sex related or is slightly higher among males compared to females [212, 249,

251, 252], although more heavy infections may be found among males than females. This is also the case for *C. sinensis* [253]. Males could therefore be more at risk of significant pathology, including cancer, as this increases in a nonlinear fashion with infection [207, 254].

As with other helminths, *O. viverrini*, and probably all of the liver flukes dealt with here, is highly overdispersed with the majority of worms being found in only a few heavily infected individuals [146]. The maximum worm load was 565 with a mean of 85 (SD = 154). Haswell-Elkins et al. [249] observed that 81 % of 11,000 worms recovered after treatment of 246 village residents were expelled by just 25 individuals (10 % of the sample population). The highest burdens were over 100 worms. Interestingly, a number of individuals who did not expel worms were nevertheless positive for eggs. In an autopsy study in Khon Kaen in which the worm burden was accurately measured, Sithithaworn et al. [62] found that 30 out of 181 cadavers contained 66 % of all the worms recovered and that only 13 people (7 %) had worm burdens greater than 400.

6.8 Treatment

Treatment programs vary considerably between countries. In general, infection with *O. felineus* is so limited in Europe where only local control is necessary if a particular community or group is infected [5]. In Thailand, a trial liver fluke control program was developed as early as 1967 in Sakon Nakhon Province [60]. This, as with other control programs in Thailand, was based on the selective treatment of infected individuals as opposed to mass treatment. One of the limitations of this approach is that although the drug of choice, praziquantel, has a high efficacy (90–95 %) and there is no evidence of drug resistance, the reinfection rate is high. This suggests that control by chemotherapy alone is unlikely to be completely successful, which is supported by the "residual" prevalence found in many areas after control measures were conducted [60]. With the advent of praziquantel in the mid-1970s, which is effective in about 90 % of cases, the duration and toxicity problems were largely eliminated [255]. The recommended daily dose for treatment of *C. sinensis* was 3 × 25 mg/kg × 1 day with cure rate of 85 % and egg reduction rate of 99.7 % [256], for *O. viverrini* the dose of 40 mg/kg with cure rate of 90 % and egg reduction rate of >99.7 [255, 257] and for *O. felineus* the dose of 3 × 25 mg/kg × 1 day with cure rate of 90 % and egg reduction rate of 100 % [258, 259].

Recent data based on higher sensitivity methods such as PCR, however, suggest that the treatment efficacy may be lower than previous estimates has suggested, an area which clearly requires urgent investigation. Nevertheless, treatment with praziquantel usually leads to the elimination of symptoms. As an alternative to praziquantel, tribendimidine has been examined and it gave an efficacy comparable to praziquantel in the treatment of *C. sinensis* infection and resulted in fewer adverse events [260] but more study is required.

6.9 Prevention and Control

Methods of prevention and control aim at breaking the transmission cycle to humans. The millions of people infected and at risk of infection, as well as the direct and indirect economic losses resulting from liver fluke infection indicate the great importance of implementing effective and long lasting prevention and control measures. There have been a number of attempts to do this both via direct treatment and also education aimed at reducing or elimination the consumption of raw or partially cooked fish. For *O. viverrini*, when fecal contamination from humans is reduced or eliminated by mass treatment and/or improved sanitation, the prevalence of infection can be substantially reduced [22]. A control program initiated in 1989 in certain provinces, particularly in the north and northeast of the country, resulted in a reduction in prevalence from 35.6 % in 1988 to 8.7–9.4 % during 2001–2009 [15, 261]. However, complete elimination of infection may not be possible if domestic cat and dog reservoir hosts maintain the source of parasite therefore playing a critical role in maintaining the life cycle. The difficulty involved in detecting infected cases with only a light infection (<1,000 EPG) and the problem of reinfection after treatment also present serious problems to effective, long-term control. At the social level, education has proven both difficult to implement and ineffective to reduce the consumption of raw or partially cooked fish in Thailand [22].

An easy and promising method of preventing infection is to kill the infective metacercariae before fish are consumed. Unfortunately, as the consumption of raw or partially cooked fish is a deeply imbedded tradition in areas where opisthorchiasis and clonorchiasis are most common, the relevant populations have proven refractory to change [41].

In particularly Thailand and Lao PDR there are three types of fish dishes which act as a source of infection with *O. viverrini*. “Koi pla” is prepared from fresh raw fish which are seasoned with lemon juice and spices and consumed without heating, posing a high risk of infection. “Pla som,” which is a dish made out of fish which have been fermented for 1–2 days is poses a moderate risk, while. “Pla ra” which undergoes less than the usual long-term, although less risky, may provide a favorable environment for metacercarial survival [41]. Other Mekong countries have their traditional potential sources of infection: fermented “pla dak” in Lao PDR, raw “pla hoc” in Cambodia which is similar to “pla som,” “goi ca mai” (raw fish salad) and slices of raw silver carp in Vietnam, a raw fish salad in China, and sushi in Korea [18]. In Russia, *O. felineus* infection may come through eating dried or salted fish or sliced raw fish (“stroganina”) which is popular among native Siberians, as well as fish pickled in vinegar.

Salted fish is generally considered ready in a day or two. It has been shown, however, that metacercariae remain viable under high salt concentrations for up to 2 weeks. The popular dishes in Russia and Eastern Europe of slightly salted fish are not safe at all. The same is true for dried fish where greater than 12 days of drying are required to kill 99 % of metacercariae. Given that the weight of fish and the temperature of drying are not constant even at fish plants or after 12 day period,

dried fish remain dangerous for consumption. Cold smoking has similar effects to those of drying and salting [262].

A recent investigation in fish farms in Lao PDR supported by Food and Agriculture Organization (FAO) demonstrated that some carp species commonly cultured in fish ponds contained *O. viverrini* metacercariae (unpublished data). This preliminary result suggests that apart from captured fish, culture fish can provide an additional source of infection to consumers and thus urgently need attention control body to ensure food safety.

Control efforts are primarily focused on the reduction and elimination of parasite transmission by ensuring proper food preparation, promoting the development of improved diagnostic techniques, providing chemotherapy, and improving sanitation. A combination of health education, mass treatment, and governmental aid could significantly reduce liver fluke infection. Emphasis on health education should be placed on the younger generation in school as a part of the conventional education curriculum.

Interrupting the life cycle of the parasite has always been regarded as a promising way of disease control, for example application of molluscicides (chemicals that kill snails) to control snail populations [34, 263]. Low concentrations of certain molluscicides (e.g., phenasal, niclosamide) are lethal for infected snails, sublethal for uninfected ones, and, presumedly, nontoxic for other animals [263]. The biggest case against this approach is that it involves interference of the ecosystem which can have dramatic consequences including potential toxicity to fish in rice fields [263, 264]. Economic factors also negate its usage as the application of molluscicides is practical only for small water bodies since the costs of treating big areas are extremely high. Additionally, it has been shown that snail populations are restored in about 5 years, hence repeated treatments are necessary. Therefore, decontamination of aquatic bodies has been abandoned in Russia.

In order to achieve overall, long-term control of liver flukes a multidisciplinary approach is necessary. This must aim at breaking the transmission cycle at the level of the first and second intermediate hosts, i.e., at the level of general hygiene as well as at the fisheries and aquaculture levels. This must also be extended to the food production and distribution industries. Of great significance, is that the population at risk must be educated as to how infection occurs and how it can thus be avoided, and to the risks of infection, particularly the development of CCA, should it occur.

The FAO recognizes the necessity of determining the importance of aquaculture in comparison with capture fisheries in the likelihood of human infection with food-borne trematodes. This is a primary requirement in food safety assurance from aquaculture at both the domestic and international trade levels. Hazard Analysis and Critical Control Point (HACCP) methodologies are already available to assist control approaches at the food production stage; however, more work is required taking into account the economics of the costs accruing through implementation of these approaches.

Thailand is considered the hot spot of opisthorchiasis and CCA and the public health importance is acknowledged. In order to prioritize the health problems associated with liver fluke infection, an estimation of burden of disease (BOD) in Thailand was instigated in 1999 and data are available from 2004 [265].

Generally BOD is based on two measurements, namely, Disability Adjusted Life Year (DALY) and, when accurate mortality data are lacking, estimated number of Years of Life Loss (YLL). DALY is a summary measure of population health for setting priorities since this measure combines both fatal and nonfatal health outcomes. BOD estimates are far from complete, especially in developing countries where resources and budget are not adequate. The BOD study in Thailand has identified a short list of the top 20 diseases based on mortality, DALY and YLL. Of these only the top ten diseases were selected. Liver cancer ranks fifth in males and eighth in females. A total of 27,500 people die every year of liver cancer, while YLL is estimated to be 400,000. Both of these figures show the high significance of opisthorchiasis, and CCA, as a public health problem in Thailand.

Thailand was one of the first nations to initiate a program of liver fluke control. This was based initially on funding supplied by USAID as early as 1950. This was followed up by a Thai government program supported by the Deutsche Gesellschaft für Technische Zusammenarbeit (German Society for Technical Cooperation, GTZ). These programs have been successful in reducing the prevalence of *O. viverrini* infection from approximately 63.6 % in 1984–1987 to 9.6 % in the year 2001 [60]. In spite of this success, a nationwide survey showed that there was still a residual prevalence of 8.7 % in 2009. The northeast of Thailand currently still has the highest prevalence with 16.6 % with northern Thailand at 10 %. Both central (1.3 %) and southern Thailand (0.1 %) have low infection prevalences [15]. These data indicate that although the control programs have been successful in reducing the prevalence, they have not eliminated the infection.

The control programs to date have relied on the use of praziquantel (40 mg/kg) curing (>95% cure rate) infected individuals. Most infected individuals involved in the control programs are now estimated to have only light infections (epg <1,000). Treatment with praziquantel, although it is successful in killing adult worms, does not prevent reinfection. Indeed, evidence is accumulating to suggest that it may compromise the immune system or cause liver complications when used repeatedly for reinfections [266]. This leads us to the conclusion that the current control programs require urgent modification.

Both short-term and a long-term program components should be reassessed. The short-term program requires a modification in the current selective treatment strategy by including traditional methods applied with increased accuracy, as well as molecular diagnostics. Multiple stool samples taken from an individual can be analyses using the Kato–Katz method or the more sensitive the formalin–ethyl acetate method. In addition, the cure rate based on the administration of praziquantel must be regularly monitored. This is particularly relevant given the different genetic groups of parasites present in different areas [20]. Control approaches must also include zoonotic cycles in carnivore reservoir hosts as they will increase in significance as human prevalences decrease.

One of the most important aspects in the long-term strategy involves an education program based on food safely. Current programs have shown that long-term, continuous education is required as raw or partially cooked fish consumption is a deeply rooted, raw attitude in the areas where it occurs [41]. Ziegler et al. [267]

recommend school-based health education for young children in order to imprint the importance of food preparation and hygiene in relation to public health. Such educational programs can be promoted through participatory activities in schools. It is anticipated that the information will not only become part of the child's background knowledge as they grow to adulthood but also be discussed at home during the period of schooling. Such a program should have a major impact at the family, extended family, and village levels. An approach of this intensity and magnitude will have the potential for a long-term impact that is not present with national and international selective treatment strategies.

Moreover, the one health approach recommended by the world Health organization is a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of health care for humans, animals and the environment is applicable for liver fluke control. This helps improving our understanding of the social, economic and ecological dimensions of the liver fluke transmission including opisthorchiasis in Thailand and Southeast Asian countries.

Long-term strategies, and indeed a good deal more research, are needed to overcome the dynamic situation caused by land use and climatic changes either taking place or predicted for the Thailand. Such changes are often coupled with dynamic changes in parasite transmission [268, 269]. Given the public health significance of *O. viverrini* infection, the Thai Ministry of Public Health has recently initiated the "Esan agenda: eradicate the liver fluke to reduce CCA" which is primarily aimed at screening for CCA patients. If the cancer is recognized sufficiently early, curative surgery may lead to an effective cure. Such a strategy, however, does not attack the problem at its roots as comparatively very few people are involved compared with the population at risk based on infection with *O. viverrini*. There are currently at least 26 million people at risk of infection in the north and northeast of Thailand. Any control program aimed at reducing the long-term burden of *Opisthorchis* infection must address this population group as the initiation point for the effective control of opisthorchiasis and its associated CCA.

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Chapter 7
Intestinal Trematode Infections

Rafael Toledo, Carla Muñoz-Antoli, and J. Guillermo Esteban

7.1 Introduction

It is estimated that more than one billion people are at risk of infection with foodborne trematodes and about 56 million people were infected in 2005 [1]. According to the target organ in the definitive host, those trematodes are classified as liver, lung and intestinal flukes. Intestinal trematodes are the largest group and about seven million people are infected worldwide [1]. About 76 of species belonging to 14 families have been recorded infecting humans. Infection commonly occurs when humans eat raw or undercooked foods that contain the infective metacercariae. A variety of aliments are involved in the transmission of intestinal flukes (Table 7.1) and the eating habits are essential to determine the distribution of these parasitic diseases. High incidence of intestinal trematodiasis is strongly associated with populations living near freshwater bodies and the practice of eating raw or undercooked aquatic products. Thus, intestinal trematode infections are commonly considered as tropical diseases with severe endemic foci in Asia, where it is estimated that more than six million people are infected [1]. However, the geographical limits and the population at risk are currently expanding and changing in relation to factors such as growing international markets, improved transportation systems, changes in eating habits in Western countries and demographic changes.

Despite the considerable public health impact and the emerging nature of intestinal trematodiases, these diseases are among the most neglected of the so-called neglected tropical diseases, and they are found predominantly in the world's poorest populations in low-income countries, and, where these diseases are common, they exacerbate poverty. This makes necessary additional efforts to gain a better

R. Toledo (✉) • C. Muñoz-Antoli • J.G. Esteban
Departamento de Biología Celular y Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicent Andrés Estellés s/n, Burjassot, 46100 Valencia, Spain
e-mail: Rafael.Toledo@uv.es; Carla.Munoz@uv.es; Jguillermo.Esteban@uv.es

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Table 7.1 Families of trematodes involved in intestinal human infections with data on the number of species involved, sources of infection and geographical distribution

Family	Number of species cited in humans	Source of infection	Geographical distribution of human cases[b]
Brachylaimidae	1	Terrestrial snails	Oceania
Cathaemasiidae	1	Not known	Asia
Diplostomidae	2[a]	Snakes, frogs, tadpoles	Asia
Echinostomatidae	20	Freshwater fish, frogs, mussels, snails, tadpoles	Africa, Asia, Europe
Fasciolidae	1	Aquatic vegetables, contaminated water	Asia
Gastrodiscidae	1	Aquatic vegetables, crustaceans, molluscs, amphibians	Africa, Asia, Europe
Gymnophallidae	1	Oysters	Asia
Heterophyidae	26	Freshwater fishes	Africa, America, Asia, Europe
Lecithodendriidae	3	Dragonflies	Asia
Microphallidae	2	Shrimps, crabs	Asia
Nanophyetidae	1	Salmonid fishes	America, Europe
Paramphistomidae	2	Aquatic plants	Africa, Asia
Plagiorchiidae	4	Insect larvae	Asia
Strigeidae	1	Not known	Asia

[a]One of them (*Fibricola cratera*) only in experimental infections
[b]Imported cases are not considered

knowledge of these diseases to facilitate their control. In this chapter, we describe the biology, medical and epidemiological features and current treatment and diagnostic tools of the main groups of intestinal flukes and the corresponding diseases.

7.2 Family Brachylaimidae

7.2.1 Background

The family Brachylaimidae contains numerous species of terrestrial trematodes that infect mammals, birds and reptiles [2]. *Brachylaima* is the most representative genus within this family. This is, however, a very problematic genus that includes many poorly known species, for which the description of the adult stage is the only information available. This problem is compounded by the morphological similarity of many of the adult worms. Species of *Brachylaima* follow a three-host terrestrial life cycle [3]. The first and second intermediate hosts of brachylaimids are either the same or two different species of terrestrial snail species. The definitive host can be either a mammal or a bird.

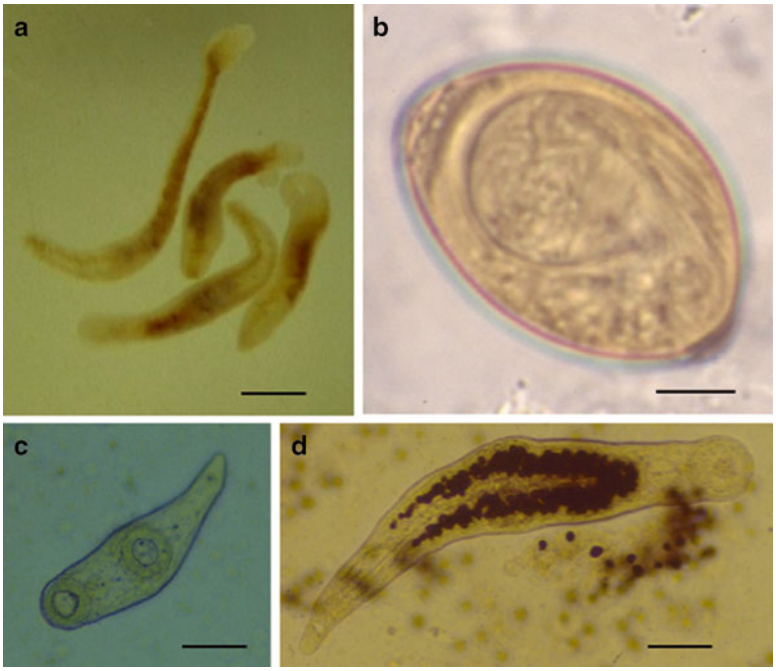

Fig. 7.1 Unstained specimens of *Brachylaima cribbi*: (**a**) adults (scale bar: 1 mm); (**b**) fertile egg (scale bar: 5 µm); (**c**) cercaria (scale bar: 25 µm); and (**d**) metacercaria (scale bar: 500 µm). Photomicrographs courtesy of Andrew R. Butcher

7.2.2 Species Reported in Humans and Geographical Distribution

Humans only have been reported in Australia as an incidental definitive host (three cases) for one species in the family, *Brachylaima cribbi* (Fig. 7.1). The first human infections with this trematode were described in two young children (21 months old) in rural South Australia in whom brachylaimid eggs were seen repeatedly in stools, but no adult worms were recovered. Both infants had been seen eating raw snails [4]. Thereafter, a 78-year-old woman presented with an 18 month history of intermittent diarrhoea was found to be infected with a brachylaimid infection. Examination of her stools revealed the presence of brachylaimid eggs. She lived in a rural area of South Australia and ate raw vegetables which had been contaminated with helicid snails. After treatment with praziquantel a degenerate adult of *Brachylaima* was recovered in her stools [5]. The parasite was identified as *B. cribbi* [6].

Infections in humans usually become chronic and can persist as long as 18 months [4, 5]. Clinical symptoms depend on the parasite load and heavy infections are associated with diarrhoea with offensive stools several times a day, abdominal pain, low-grade fever and fatigue [4, 5]. There are no further studies on the pathology of infections with brachylaimids.

7.2.3 Host–Parasite Relationships and Immunology

The complexity of the life cycles of *Brachylaima* spp. may explain the scarcity of the data on host–parasite relationships in infections with brachylaimids. However, the use of different strains of mice as experimental hosts has allowed for studies on several aspects of the development of these flukes and the immunology of the infections. These studies have been focused on *B. cribbi*. However, there was variability in susceptibility in relation to gender and maturity of mice [7]. Mature female CB57BL/6J mice were significantly more resistant to *B. cribbi* infection that older mature females and adolescent females. These differences in susceptibility were attributed to physiological factors. Butcher, Palethorpe and Grove [7] suggested that sex hormones provided a significant level of protection to *B. cribbi*.

B. cribbi evokes significant antibody responses as determined by indirect ELISA. Butcher, Palethorpe and Grove [8] showed that humoral and/or cellular mediated immunity are important in mediating resistance and influencing the fertility of adult worms. The course of the infection in immunocompetent CB57BL/6J and immunodeficient NOD SCID mice reinfected with *B. cribbi* was assessed. In the case of CB57BL/6J mice there were significant differences in the mean faecal eggs per gram of faeces and worm fecundity, having lower egg counts and reduced fecundity with the challenge infections. In contrast, no significant differences were observed in NOD SCID mice between primary and challenge infections. The immunology of the infection in humans has not been studied.

7.3 Family Diplostomidae

7.3.1 Background

The family Diplostomidae Poirier, 1886 comprises digenean parasites of numerous orders of birds and mammals. Recently, Niewiadomska [9] accepted a total of 41 genera within this family grouped into subfamilies according to host specificity. A total of the 11 genera reported in mammals were included in the subfamily Alariinae Hall and Wigdor, 1960.

In general, species of the Diplostomidae have a three-host life cycle. Fork-tailed cercariae are produced in sporocysts in the gastropod first intermediate host. The cercariae emerge from the snails and penetrate and form metacercariae in fishes, amphibians, molluscs and annelids [10]. Definitive hosts become infected by the ingestion of the second intermediate host harbouring metacercariae. Eggs typically hatch and penetrate the first intermediate host [11]. In some Diplostomidae the life cycle is expanded to incorporate four hosts by inclusion of an unencysted larval stage known as mesocercariae (a form between the cercariae and the metacercariae). In this case the definitive host becomes infected after ingestion of the second intermediate host or the paratenic host harbouring mesocercariae.

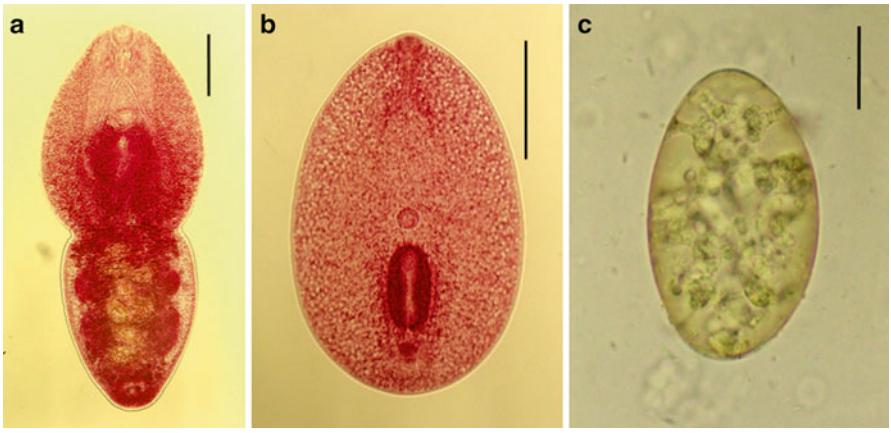

Fig. 7.2 *Neodiplostomum seoulense*: (**a**) adult specimen (scale bar: 250 μm); (**b**) mesocercaria collected from a snake (scale bar: 250 μm); and (**c**) egg (scale bar: 25 μm). Photomicrographs courtesy of Woon-Mok Sohn

7.3.2 Species Reported in Humans and Geographical Distribution

At least members of three genera of Diplostomidae (*Alaria*, *Neodiplostomum* and *Fibricola*) are known to parasitize man. However, in the case of *Alaria* spp., humans serve as a paratenic host harbouring metacercariae in different tissues. Humans become infected after eating tainted frog meat [12]. At the intestinal level, only *Fibricola cratera* and *Neodiplostomum seoulense* serve as parasites of humans.

Human infections with *F. cratera* can be considered as anecdotal since the only one report is an experimental infection of a human volunteer [13]. This species is a parasite of wild mammals in North America. Frogs are the second intermediate host, and snakes act as paratenic hosts [13]. A total of 100 metacercariae were inoculated in a human volunteer producing a patent infection that lasted 40 months. Symptoms of epigastric discomfort, loose stools and flatulence occurred over the first year of infection, but ameliorated thereafter [13]. No further studies have been conducted in relation to the human studies with this species.

N. seoulense, formerly named *F. seoulensis* [14], is a relatively common parasite of humans and animals in Korea [15]. Morphologically it is characterized by a bisegmented body, a tribocytic organ, butterfly-shaped testes and a wide distribution of the vitellaria in the anterior body to the level of the ventral sucker (Fig. 7.2a) [16–18]. The freshwater snails *Hippeutis (Helicorbis) cantori*, *Segmentina (Polipylis) hemisphaerula* and *Austropeplea ollula* serve as the first intermediate host [17, 19, 20]. Second intermediate hosts are tadpoles and frogs harbouring metacercariae, and snakes may act as a paratenic host harbouring the mesocercariae (Fig. 7.2b) [10, 17]. The site of infection in the definitive host is the duodenum, but parasites may extend to the jejunum and ileum in heavy infections [21] (Fig. 7.2).

Human infections with *N. seoulense* have been recorded only in Korea [18]. This species was first implicated when a 25-year-old male suddenly suffered severe gastrointestinal symptoms [22]. Another 26 cases were also reported among Korean soldiers, probably infected during their survival training. In all the cases, the infections were related with a history of consuming improperly cooked snakes and/or frogs [22–24]. Chai and Lee [15] estimated the total number of human cases as 1,000 in the Republic of Korea.

7.3.3 Clinical Manifestations and Pathology

The symptomatology of *N. seoulense* infections in humans has been scarcely studied, and most of the current knowledge is derived from studies on experimentally infected rodents [25].

The clinical manifestation and the pathology induced by *N. seoulense* are markedly related with the worm burden. It has been shown that the severity of the clinical symptoms and the mortality in experimentally infected mice is proportional to the cyst inoculum [26, 27]. In human infections, the symptomatology also has been shown to be dependent on the worm burden. Severe clinical manifestations only were reported in the first patient [22]. This patient rapidly developed epigastric discomfort, fullness and pain and anorexia. Thereafter, diarrhoea, fever and tenderness also appeared. After treatment with bithionol and magnesium purgation, a total of 79 adult worms were collected [22]. In contrast, the remainder human cases had no clinical symptoms. The absence of clinical signs was attributed to the chronic and repeated infection with small amounts of metacercariae and a relatively low numbers of adults in the intestine [23].

The parasite may cause mechanical and chemical damage. Each worm embraces a villous with the forebody which cause injury in the intestinal mucosa [28]. Moreover, the tribocytic organ may pierce the host villi, and secretes alkaline phosphatase which can lyse the villi [29, 30]. The changes induced by *N. seoulense* were studied by Lee et al. [28] in mice and rats. Shortening, widening and fusion of the villi were observed. There was also a reduction in the number of goblet cells in the areas surrounding the worms, capillary congestion, lymphatic dilation and inflammatory cell infiltration including lymphocytes, plasma cells, eosinophils and occasional giant cells. In heavy infections, the changes were extended to the jejunum and gross bleeding was also observed [28]. Recently, Pyo et al. [31] showed that infection with *N. seoulense* downregulates the expression of the neuronal growth associated protein (GAP)-43 in mice suggesting that neuronal damage is induced by the parasite.

7.3.4 Host–Parasite Relationships and Immunology

There are some evidences supporting the existence of mechanisms of host protection against *N. seoulense*. For example, a significant reduction in worm recovery has been demonstrated in secondary infections in rats [32]. In mice, it has been shown

that worm survival is markedly dependent upon the genetic background of the host. The worm survival was markedly higher in BALB/c mice than in the C3H strain [33]. However, little is known about the effector mechanisms of the host immune response in *N. seoulense* infections. Several studies have shown that histamine and macrophages may have an important role on worm rejection. Histamine released by mast cells could facilitate worm expulsion by the increasing of intestinal motility and macrophages have been shown to kill worms in vitro [34, 35]. *N. seoulense* induces a mixed Th1/Th2 phenotype with overexpression of IFN-γ and IL-4 in mice [35]. Antibody responses in mice are characterized by elevated levels of IgG, IgG2a and IgA [35, 36]. Major worm antigens appear to be located in the tribocytic organ, seminal vesicle, caeca and vitelline follicles [37]. Han et al. [36] detected by immunoblotting that the major antigens in the crude extract of adult worms had a molecular weight ranging from 26 to 94 kDa. Kim et al. [38] identified two cystatin-binding cysteine proteinases, weighing 50 and 60 kDa, were recognized by the sera of humans infected with *N. seoulense*.

7.3.5 Diagnosis

Diagnosis of *N. seoulense* is usually done by detection of eggs in faeces using traditional methods. The eggs are ellipsoid, thin-shelled, with an inconspicuous operculum and measuring 86–99 × 55–63 μm [22] (Fig. 7.2c). They can be differentiated from other similar eggs, such as those of *Echinostoma* spp. by their clean shell surface and the absence of abopercular wrinkles at the posterior end [18]. Although immunological or DNA-based methods for diagnosis have not been developed, Kim and colleagues [38] postulated that cystatin-binding cysteine proteinases could be putative antigens for serodiagnosis of *N. seoulense* infections in humans.

7.4 Family Echinostomatidae

7.4.1 Background

The family Echinostomatidae includes digeneans characterized by the presence of a prominent cephalic collar of spines (Fig. 7.3a). Adult echinostomatids are also characterized by the presence of spine-like structures, two post-ovarian testes in tandem located in the posterior part of the body and oral and ventral suckers that are close to each other.

Echinostomatids constitute a heterogeneous group of hermaphrodite trematodes that parasitize, as adult worms, numerous vertebrate hosts of all classes. The typical location is the intestine, though species that parasitize other sites also exist [39]. Echinostomatids follow a three-host life cycle. The first intermediate hosts are aquatic snails in which a sporocyst, two generations of rediae and cercaria develop. Emerged cercariae freely swim and infect the second intermediate hosts, which may

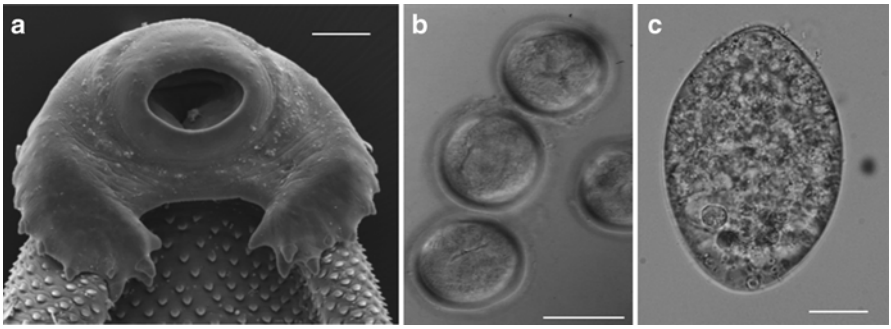

Fig. 7.3 (**a**) SEM microphotograph of the cephalic collar of spines of *Echinostoma* spp. (scale bar: 100 μm); (**b**) metacercarial cysts of *Echinostoma* spp. (scale bar: 100 μm); and (**c**) egg of *Echinostoma hortense* (Photomicrograph courtesy of In Sik Kim) (scale bar: 25 μm)

be several species of aquatic organisms such as snails, frogs, clams and fishes. The definitive host, including humans, become infected after ingestion of the second intermediate host harbouring the encysted metacercariae (Fig. 7.3b). Finally, adults produce eggs that are released with the host's faeces (Fig. 7.3c) [39].

There is considerable confusion in relation to the taxonomy within the Echinostomatidae. This family has been viewed as a monophyletic taxon, though the morphological similarity between its members and the diversity of the criteria adopted by the different authors for the classification have led to its division into an impressive number of taxa [40–43].

7.4.2 Species Reported in Humans and Geographical Distribution

Echinostomes commonly parasitize waterfowls and mammals associated with freshwater habitats. However, the specificity toward the definitive hosts is low determining that humans may become infected. Human echinostomiasis may occur worldwide, though the distribution is usually focal and occasional cases are cited. Most of the cases are reported from East and Southeast Asia in relation to the eating habits in these areas [44]. Metacercariae, the infective stage, are ingested by humans in raw or undercooked freshwater fresh or brackish water molluscs, fishes, crustaceans and amphibians (tadpoles or frogs). As a consequence, the infections are more prevalent in areas where traditional eating practices encourage the consumption of these types of foods. For example, in the Philippines, human echinostome infections are related to eating raw fish or snails dipped in a salt and vinegar mixture, known as *kinilaw* and other methods of local fish preparations. In Cambodia, similar types of local food are commonly eaten which determine the prevalence observed in these areas [45–47]. Moreover, it has been suggested that drinking untreated water containing echinostome cercariae can be a source of human infection [48].

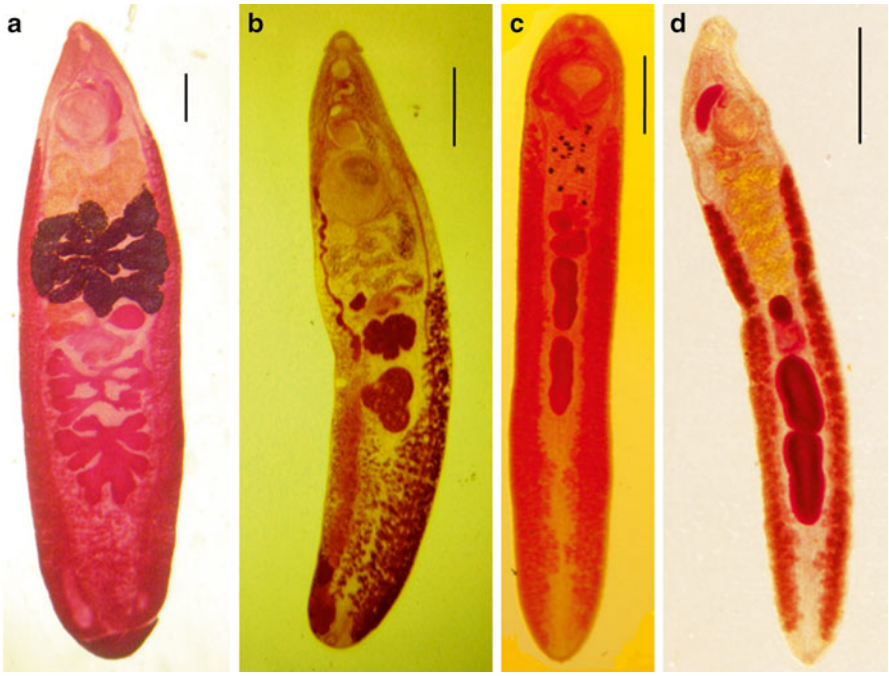

Fig. 7.4 Adult worms of (**a**) *Artyfechinostomum malayanum* (syn. *Echinostoma malayanum*) (Photomicrograph courtesy of Weerachai Saijuntha); (**b**) *Echinostoma hortense* (Photomicrograph courtesy of Woon-Mok Sohn); (**c**) *Hypoderaeum conoideum*; and (**d**) *Echinoparyphium recurvatum* (scale bars: 1 mm)

The number and identity of the species causing human echinostomiasis is uncertain due to the absence of systematic surveys which determine that most of the available information is based on occasional case reports. Furthermore, the problematical taxonomy of the group complicates the situation since misidentifications are common. For example, *Artyfechinostomum malayanum* (Fig. 7.4a) was originally described in Malaysia as *Echinostoma malayanum*. Thereafter, *Artyfechinostomum sufrartyfex* has been considered by several authors as a synonym of *A. malayanum*. However, a recent study has suggested that the synonymy of these species is not valid [49]. Herein both species will be considered as separate taxa. Haaseb and Eveland [50] listed a total of 21 species of echinostomes infecting humans, while Chai [51, 52] compiled 20 species and their identity differed with those reported by Haaseb and Eveland [50]. Table 7.2 compiled the main features of the echinostome species involved in human infections. Further details on the morphology and biology of these species can be found in the works by Chai [18, 52].

The highest incidence of echinostomiasis occurs in Asia, mainly in Southeast Asia. In fact, all the species reported by Chai [52] as causative of human echinostomiasis, with the exception of *Himasthla muehlensi*, have been reported in this area [18].

Table 7.2 Species of Echinostomatidae involved in human infections

Acanthoparyphium tyonense	Korea
Artyfechinostomum malayanum[a]	India, Indonesia, Lao PDR, Malaysia, the Philippines, Singapore, Thailand
Artyfechinostomum oraoni	India
Echinochasmus fujianensis	China
Echinochasmus japonicus	China, Korea, Japan[b], Lao PDR
Echinochasmus jiufoensis	China
Echinochasmus liliputanus	China
Echinochasmus perfoliatus	China, Japan
Echinoparyphium recurvatum	Egypt, Indonesia, Taiwan
Echinostoma angustitestis	China
Echinostoma cinetorchis	Japan, Korea, Taiwan
Echinostoma echinatum	Indonesia
Echinostoma hortense	China, Japan, Korea
Echinostoma ilocanum	Cambodia, India, Indonesia, Malaysia, the Philippines, Thailand
Echinostoma macrorchis	Japan
Echinostoma revolutum	Cambodia, China, Egypt, Indonesia, Lao PDR, Russia, Thailand, Europe
Episthmium caninum	Thailand
Himasthla muehlensi	The USA[c]
Hypoderaeum conoideum	Thailand
Isthmiophora melis	China, Rumania, Taiwan

[a]Syn. *Echinostoma malayanum*
[b]Experimental infection
[c]Imported infection

A total of nine species have been reported infecting humans in China. Most of the cases occurred in the provinces of Fujian, Guandong, Yunnan, Anhui and Hubei. The mode of transmission could be the use of human excrements collected from latrines for fertilization of fish pond in these areas. Among the five members of the genus *Echinochasmus* reported in China (*E. fujianensis*, *E. perfoliatus*, *E. jiufoensis*, *E. liliputanus* and *E. japonicus*), *E. fujianensis* is the most common. In several areas, the prevalence among residents reached the 3.2 %, with the highest rates in children from 3 to 15 years [53, 54]. The prevalence of *E. japonicus* in some counties of Guandong and Fujian was 4.9 %, whereas the prevalence of *E. perfoliatus* in these areas was lower (1.8 %) [54]. Interestingly, prevalence of 13.4 % of *E. liliputanus* was reported in Anhui [55]. A single case of human infection of *E. jiufoensis* has been reported during an autopsy of a 6-month-old girl in Guangzhou [56]. A total of three members of *Echinostoma* (*E. revolutum*, *E. hortense* and *E. angustitestis*) have been also detected in China. *E. revolutum* has been reported in Yunnan and Guandong provinces and two cases of *E. japonicus* infection were recorded in Fujian [52, 53]. In Liaoning province, six patients with hepatitis were found to be infected with *E. hortense* (Fig. 7.4b) [57]. More anecdotal is the infection with *Isthmiophora melis* [52].

A total of five species of echinostomes have been reported in Indonesia infecting humans (*A. malayanum*, *Echinoparyphium recurvatum*, *Echinostoma echinatum, E. ilocanum* and *E. revolutum*), mainly focused in the major Islands (Sumatra, Java and Sulawesi) [44]. A high prevalence of *E. echinatum* was detected from 1937 to 1956 in the Lake Lindu Valley (Sulawesi). The average prevalence was 43 %, but it reached 96 % in some areas [58]. Human infections appeared to be due to the habit of eating raw or insufficiently cooked lake mollusks, particularly bivalves (*Corbicula* spp.). Elimination of *Corbicula* clams from the diet significantly reduced the prevalences and a survey in the 1970s only revealed occasional presence of eggs [59]. In other areas an increasing number of cases were reported in relation to the growing popularity of exotic food available in Korean and Japanese restaurants in Indonesia [44, 60]. Moreover, in the Indonesian area of Borneo (West Kalimantan), echinostome eggs are frequently observed in faces of the residents [61]. Recently a prevalence of 6 % has been found in southern Lao PDR [62].

A total of four species have been recorded in Japan (*Echinochasmus perfoliatus*, *Echinostoma cinetorcis*, *E. hortense* and *E. macrorchis*), Korea (*A. tyosenense*, *E. hortense*, *E. cinetorchis* and *E. japonicus*), Thailand (*E. revolutum*, *E. ilocanum*, *Episthmium caninum* and *Hypoderaeum conoideum*), Lao PDR (*A. malayanum, E. japonicus*, *E. revolutum* and *Euparyphium* sp.) and India (*A. malayanum*, *A. oraoni*, *A sufrartyfex* and *E. ilocanum*). However, some of these species only have been found occasionally. For example, in Japan only one case has been detected for *E. perfoliatus* and *Echinostoma macrorchis* in Japan [63, 64], while three cases were reported for *Episthmium caninum* in Thailand [65, 66]. In the case of *A. tyosenense* and *A. oraoni* a total of 10 and 20 cases were reported in Korea and India, respectively [18, 67, 68]. Human infections with *E. cinetorchis* were first reported in Japan and the in the Republic of Korea [69–73]. Human infections with *E. hortense* have been reported both in Japan and Korea [52]. An endemicity of this echinostome species has been reported among residents of Cheongsong-gun (Republic of Korea) with 22 % of prevalence [73]. *Hypoderaeum conoideum* (Fig. 7.4c) in humans only has been recorded in the northeast area of Thailand with a prevalence of 55 % among the residents [74].

A recent study in Lao PDR among 2,074 residents in riparian villages along the Mekong River in the Khammouane province demonstrated the presence of echinostome eggs in the faeces of 1.1 % of residents. A total of 55 specimens belonging to *E. revolutum*, *A. malayanum*, *E. japonicus* and *Echinoparyphium* sp. were recovered after treatment and purgation [75]. Recently, Sayasone and colleagues [76] detected three human cases of *E. japonicus*. Three species (*E. revolutum*, *E. recurvatum* and *I. melis*) were reported in humans from Taiwan [77] and the prevalence of infection varied from 11 to 65 % in some areas [78]. In Cambodia, *E. revolutum* and *E. ilocanum* have been recently reported. Sohn and colleagues [46] demonstrated a prevalence of 1.0 % of *E. ilocanum in* the Oddar Meanchey province. Sohn and colleagues [47] determined a prevalence of 7.5–2.4 % of *E. revolutum* in children from the Pursat province. In the Philippines *E. ilocanum* and *A. malayanum*

infect humans with an overall prevalence of 3 %, though it reached 44 % in some areas [79]. In Malaysia and Singapore, only human infections with *A. malayanum* have been recorded [52].

The number of known reports outside of Asia is very limited. Only four species have been occasionally recorded. A human infection with *I. melis* was detected in 1916 in a diarrheic patient in Rumania [80] and *E. recurvatum* (Fig. 7.4d) and *E. revolutum* have been sporadically reported in Egypt and Russia, respectively [52]. Imported cases in the USA also have been reported. *H. muehlensi* was originally described on the basis of five adult specimens from a German patient who lived in Colombia and travelled to New York where he had eaten raw clams [51]. DeGirolami and Kimber [81] recorded *Echinostoma* spp. from Asian refugees in the USA. Poland and colleagues [82] reported 18 cases of imported echinostomiasis among a total of 20 American tourists to Kenya. A total of ten of the patients showed moderately severe abdominal cramps and loose or watery stools.

7.4.3 Clinical Manifestations and Pathology

The clinical symptoms of human echinostomiasis may be more severe than those produced by other intestinal trematodes, though the clinical features greatly depend on the parasite load [44, 52]. Human morbidity and mortality due to echinostomiasis depend on a number of factors such as a prolonged latent phase, limited acute phase and similarity with the symptomatology of other intestinal pathologies and, even, the existence of asymptomatic presentations [44].

Epigastric and abdominal pain, easy fatigue, diarrhoea and weight loss are the most common symptoms in human echinostomiasis [15, 25, 44]. Besides these, other symptoms also can be detected. Several studies have shown that patients infected with *E. hortense* additionally suffered acid belching, anorexia, headache, nausea and vomiting, and urinary incontinence [83–86]. Peripheral blood eosinophilia has been commonly reported [73, 82]. However, the levels of eosinophilia appear to be markedly dependent upon the worm load and ranged from 2 to 24 % as demonstrated in *E. hortense* infections [73].

The intestinal pathology induced by echinostomes in humans has been poorly studied and the most of the known data were obtained by gastroduodenal endoscopies in *E. hortense* infections. In general, the patients showed mucosal erosion and ulceration, bleeding in the stomach and the duodenum, signs of chronic gastritis and infiltration of inflammatory cells including neutrophils [84, 85, 87]. Interestingly, stage IIc or stage III early gastric cancer was determined by gastroduodenal fiberscopy [84].

Further details on the pathology of echinostome infections were obtained using laboratory rodents. The pathological effects of echinostomiasis are dependent on a wide variety of factors including the echinostome species, the host species and the intensity of the infection [25]. Echinostomes provoke inflammatory responses in the attachment sites. The surrounding areas showed marked dilation, erosion of the villi and lymphocytic infiltration [39, 88–91]. Moreover, goblet cell hyperplasia, neutrophilia and

infiltration of inflammatory cells, together with crypt hyperplasia with increased mitotic rates also occur [90, 92]. Cellular infiltration of lymphocytes, eosinophils and plasma cells also were observed in the lamina propria and submucosa [90, 93].

7.4.4 Host–Parasite Relationships and Immunology

Immunology of echinostomiasis has been extensively studied in laboratory rodents. It has been shown that the rodent hosts are able to express various types of resistance to echinostome infections which suggest that the parasites can be spontaneously expulsed or, in contrast, develop a chronic infection depending on the host–parasite combination [25, 39, 94].

Although it has been shown that echinostomes alter several immunological parameters the role of these alterations in the course of the infections remains unclear [39, 52, 95]. *Echinostoma* spp. induces changes at the cellular level and in the expression of certain glycoconjugates in the intestinal mucosa [25]. Mastocytosis, eosinophilic infiltration and increase in the goblet cells and mast cell populations have been commonly observed, though there are conflicting data in relation to their effect on the echinostome infections [90–92, 96–102]. Furthermore, several studies have suggested that the alterations of the terminal sugar of the mucins produced by goblet cells may regulate the worm expulsion [92, 99, 101].

Apart from the changes in cell populations, echinostomes also may induce energic antibody responses [95]. However, they do not appear to alter the course of the infections. For example, *Echinostoma caproni* induces elevated responses of IgM, IgG, IgG1 and IgG3 in the serum of mice in which the parasite survives for more than 25 weeks [103–105]. In contrast, low levels of antibodies were detected in the serum of *E. caproni*-infected rats concomitantly with an early expulsion of the worms [105]. At the intestinal level, increases in the IgM, IgA, IgG1 and IgG2a levels were detected in mice [105]. The response against *E. hortense* is characterized by an elevation of the serum levels of IgG1, IgE and IgA [106]. The target antigens of these responses in *E. caproni* infections were studied by Sotillo and colleagues [107]. A total of four proteins (enolase, actin, HSP-70 and aldolase) appeared to be the major antigens in the *E. caproni* adult worms.

The cytokine profile in echinostome infections has been poorly studied. However, new information about this topic has been obtained in recent years. The production of cytokines in the splenocytes of mice infected with *E. hortense* has been studied [102, 106, 108]. These studies detected a predominance of the Th2 responses with elevated expression of IL-4 and IL-5. In *E. caproni* infections, Brunet and colleagues [109] observed an elevated production of IFN-γ in the spleen cells of experimentally infected mice. Comparative studies using hosts of different compatibility with *E. caproni* have provided further insight in the responses determining the course of the infection. The development of chronic infections appears to be related with the development of local Th1 responses, whereas the early worm rejection is mediated by the development of a biased Th2/Th17 local phenotype [110, 111].

7.4.5 Diagnosis

Clinical diagnosis of human echinostomiasis is difficult since the infection may remain unapparent for a while or the symptoms, if present, are often unspecific. Laboratory diagnosis is based on the demonstration of eggs in faeces. The eggs are oval, yellowish, thin shelled with an operculum at the anterior end which may be difficult to see. The size of the most human infecting echinostomes are in the range 66–145 × 43–90 mm, though the eggs of several species may fall outside this range [52]. However, the difficulty entailed in the specific characterization of the eggs strongly recommends recovering the adult worms.

Although immunological methods for the diagnosis of human echinostomiasis have not been developed, several studies using laboratory rodents have shown that conventional ELISA and capture ELISA may be promising methods for the detection of human infections [104–106, 112–115].

7.5 Family Fasciolidae

7.5.1 Background

The family Fasciolidae comprises large trematodes that inhabit the liver and bile ducts but members of two genera (*Fasciolopsis* and *Parafasciolopsis*) are intestinal parasites. The life cycle of the members of this family includes a metacercarial stage that encysts on pasture and other vegetation.

7.5.2 Species Reported in Humans and Geographical Distribution

Fasciolopsis buski is the only fasciolid species reported infecting the intestine of humans. This is the largest trematode parasitizing humans (8–10 × 1–3 cm) (Fig. 7.5a) and a common intestinal parasite of humans and pigs in Asia [12].

In humans, *F. buski* inhabits the duodenum and the jejunum, though it can extend to almost the complete intestine and, even, the stomach in heavy infections. Adult worms produce over 25,000 eggs every day. Unembryonated eggs are discharged into the intestine and stool. Eggs become embryonated in water and release miracidia, which invade a suitable snail intermediate host. Several species of genera *Segmentina* and *Hippeutis* serve as intermediate hosts. In the snail the parasite undergoes several developmental stages (sporocysts, rediae and cercariae). The cercariae are released from the snail and encyst as metacercariae on aquatic plants such as water chestnut, water caltrop, lotus, bamboo and other edible plants. The mammalian final host becomes infected by ingesting metacercariae on the aquatic plants.

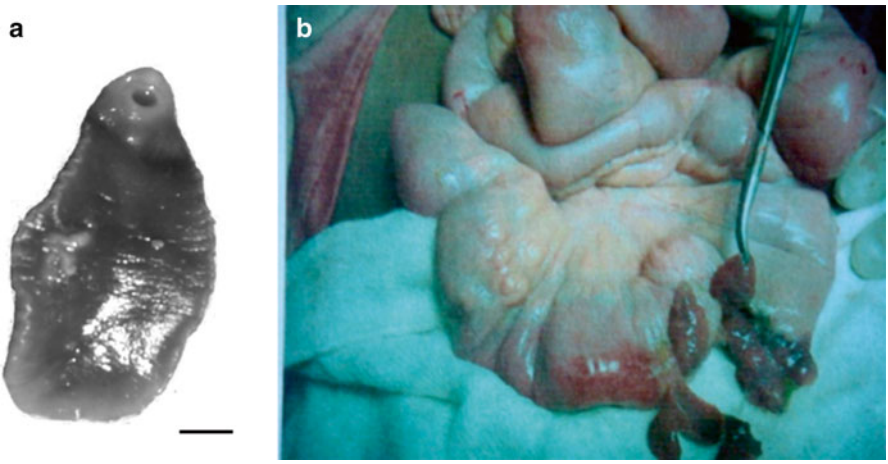

Fig. 7.5 (**a**) Unstained specimen of *Fasciolopsis buski* (scale bar: 0.5 mm) (Photograph courtesy of Deepak Bagga); (**b**) *Fasciolopsis buski* emerging from perforation site of the ileum (scale bar: 5 μm) (Photograph courtesy of Umesh Chandra Singh)

After ingestion, the metacercariae excyst in the duodenum in about 3 months and attach to the intestinal wall. There they develop into adult flukes in approximately 3 months, attached to the intestinal wall of the mammalian hosts (humans and pigs). The adults have a life span of about 1 year.

Fasciolopsiasis is confined and endemic in Far East and Southeast Asia [12]. The disease occurs focally and is linked to freshwater habitats and is associated with common social and agricultural practices and promiscuous defecation [116]. Humans commonly become infected by eating raw or undercooked aquatic plants, but infection can be also contracted by the drinking or use of contaminated water or processing of the water-derived plants, e.g. using teeth to peel plants [12, 117, 118]**.** Fasciolopsiasis can be aggravated by social and economic factors such as poverty, malnutrition and uninspected and poorly sanitized food markets [116]. In fact, differences in the incidence within the same area have been found in relation to the economic status, educational background or the standard of health and/or way of life [119]. The infection predominantly occurs in children and the worm burden may exceed 800 flukes/child [117, 118]. In foci of transmission, the prevalence of infection in children ranged from 10 to 60 % in countries such as China, Taiwan, India, Bangladesh or Thailand [118, 120–123]. Pigs may play an important role in the transmission of the parasite. The pig is the main reservoir and different infection rates have been reported, ranging from 10 % in China to 52 % in Taiwan [116]. Fresh aquatic green fodder and untreated water used to raise pigs appear to be the source of infection in farm animals [118, 124].

In China, infections have been reported in ten provinces reaching 85 % of prevalence in some of them [118]. In Bangladesh, the prevalence in schoolchildren in an endemic focus reached 50 % [117]. In India, 60 % of people were found to be

infected and harbouring 1–57 worms in Assam [125]. In Thailand, the central area is the main endemic area with an estimation of 20 % of infected people [126]. Infection has been also reported from Lao PDR, Vietnam, Cambodia, Indonesia, the Philippines, Kampuchea, Burma and Malaysia [116]. Moreover, imported cases from the Far East have been recorded in Korea, Japan, the USA, Venezuela, Australia, Guatemala, Israel and Cuba [116].

Although several studies showed that human fasciolopsiasis decreased in the 1980s, this tendency was not maintained since people continue eating raw vegetables. Recently, Quang and colleagues [127] suggested the possibility of an emergence of human fasciolopsiasis in Lao PDR.

7.5.3 Clinical Manifestations and Pathology

Clinical symptoms in *F. buski* infections in humans are related to parasite load and can be fatal in heavy infections [117, 118]. In light infections, symptomatology may include anaemia, eosinophilia, dizziness and gastrointestinal symptoms. In moderate and heavy infections there may appear severe epigastric and abdominal pain, diarrhoea or bowel obstruction, nausea, acute ileus, anasarca, and eosinophilia and leucocytosis [12, 117]. Eventually, it may cause intestinal perforation (Fig. 7.5b) [128].

Moreover, adult flukes damage the intestinal mucosa and cause extensive duodenal erosions, ulceration, haemorrhage, abcesses and catarrhal inflammation. Absorption of toxic and allergic worm metabolites causes ascitis, general oedema and facial oedema [12, 119].

7.5.4 Diagnosis

Laboratory diagnosis is based on the demonstration of eggs in faeces. The eggs are ellipsoidal, operculated, non-embryonated, measuring 130–140 × 80–85 mm [117]. Immunological or molecular methods have not been developed.

7.6 Family Gastrodiscidae

7.6.1 Background

The family Gastrodiscidae contains trematodes relatively large, i.e. approximately 8–14 mm in length. They are intestinal parasites of terrestrial mammals, including man, and have been distinguished by a dorsoventrally flattened body, which has the appearance of being divided into two parts.

7.6.2 Species Reported in Humans and Geographical Distribution

Only one species of Gastrodiscidae, *Gastrodiscoides hominis*, has been found infecting humans. Adult of *G. hominis* are large flukes (8–14×5.5–7.5 mm). Moreover, this species is characterized by a short and cylindrical anterior part, large and discoidal posterior part, subterminal pharynx, testes lobed and in tandem, a post-testicular ovary, an ascending uterus and a ventral genital pore.

The life cycle is not completely understood. Adult worms inhabit the caecum and colon of humans, pigs, small, monkeys and other mammals. Unembryonated eggs are laid and, in a freshwater environment, the miracidium hatchs and infects the first intermediate host. Only the snail *Helicorbis coenosus* is known to act as the first intermediate host. After the development of mother and daughter rediae, the cercariae emerge and encyst in aquatic plants, snails, tadpoles, frogs or crayfish. Definitive host becomes infected after swallowing metacercariae with tainted vegetables or raw or undercooked crustaceans, molluscs or amphibians [129].

G. hominis has been detected infecting humans in India, Burma, Pakistan, Myanmar, Vietnam, the Philippines, Thailand, China, Kazakhstan, and Indian immigrants in Guyana, Zambia, Nigeria and the Volga Delta in Russia [116]. Although *G. hominis* is mainly a parasite of pigs, high prevalence in humans has been detected is some areas. For example, Buckley [125] detected a prevalence of 41 % in children from Assam (India).

Pathology and symptomatology of humans *G. hominis* infections are not well known. Heavy infections may induce headache, epigastric pain and diarrhoea that may be a reaction to metabolites released by the parasite [130]. Acetabulum of the adult worm is found to drag the mucosa like a plug causing inflammation [116, 129]. In human infection a picture similar to that detected in pigs might be expected. Surface desquamation, infiltration with eosinophils, lymphocytes and plasma cells appear in sections of the lesions caused by the fixation of the parasites to the mucosa. Hypersecretion of mucus and necrosis of the mucuos glands are also observed. The lamina propria shows infiltration of eosinophiles, lymphocytes, macrophages and plasma cells [116, 129].

Diagnosis of human gastrodiscoidiasis is feasible by detection of eggs in faeces. Egg size is about 4–6×5–10 mm and it is deep yellow, operculated, non-embryonated, measuring about 150×70 mm [129].

7.7 Family Gymnophallidae

7.7.1 Background

Gymnophalloidiasis is the intestinal infection caused by *Gymnophalloides seoi*, belonging to the family Gymnophallidae. This family consists of a small group of marine digeneans. Most members use mollusks as the first intermediate host, the

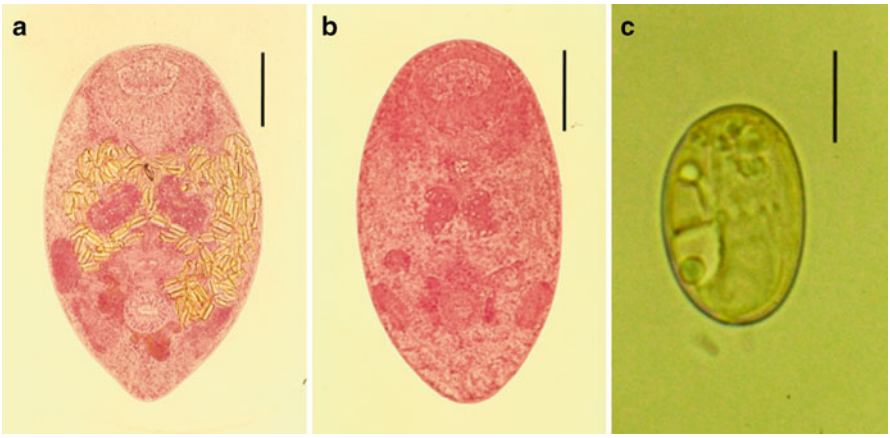

Fig. 7.6 *Gymnophalloides seoi*: (**a**) adult worm (scale bar: 100 μm); (**b**) metacercaria collected from a naturally infected oyster (scale bar: 100 μm); and (**c**) egg (scale bar: 10 μm). Photomicrographs courtesy of Woon-Mok Sohn

metacercariae never encysts and are usually parasitic in bivalves. With rare exceptions, charadriiform and anseriform birds are the definitive host which becomes infected after ingestion of the second intermediate host harbouring the metacercariae [131]. Although a total of five genera are accepted in the family, only *G. seoi* has been found infecting humans.

7.7.2 *Species Infecting Humans and Geographical Distribution*

Human infections with *G. seoi* only have been recorded in the Republic of Korea [12, 18, 132]. This fluke was first discovered in 1988 in a Korean woman suffering pancreatitis and gastric discomfort [133, 134]. This woman lived in the southwestern coastal island of Aphae in the Shinan County, which was subsequently found to be an endemic area. Then, more than 25 villages in western and southwestern coastal islands and 3 mainland coastal villages have been reported as endemic foci with prevalences ranging from 9.3 to 57.9 % [12, 51, 135–139].

The adult parasite is small (0.4–0.5 × 0.2–0.3 mm) and characterized by a large oral sucker and small ventral sucker, short caeca and a unique ventral pit (Fig. 7.6a) [134]. The first intermediate host remains unknown but the second intermediate host was found to be the oyster *Crassostrea gigas* [140, 141]. Humans become infected by eating raw or undercooked oysters harbouring the metacercariae (Fig. 7.6b). The parasite inhabits the small intestine, though a case of mucosal tissue invasion has been reported. An adult worm was incidentally found in a surgical pathology specimen of the lymph node around the colon of a 65-year-old Korean patient [142].

The symptomatology induced by *G. seoi* may vary greatly between individual patients. Gastrointestinal discomfort and indigestion are the most common symptoms. Moreover, fever, anorexia, weight loss, easy fatigue and weakness can also appear [134]. It has been also suggested that gymnophalloidiasis increases the levels of amylase in the serum and urine. In two patients, the gymnophalloidiasis was accompanied by diabetes mellitus suggesting a relationship between both diseases [140].

7.7.3 Host–Parasite Relationships and Immunology

Apparently, *G. seoi* induces less pathological damage than other intestinal trematodes. Adult worms inhabit the duodenum and jejunum where they are attached by grasping or pinching the epithelial layer with the oral sucker. This induces villous atrophy and crypt hyperplasia with inflammation of the villous stroma and the crypt [25, 143]. A marked goblet cell hyperplasia dependent on CD4+ T-helper cells along the villous epithelia was observed, mainly in the jejunum [143, 144]. These changes were resolved at 2–3 weeks post-infection. However, it is suspected that *G. seoi* invades the pancreatic duct of humans which may cause further complications which may require medical attention. Acute pancreatitis was diagnosed in one patient of gymnophalloidiasis and diabetes mellitus was accompanied in other two patients [133, 140].

In mice, *G. seoi* infections are spontaneously expelled within 3 weeks post-infection but the course of the infection differs depending on the strain of mice [145, 146] indicating a genetic background in the resistance to infection. Moreover, comparative studies on the development of *G. seoi* in immunocompetent and immunosuppressed mice indicated that the differential susceptibility is dependent on the host immune response [145–147]. Little is known about the immune effector mechanisms responsible for the short-term parasitism of *G. seoi* observed in mice. However, the observation of a strong proliferation of goblet cells previous to the worm expulsion in *G. seoi*-infected mice suggested that this can be one of the effector mechanisms involved in worm rejection [143]. However, recent studies have shown that T-cell independent mechanisms also mediate in the expulsion of *G. seoi*. In this context, alteration of the mucin quality with changes in the terminal sugar chain and elevated levels of IL-4 and IL-5 expression appears to be of importance [144]. Furthermore, it has been demonstrated that *G. seoi* antigens upregulate Toll-like receptors and mucin related genes (MUC2) in human intestinal epithelial cells via IFN-γ [148].

7.7.4 Diagnosis

Diagnosis can be done by detection of eggs in the faeces. However, the detection and identification may be difficult because of (1) low-laying capacity of the parasite and (2) identification of *G. seoi* eggs. The daily egg output/worm is estimated in 2–84 in

the human host [134] which makes difficult the isolation of the eggs unless heavy infections occur. Another difficulty entailed with the diagnosis of the gymnophalloidiasis is the identification of the eggs. They are very small (approximately 23 × 13 μm) and thin and transparent shelled (Fig. 7.6c) [149]. Due to their small size, the eggs can be overlooked by an inexpert analyst or misdiagnosed as a bubble or other artefacts. Moreover, differential diagnosis with other digeneans may be difficult [149].

7.8 Family Heterophyidae

7.8.1 Background

Heterophyasis is caused by the members of the family Heterophyidae. This family contains small egg-shaped trematodes with infective metacercariae that are usually encysted in fish second intermediate hosts. The heterophyids are characterized by the possession of a gonotyle or genital sucker [150]. This author accepted 13 subfamilies within the family which may be differentiated on the basis of the body morphology, presence of a circumoral crown of spines, extension of the vitellaria and uterus, morphology of the testes and location of the genital pore.

The definitive host becomes infected by eating raw or poorly cooked fish harbouring metacercariae. The adult worms live between the villi of the anterior region of the small intestine and release fully embryonated eggs into water. The eggs are then ingested often by littorine snails (species of *Melanoides*, *Semisulcospira* and others), and hatch within the snail's intestine. Intramolluscan development comprises sporocyst and redial stages and cercariae are released into the water where they typically penetrate shrimps or shore-fish, such as cunners, gudgeon and charr, and encyst on the surface or muscles of the fish. Metacercariae may remain viable for years [12, 25, 151].

7.8.2 Species Infecting Humans and Geographical Distribution

A total of 26 species of heterophyids have been reported infecting humans (Table 7.3). Humans become infected by eating fish or shrimps harbouring viable metacercariae, which mature into adults in 5–10 days. Adult heterophyids attach and live embedded the intestinal mucosa producing embryonated eggs that reach water reservoir due to the lack or non-use of sanitary latrines which allow for the maintenance of the life cycle.

Probably, *Heterophyes heterophyes* and *Metagonimus yokogawai* (Fig. 7.7a, b) are the most relevant species from the standpoint of human disease. *H. heterophyes* was first discovered in an Egyptian and is a common parasite in the Nile Delta

Table 7.3 Species of Heterophyidae involved in human infections

Genus	Species	Geographical distribution of human cases
Apophallus	*A. donicus*	The USA
Ascocotyle	*A. longa*[a]	Brazil[a]
Centrocestus	*C. armatus*	Japan, Korea
	C. caninus	Thailand
	C. cuspidatus	Egypt
	C. formosanus	Japan, Taiwan, Vietnam
	C. kurokawai	Japan
Cryptocotile	*C. lingua*	Greenland
Haplorchis	*H. pleurolophocerca*	Egypt
	H. pumilio	China, Egypt, Korea, Lao PDR, the Philippines, Taiwan, Thailand, Vietnam
	H. taichui	China, Lao PDR, the Philippines, Thailand, Vietnam
	H. vanissimus	The Philippines
	H. yokogawai	China, Egypt, India, Indonesia, Lao PDR, Malaysia, the Philippines, Taiwan, Thailand
Heterophyes	*H. dispar*	Korea[b]
	H. heterophyes	Egypt, Iran, Korea[b], Sudan
	H. nocens	China, Japan, Korea
Heterophyopsis	*H. continua*	Japan, Korea
Metagonimus	*M. miyatai*	Japan, Korea
	M. takahashii	Korea
	M. yokogawai	All Far East, India, Balkan states, Israel, Siberia, Spain
Procervum	*P. calderoni*	Africa, China, the Philippines
Pygidiopsis	*P. summa*	Japan, Korea
Stellanchasmus	*S. falcatus*	Hawaii, Japan, Korea, Palestine, the Philippines, Thailand, Vietnam
	S. pseudocirratus	Hawaii, the Philippines
Stictodora	*S. fuscata*	Korea
	S. lari	Korea

[a]Referred to as *Phagicola* sp. by Chieffi and colleagues [205] in Brazil
[b]Imported cases

around the lakes Manzala, Borollos and Edco [54]. In the period 1984–1991, the prevalence in the five governorates of the Delta ranged from 0.001 to 1 % and the population at risk was estimated as 933,000. People became infected by consuming recently salted or insufficiently baked fish [152]. In the villages of Khuzestan (Iran) the prevalence in humans ranged from 2 to 24 % [54]. In Asia, several foci have been identified but this parasite could be confused with *Heterophyes nocens* [18]. Imported cases in Japan were reported from people who returned from Egypt, Saudi Arabia and Sudan [18]. In Western Europe, human infections with *H. heterophyes* have been recorded sporadically. For example, Martínez-Alonso and colleagues [153] described the infection of a woman after eating raw fish in a Chinese restaurant in Spain. *M. yokogawai* is probably the most common intestinal fluke infecting humans in the Far East. It has been recorded in China, Japan, Korea,

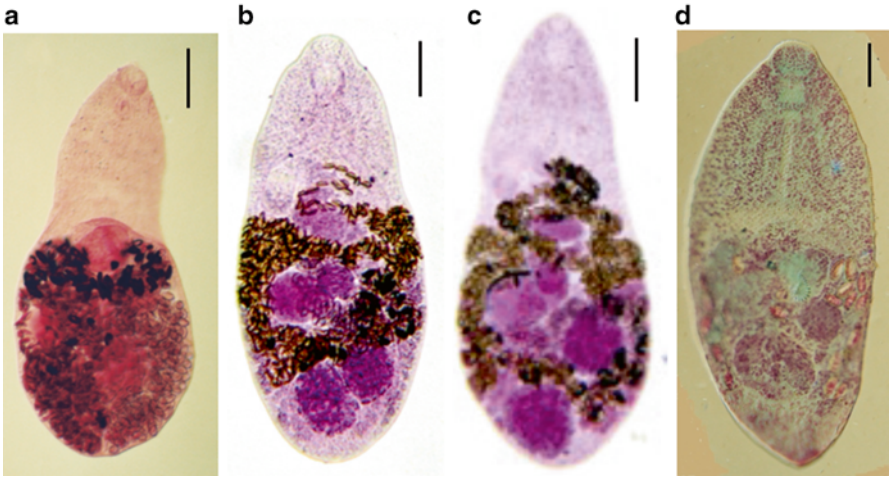

Fig. 7.7 Adult worms of (**a**) *Heterophyes heterophyes* (scale bar: 50 μm); (**b**) *Metagonimus yokogawai* (scale bar: 150 μm) (Photomicrograph courtesy of Hideto Kino); (**c**) *Metagonimus miyatai* (scale bar: 75 μm) (Photomicrograph courtesy of Hideto Kino); and (**d**) *Haplorchis taichui* (scale bar: 100 μm)

Taiwan and Indonesia [54]. In Korea, the prevalence reached 4.8 % [154], with special incidence in riverside communities where raw sweetfish is commonly eaten [155]. In particular, the eastern parts of Gyeongbuk province, Gangjin-gun (Tamjin River), Boseong (Boseong River), Hadonggun (Seomjin River) and Samcheok (Osip Stream) were the most important endemic areas with a prevalence ranging from 20 to 70 % [15, 156]. In China, *M. yokogawai* is distributed in Guandong, Anhui, Hubei and Zheijang [18]. In India, only three cases have been reported, The first two cases occurred with a Muslim family in upper Assam [157] and more recently an isolated case was found in 6-years old female in New Delhi [158].

Another ten species of Heterophyidae have been recorded in the Republic of Korea (Table 7.3). *H. nocens* commonly infects humans, though the identity of this species is confused and may be a synonym of *H. heterophyes*. Prevalence ranging from 17 to 70 % was detected among residents in the southwestern coastal areas [159]. Human infections with *Metagonimus miyatai* (Fig. 7.7c) have been detected in people from Gum, Namhan and Hantaan rivers [18]. In the upper reaches of Namhan River have also recorded human infections with *Metagonimus takahashi* [160]. *Pygidiopsis summa* is widely distributed in the western and southwestern coastal areas of Korea [161]. Recently a prevalence of 4.9 % of heterophyid infections was described in South Jeolla province [139]. Sporadic cases of *Centrocestus armatus* (1 case), *Haplorchis pumilio* (1) *Heterophiopsis continua* (10), *Stellantchasmus falcatus* (4), *Stictodora fuscata* (14) and *S. lari* (6) have been also recorded [15, 18, 162]. Furthermore, two imported cases of *Heterophyes dispar* infections were reported in men returning from Saudi Arabia [163]. A recent study demonstrated that intestinal trematodiasis constituted an unrecognized food safety risk in Vietnam and heterophyids were the most

prevalent intestinal trematodes. Elevated prevalence was detected for *H. pumilio*, *Haplorchis taichui* (Fig. 7.3d), *Haplorchis yokogawai* and *S. falcatus* in several areas [164].

In Japan, seven additional species of heterophyids have been recorded infecting humans (Table 7.3) [18, 54]. Of particular interest are the endemic foci of *H. nocens* detected in Shizouka prefecture [165]. In Lao PDR and Vietnam, several studies have shown the *Haplorchis* spp. (*H. pumilio*, *H. taichui* and *H. yokogawai*) and *Centrocestus formosanus* have a significant impact among residents [137, 164, 166, 167].

7.8.3 Clinical Manifestations and Pathology

Commonly, low-grade infections with heterophyids are of no clinical consequences. Heavy infections are associated with diarrhoea, mucus-rich faeces, abdominal pain, dyspepsia anorexia, nausea and vomiting [25]. Symptoms frequently subside spontaneously after 1 month, although the flukes may remain for more than 1 year, and only episodic diarrhoea may appear [168]. Several complications are associated with heterophyasis. Anaphylaxis was developed by a woman after infection with *H. heterophyes* after eating raw fish in Spain [153]. Occasionally worm eggs may enter the circulatory system via the crypts of Lieberkhün which may be fatal [130, 168]. Eggs can be transported to other organs and they have been found in cardiac muscles in autopsies, spinal cord or brain, where they become trapped and elicit granulomatous lesions and fibrosis. Signs of heterophyid myocarditis may include subepicardial oedema, epicardium haemorrhage, fragmentation of muscle fibres, cardiac enlargement, embolism of the capillaries, cough, dyspnea, cyanosis, fatigue, oedema and ascites, palpitation, loss of reflexes and abnormal heart sounds. Eggs or worms in the spinal cord or brain may cause neurological disease, transverse myelitis and loss of sensory and motor function [168]. Recently, *H. taichui* has been identified as a possible etiologic causative agent of irritable Bowel syndrome-like symptoms [169].

The adult worms parasitize mainly the duodenum, though they may extend more distally in heavy infections [170]. The pathology induced by heterophyids has been mainly studied in laboratory animals. Adult worms live embedded in the intestinal mucosa causing villous atrophy, hypertrophy of crypts of Lieberkhün, enlargement of mesenteric lymph nodes and inflammatory responses with cell infiltration [25]. In humans, Chi and colleagues [171] studied the pathology of a human case of metagonimiasis. The parasitism was incidentally detected in an intestinal segment that was removed surgically for treating intestinal perforation related to a malignant histiocytosis. The adult worms of *M. yokogawai* were found free in the jejunal lumen as well as impacted in the intervillous spaces. The pathological findings in this human case were similar to those in the recovering phase of infection in experimental animals. The main histological lesions were massive lymphoplasmacytic and eosinophilic infiltration in the stroma, erosion of the enterocytes in the areas surrounding the worms, goblet cell depletion and occasional villous oedema. Sukontason and colleagues [172] studied the pathology

induced by *H. taichui* on three human cases revealing mucosal ulceration, mucosal and submucosal haemorrhages, fusion and shortening villi, chronic inflammation, and fibrosis of the submucosa.

7.8.4 Host–Parasite Relationships and Immunology

The fact that pathology induced by heterophyids is spontaneously restored indicated the development of host protective immunity. Several effector mechanisms have been suggested to act against heterophyids [25]. Intraepithelial lymphocytes and the lamina propria lymphocytes acting as CD8+ cytotoxic and IgA producing cells, respectively, may be of importance. Moreover, mucosal mast cells and goblet cells appear to be also involved in the worm expulsion in rodents [18]. Regarding antibody responses, it is known that both systemic and local antibody responses occur in these infections. The specific IgG levels in the serum of *M. yokogawai*-infected cats rose from 7 dpi and the maximum levels were observed at 2–4 wpi with both antigens [173]. Elevated levels of IgG, IgM and IgE have been detected in the serum of humans infected with *H. heterophyes* [153, 174, 175]. In the intestine, the levels of IgG, IgM and IgA were increased [174]. Systemic IgG response has been also detected in humans infected with *H. taichui* and *M. yokogawai*, respectively [176, 177]. There is some controversy on the potential role on the antibody response in the course of heterophyid infections. An inverse relationship between the antibody levels and the load of the infection has been often observed, suggesting that humoral response may be involved in worm expulsion [174, 176]. In contrast to these findings, Cho and colleagues [173] detected that specific IgG levels were directly related to the number of *M. yokogawai* adult worms in experimentally infected cats.

7.8.5 Diagnosis

Diagnosis can be done by detection of eggs in the faeces. However, the detection and identification may be difficult because of (1) similarity of the eggs of the heterophyids, (2) low-laying capacity of the parasite and (3) existence of extraintestinal cases. Due to the similarity of the eggs of heterophyids, when eggs are detected it is usually referred to as “heterophyid infection”. The specific diagnosis may require the recovery of adult worms after treatment and purgation [18]. Because of this fact, close examination of the egg sizes may be of help [149]. Moreover, light infections can be easily missed since the egg-laying capacity of the heterophyids is low [168]. For example, the daily egg output of *H. taichui* is estimated as 82 eggs/worm [178]. More problematic is the diagnosis of the extraintestinal cases which only can be detected by surgery or autopsy.

Few studies have been done concerning the immunodiagnosis of heterophyid infections. ELISA and western-blot methods showed a good level of sensitivity but cross-reactivity with other trematodes occurred [176, 177]. Similarly, several molecular-based methods for the diagnosis have been developed recently [178–183].

7.9 Other Families of Intestinal Trematodes Infecting Humans

7.9.1 Family Cathaemasiidae

Only one species belonging to this family, *Cathaemasia cabrerai*, has been recorded infecting humans. A single case was reported in the Philippines [184]. No further information about this parasite is available.

7.9.2 Family Lecithodendriidae

The lecithodendriids are characterized by possessing an oval body and a spinose tegument. The testes are opposite and a cirrus sac is present. The position of the genital pore is variable, the vitellaria are in lateral clusters, and the eggs are operculate and embryonated when laid. Adults of these digeneans are parasites of the digestive tract of amphibians, birds or mammals (typically bats). Three species of this family have been found infecting humans: *Prosthodendrium molenkampi*, *Phaneropsolus bonnei* and *P. spinicirrus* [12].

P. molenkampi was first discovered infecting humans in Indonesia, but thereafter, high prevalence was detected in Thailand reaching 19 % [185]. Moreover, human infections have been reported in Lao PDR [137, 166]. *P. bonnei* was first discovered concurrently with *P. molenkampi* in humans in Indonesia. Later, it was recorded in Malaysia, India, Thailand and, more recently, Lao PDR [18, 166]. Metacercariae of both species have been detected in dragonflies and it is thought that people become infected after eating this odonat which is common in these areas [18]. This may explain that concurrent infections have been commonly reported [166]. Only one human infection by *P. spinicirrus* has been recorded in Thailand [186]. In general lecithodendriids do not appear to produce clinical disease in humans [12].

7.9.3 Family Microphallidae

This family includes trematode parasites of vertebrates and is characterized by possession of a pryriform body, spinose tegument, short intestinal caeca, testes opposite in the hindbody, vitellaria posterior to the testes and the uterus confined to the hindbody. Eggs are operculated and non-embryonated. The life cycle includes three hosts: a mollusk, an arthropod and a vertebrate. In some species, the cycle is abbreviated by eliminating the second intermediate (arthropod) host. In these species, the cercaria encysts within the sporocyst and the infected mollusk is eaten by the definitive host [12].

Two species of this family have been recorded in humans: *Spelotrema brevicaeca* and *Gynaecotyla squatarolae*. In *S. brevicaeca*, cercariae encyst in the abdomen and cephalothorax of shrimps or crabs, which appears to be the source of human infections.

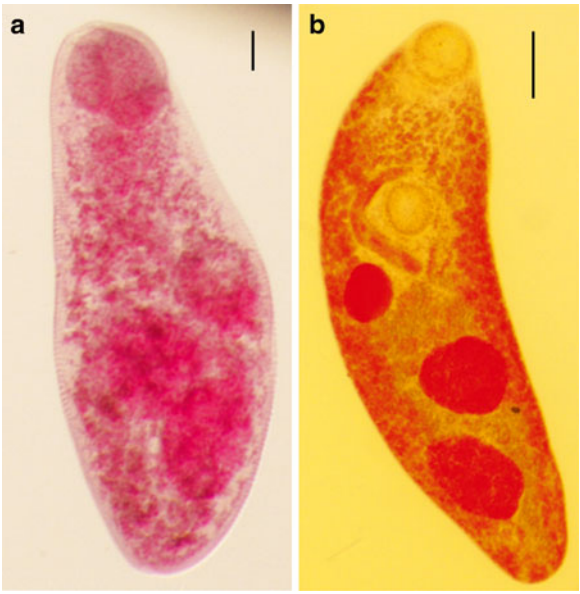

Fig. 7.8 Adult worms of (**a**) *Nanophyetus salmincola* (scale bar: 100 μm); and (**b**) *Plagiorchis muris* (scale bar: 200 μm)

This species was reported as the cause of fatal involvement in the heart and spinal cord of humans in the Philippines. Fluke eggs were found in the heart, brain and spinal cord of persons who died of acute cardiac dilatation [12, 18].

The first human infection of *G. squatarolae* has recently been reported in Korea [162]. A 50-year old female was found to be infected with this trematode concomitantly with other trematodes such as *G. seoi* and *H. pumilio*. It is known that *G. squatarolae* metacercariae encyst in crabs (*Macrophthalmus* spp.) and the woman had usually eaten brackish crabs in soy sauce.

7.9.4 Family Nanophyetidae

Only one species belonging to this family, *Nanophyetus salmincola*, has been recorded in humans. This parasite is characterized by a pyriform body and the presence of two large testes in the posterior half of the body (Fig. 7.8a). The life cycle of *N. salmincola* involves the release of non-embryonated eggs in the faeces of the mammalian host. The miracidium hatches and penetrates the freshwater snail, *Oxytrema silicula*. Cercariae emerge from snails, penetrate the skin and gills of salmonid fishes, and encyst in the muscles and connective tissue. The adults develop in the intestine of mammals following ingestion of the infected fish [12].

Human nanophyetiasis is endemic in the far-eastern part of Russia with an average prevalence of 5 % in some areas [54]. In local ethnic minorities, the prevalence is higher and reaches 60 % in some localities [159]. In the northwest USA, Eastburn and colleagues [187] reported ten human infections. Patients became infected after

eating improperly cooked salmon or trout. The main symptoms were gastrointestinal complaint of abdominal discomfort, diarrhoea, nausea and vomiting. Peripheral blood eosinophilia was also observed.

It should be noted that *Nanophyetus schikhobalowi* was described from natives of far-eastern Siberia though this fluke is currently considered as a subspecies of *N. salmincola* [51].

7.9.5 Family Paramphistomidae

The family Paramphistomidae is restricted to paramphistomoid digeneans, parasitic in mammals, which lack pharyngeal sacs, a cirrus sac and a ventral pouch. However, this family has often been used as a repository with a varying number of subfamilies, and there is confusion on its taxonomy.

Members of Paramphistomidae rarely infect humans. Only two species, *Fichoederus elongatus* and *Watsonius watsoni*, have been recorded. Information about *F. elongatus* is scarce. This is a parasite of humans in China [54]. *W. watsoni* was first discovered in a patient from West Africa who died of severe diarrhoea [80]. In both species the source of infection is suspected to be the ingestion of aquatic plants.

7.9.6 Family Plagiorchiidae

The members of this family are characterized by having a spinose tegument, the ventral sucker in the anterior half of the body, long intestinal caeca, testes opposite, tandem or oblique and located in the hindbody. A cirrus sac is present and the genital pore is anterior to the acetabulum. The ovary is pretesticular and the vitelline follicles are adjacent to the intestinal caeca and are variable in length (Fig. 7.8b). The eggs are operculate and embryonated [188]. The life cycle typically involves embryonated eggs being eaten by pulmonate snails, in which sporocysts develop and produce xiphidiocercariae. Emerged cercariae encyst in insect larvae, but encystment in freshwater fishes also occurs. Definitive hosts become infected after eating the second intermediate host harbouring the cysts.

Occasional human infections with *Plagiorchis harinasutai* (in Thailand), *P. javensis* (in Indonesia), *P. muris* (Fig. 7.8b) (in Japan and Korea) and *P. philippinensis* (in the Philippines) have been recorded [54, 189–191].

7.9.7 Family Strigeidae

The family Strigeidae includes trematodes with a cup-shaped forebody and a tribocytic organ (an accessory sucker). The hindbody is cylindrical or ovoid and contains the reproductive organs. The short uterus contains operculate, non-embryonated eggs. The adult flukes are parasites of the intestine of birds and mammals [12].

Within this family only *Cotylurus japonicus* has been recorded parasitizing the intestine of humans. The first infection was reported from a 13-year-old girl in China [192]. Further details about the human infections are scarce, and the source of the infection is unknown [130].

7.10 Treatment of Intestinal Trematode Infections

At present, the drug of choice for the treatment of intestinal trematode infections is praziquantel. Although the exact mechanism of action of praziquantel has not been elucidated, it has been postulated that a disruption of Ca^{2+} homeostasis occurs as praziquantel induces a rapid contraction of the trematodes [193]. Praziquantel exhibits a broad spectrum against trematodes and has an excellent safety profile [194]. All treatment schedules with praziquantel are well tolerated, with only few adverse events including abdominal pain, dizziness, headache, vomiting, nausea and urticaria and less commonly may also appear rash, hypotension and sudden expulsion of the worms which may produce obstruction [194, 195]. Furthermore, there are no reports of food-borne trematodes resistant to praziquantel. All the used treatments consist of a single dose of 25 mg/kg for echinostomiasis and heterophyasis and 10 mg/kg for gymnophalloidiasis [132, 194, 196, 197]. Other drugs such as mebendazole, thymol, carbon tetrachloride and tetrachloroethylene also have been used.

The limited number of available drugs for food-borne trematodiases, together with the concerns in relation to the development of resistance against these drugs, has encouraged the search for alternative drugs. In this context, preliminary studies have shown that a number of compounds might be further developed for the treatment of intestinal trematodiases using *Echinostoma caproni* as an experimental model [196]. Artemisinin, the active constituent of the herb *Artemisia annua*, is a sesquiterpene lactone that contains an unusual peroxide bridge. In recent years, the activity of artemisinin and its derivatives including artemether, artesunate and dihydroartemisinin against food-borne trematodes has been investigated in vitro and in vivo. In trematodes it has been shown that artemether disrupts the tegument [198]. Complete healing of *E. caproni* infections was achieved in mice using a single oral dose of 200 mg/kg [199]. Recently, artesunate has successfully studied against heterophyids in experimentally infected mice [200]. Due to the problems that artemisinins have in relation to their bioavailability, preparation and pharmacokinetics, many synthetic peroxide analogues have been prepared and investigated. One of them, ozonide OZ78, has shown to be an effective flukicide in the rodent model. Complete *E. caproni* worm burden reduction was achieved in acute and chronic infections in mice using a dose of 1,000 mg/kg [201]. The increasing interest in medicinal plants as new sources of antiparasitic drugs has led to study several extracts as flukicides. Recently, Ferreira and colleagues [202] showed that ethanolic extracts of *Artemisia annua*, *A. absinthium and Asimina triloba* kill *E. caproni,* probably in relation to their elevated content in artemisinin and acetogenins.

7.11 Control of the Intestinal Trematode Infections

Eating raw or improperly cooked freshwater fish and fresh or brackish water snails, snakes, bivalves or aquatic vegetables should be avoided to prevent intestinal trematode infections. There is evidence that various types of marinades and food preparations commonly used may not affect the viability of the metacercariae. Various physical and chemical factors have been studied to determine their effects on the viability of encysted metacercariae of *E. caproni*. Viability was equated with chemical excystation in an alkaline trypsin-bile salts medium. Of numerous marinades tested, the one that was most harmful to isolated and in situ cysts was vinegar. Concentrated solutions of NaCl and sucrose had no effect on the viability of isolated and in situ cysts, suggesting that their use in food preparations for molluscs would not be effective in killing echinostomatid cysts in tainted snail tissues [203]. Similarly, Wongsawad and colleagues [204] studied the effect of several factors on the viability of the metacercaria of *S. falcatus*. The authors concluded that the worms were killed in NaCl at 20 %, 30 % and 40 % within 12 h, 6 h and 2 h, respectively. Acetic acid at 5 and 10 % killed the metacercaria within 12 and 6 h while at 20 % and 30 %, within 2 h. The killing effect of 3 % vinegar was found within 18 h and of 5 % vinegar within 12 h. Lemon juice showed no killing effect.

The nature of intestinal trematode infections does not justify the establishment of a separate control programme, because it can be controlled along with other foodborne diseases for which there are sustained WHO control programmes. The control of human intestinal trematodiasis via blocking or interruption of the life cycle can be achieved through proper diagnosis, followed by pharmacologic treatment and prevention of reinfection. The control should be focused predominantly on a reduction or elimination of the transmission of the disease. In theory, the means of control in endemic regions can include: a reduction of the sources of infection, particularly human beings, through effective treatment; the protection of fish ponds and aquaculture systems from contamination with faeces from people and other definitive hosts; the treatment or sterilization of faeces; the control of snail host populations; and the implementation of education campaigns. Therefore, in principle, the prevention and control is relatively simple. Since infection of the definitive host is only contracted through the ingestion of metacercariae, the most practical measure for preventing and controlling human infection is to eliminate the consumption of raw, undercooked aliments susceptible to harbour the metacercariae. However, this strategy may be difficult to implement in some endemic regions because of the ancient eating traditions. Thus, only adequate cooking will render safe for human consumption. Nonetheless, together with education awareness programmes focused particularly on teaching young children about the parasites, its life cycle, and the disease it causes, prevention and control could be successful. Targeting children may have the advantage that they are less entrenched than adults in their customs and eating habits. Significant changes for the control of intestinal trematodiasis may include (1) the development of effective broad-spectrum anthelmintic, (2) an understanding by WHO of the differences between intestinal helminths and arthropod-borne

infectious agents and (3) the implementation of control programmes in school-age children, with strong community therapy programmes delivering multiple treatments against concurrent helminthic infections. A decreasing pattern in some food-borne trematode diseases along with industrialization, health education, and alteration of environment has been observed in certain areas of Southeast Asia. This is particularly true for Taiwan and mainland China, where industrial developments and wastewater discharges pollute streams and rivers, practically destroying those aquatic animals involved in trematode life cycles. The WHO control programmes operating through the essential components of diagnosis, treatment, and prevention for the control of human zoonotic trematodiases have not been successful against several intestinal trematodiases, although they have been for other trematode diseases.

7.12 Concluding Remarks

Despite the significant public health impact of the intestinal trematodiases, these diseases are among the most neglected tropical diseases. There was a general lack of knowledge about these infections and their causative species. Traditionally, intestinal trematode infections were considered as minor diseases confined to low-income countries, mainly in Asia, and presence in the press media and funding research were practically inexistent. In recent years, this picture is changing by a number of factors. The number of infected is counted in the millions and an elevated percentage of the world's population is considered to live at risk for infection. Currently, the geographical limits and the population at risk are currently expanding and changing in relation to factors such as growing international markets, improved transportation systems, new eating habits in developed countries and demographic changes. Moreover, the number of species causing human intestinal trematode infections is high which makes difficult their correct knowledge, several severe endemic foci have been described and some species can cause serious health problems. This is aggravated since, as it occurs with other neglected tropical diseases, there are several gaps in our knowledge on the intestinal trematode infections.

In this context, new approaches to these diseases are needed. Current control strategies are reasonable and logical, though approaches based on new technologies for training of health workers, treatment, diagnosis and control of these diseases should be implemented. At present, a limited number of drugs are available, which is aggravated by the little incentive to invest in the discovery and development of new trematodicidal drugs. Moreover, the identification of parasite-specific proteins could clearly facilitate the design of new tools for rapid and cheap diagnosis, which may help to control the transmission of the parasite. In this context, the identification of potential targets for vaccination seems to be one of the best ways to control these parasite infections. The emergence of the intestinal trematodiases associated with the new risks of transmission introduced by the "globalized world" makes necessary the development of updated maps of distributions and risk of transmission and all these measures should be also accompanied by programmes of training

of health personnel and the population in general to change risk eating habits. These approaches can provide the tools necessary for a proper understanding and control of the intestinal trematode infections.

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Chapter 8
Epidemiology of Trematode Infections

Jong-Yil Chai

8.1 Introduction

Parasites of the Class Trematoda (Phylum Platyhelminthes) are taxonomically diverse and largely consist of Subclass Monogenea, Aspidogastrea, and Digenea [1]. Only those of the Subclass Digenea are endoparasites of humans and animals. Species of trematodes important for human infections can be divided into four groups according to their habitat in the definitive host; blood flukes, liver flukes, lung flukes, and intestinal flukes (including those parasitic in the throat and pancreatic duct). Blood flukes comprise of more than five species of *Schistosoma* which parasitize in the mesenteric venules or vesical or pudental plexus of the urinary bladder [1]. The major liver flukes infecting humans include four species, *Clonorchis sinensis*, *Opisthorchis viverrini*, *O. felineus*, and *Fasciola hepatica*. They usually inhabit in the bile duct of humans or animals, although *F. hepatica* can also be found in ectopic foci such as the eye, intestinal wall, and muscles. More than eight species of *Paragonimus* are currently acknowledged to be able to infect the lungs of human patients; *Paragonimus westermani*, *P. africanus*, *P. heterotremus*, *P. skrjabini*, *P. skrjabini miyazakii*, *P. kellicotti*, *P. mexicanus*, and *P. uterobilateralis* [2]. Intestinal flukes are more diverse, including heterophyids (*Metagonimus yokogawai*, *Heterophyes nocens*, and *Haplorchis taichui*), echinostomes (*Echinostoma revolutum*, *E. ilocanum*, *Echinochasmus japonicus*, *Artyfechinostomum malayanum*, and *Acanthoparyphium tyosenense*), gymnophallids, lecithodendriids, microphallids, neodiplostomes, and plagiorchiids [3, 4]. Epidemiological characteristics of these trematodes that include the geographical distribution, prevalence and intensity of infection, mode of transmission and infection source, and others related to prevention and control are highly variable according to each trematode species (Table 8.1).

J.-Y. Chai (✉)
Department of Parasitology and Tropical Medicine, College of Medicine,
Seoul National University, Seoul 110-799, South Korea
e-mail: cjy@snu.ac.kr

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Table 8.1 Source of infection and geographical distribution of human-infecting trematodes

Species	Source of infection	Geographical distribution
Schistosoma japonicum	Contact with water	China, Taiwan, the Philippines, Indonesia
Schistosoma mekongi	Contact with water	Laos, Cambodia
Schistosoma mansoni	Contact with water	Egypt, Sudan, Ethiopia, Cameroon, Congo, Uganda, Kenya, Tanzania, Zimbabwe, Zambia, South Africa, Madagascar, Mozambique, Malawi, Gambia, Guinea, Sierra Leone, Liberia, Mali, Mauritania, Senegal, Ghana, Nigeria, Saudi Arabia, Yemen, Brazil, Puerto Rico, Surinam, Venezuela, Vieques, Dutch Guiana, Sergipe
Schistosoma haematobium	Contact with water	Sudan, Ethiopia, Central Africa, Chad, Nigeria, Angola, Ghana, Senegal, Egypt, Morocco, Tanzania, Kenya, Rwanda, Brundi, Zambia, Mozambique, Zimbabwe, Madagascar, Mauritius, Réunion, Lebanon, northern Syria, Arabia, Iraq, Iran, India
Schistosoma intercalatum	Contact with water	Cameroon, Equatorial Guinea, Gabon, Chad, Nigeria, Central African Republic, D.R. Congo, Sao Tome, Egypt, and Madagascar
Opisthorchis viverrini	Freshwater fish	Thailand, Laos, Cambodia, Vietnam
Clonorchis sinensis	Freshwater fish	Korea, China, Taiwan, Vietnam, Russia
Opisthorchis viverrini	Freshwater fish	Thailand, Laos, Cambodia, Vietnam
Opisthorchis felineus	Freshwater fish	Spain, Italy, Albania, Greece, France, Macedonia, Switzerland, Germany, Poland, Russia, Turkey
Metorchis conjunctus	Freshwater fish	Canada, the USA
Metorchis bilis	Freshwater fish	Central and eastern Europe, Russia
Metorchis orientalis	Freshwater fish	East Asia (human; in China)
Fasciola hepatica	Aquatic vegetation	Ecuador, Bolivia, Chile, Peru, Cuba, Egypt, Portugal, France, Spain, Iran, Turkey, Korea, Japan, China, Thailand, Vietnam
Fasciola gigantica	Aquatic vegetation	Japan, Korea, China, Russia, Vietnam, Thailand, Malaysia, India, Iran, Sudan, Senegal, Chad, Ghana, Niger, Central African Repub., Tanzania, Kenya, Hawaii
Dicrocoelium dendriticum	Ant	Europe, Northern coast of Africa, Russia, Turkey, Syria, Iran, India, China, the Philippines, Japan, the USA, Canada, Cuba, Colombia, Brazil
Dicrocoelium hospes	Ant	Africa
Paragonimus westermani	Crab, crayfish	China, Taiwan, Korea, Japan, Southeast Siberia, the Philippines, Malaysia, Thailand, Cambodia, Laos, Vietnam, Sri Lanka, India, Nepal, Pakistan, Papua New Guinea, the USA
Paragonimus heterotremus	Crab	China, Vietnam, Laos, Thailand, India, China, Vietnam, Laos, Thailand, India
Paragonimus skrjabini	Crab	China, India

Paragonimus miyazakii	Crab	Japan
Paragonimus kellicotti	Crab, crayfish	The USA, Canada
Paragonimus mexicanus	Crab	Peru, Ecuador, Costa Rica, Panama, Guatemala, possibly in Colombia and Brazil
Paragonimus africanus	Crab	Cameroon, Nigeria
Paragonimus uterobilateralis	Crab	Liberia, Nigeria, Cameroon, Gabon
Metagonimus yokogawai	Freshwater fish	Far Eastern Asia (Korea, Japan, China, Taiwan, Russia, Indonesia), Israel, Spain
Metagonimus takahashii	Freshwater fish	Japan, Korea
Metagonimus miyatai	Freshwater fish	Japan, Korea
Heterophyes nocens	Brackish water fish	Japan, China, Korea
Heterophyes heterophyes	Brackish water fish	Egypt, Sudan, Palestine, Bangladesh, Iran, India, Turkey, Russia
Heterophyes dispar	Brackish water fish	Egypt, Saudi Arabia, northern Africa, eastern Mediterranean
Haplorchis taichui	Freshwater fish	Taiwan, the Philippines, Bangladesh, India, Sri Lanka, Palestine, Iraq, Egypt, Malaysia, Thailand, Laos, Vietnam, China
Haplorchis pumilio	Freshwater fish	The Philippines, Thailand, Laos, Vietnam, South China, Taiwan, Malaysia, India, Sri Lanka, Iraq, Egypt, Korea
Haplorchis yokogawai	Freshwater fish	The Philippines, South China, Malaysia, Indonesia, Thailand, Laos, Vietnam, India, Australia, Egypt
Centrocestus formosanus	Freshwater fish	Taiwan, China, Japan, the Philippines, Thailand, Lao PDR, Vietnam, India, Turkey, the USA, Mexico, Brazil
Centrocestus armatus	Freshwater fish	Korea, Japan
Centrocestus kurokawai	Freshwater fish	Japan
Pygidiopsis summa	Brackish water fish	Korea, Japan
Stellantchasmus falcatus	Fish	The Philippines, Hawaii, Japan, Palestine, Thailand, Vietnam, Korea
Stellantchasmus pseudocirratus	Fish	Palestine, Taiwan, the Philippines, Hawaii
Stictodora fuscata	Brackish water fish	Korea, Japan
Stictodora lari	Brackish water fish	Korea, Japan, Australia
Echinostoma revolutum	Freshwater snail	Asia, Europe, Africa, Australia, New Zealand, North and South America
Echinostoma echinatum	Freshwater snail	European countries (esp. Germany), Asia (Indonesia), South America (Brazil)
Echinostoma cinetorchis	Freshwater fish, snail	Japan, Korea, China
Echinostoma hortense	Freshwater fish	Japan, Korea, China

(continued)

Table 8.1 (continued)

Species	Source of infection	Geographical distribution
Echinostoma ilocanum	Freshwater snail	The Philippines, Indonesia, China, Thailand, India, Cambodia
Echinochasmus japonicus	Freshwater fish	Japan, Korea, China
Echinochasmus liliputanus	Freshwater fish	Egypt, Syria, Palestine, China
Echinochasmus perfoliatus	Freshwater fish	Hungary, Italy, Romania, Russia, Japan, China, Taiwan, Denmark
Echinochasmus fujianensis	Freshwater fish	China
Echinochasmus jiufoensis	Freshwater fish	China
Artyfechinostomum malayanum	Freshwater snail	Malaysia, Singapore, Thailand, Indonesia, India, the Philippines, Laos
Artyfechinostomum oraoni	Freshwater snail	India
Acanthoparyphium tyosenense	Brackish water snail	Korea
Echinoparyphium recurvatum	Freshwater snail	Cosmopolitan, especially Taiwan, Indonesia, Korea, Egypt
Hypoderaeum conoideum	Freshwater snail	Europe (including Spain), Russia (Siberia), Japan, Thailand
Isthmiophora melis	Freshwater fish, tadpole	Europe (including Romania), Russia, China, Taiwan, North America
Fasciolopsis buski	Aquatic plants	China, Taiwan, Thailand, Vietnam, Laos, Cambodia, Bangladesh, India, Indonesia, Myanmar, the Philippines, Singapore, Malaysia
Gymnophalloides seoi	Oyster	Korea
Spelotrema brevicaeca	Crab, shrimp	The Philippines
Gynaecotyla squatarolae	Brackish water crab	Korea, Japan
Neodiplostomum seoulense	Snake, frog	Korea, China
Prosthodendrium molenkampi	Aquatic insect	Indonesia, Malaysia, India, Thailand, Laos
Phaneropsolus bonnei	Aquatic insect	Indonesia, Thailand, Laos
Plagiorchis muris	Aquatic insect, fish	The USA, Japan, Korea
Plagiorchis harinasuta	Unknown	Thailand
Plagiorchis javensis	Insect larva	Indonesia
Plagiorchis philippinensis	Insect larva	The Philippines
Plagiorchis vespertilionis	Insect larva	Korea, Europe

8.2 Blood Flukes

Human-infecting schistosomes are five species that include *Schistosoma japonicum*, *S. mekongi*, *S. mansoni*, *S. haematobium*, and *S. intercalatum*. About 200–300 million people in 77 countries are affected by these blood flukes [5].

8.2.1 Schistosoma japonicum and S. mekongi

Schistosoma japonicum is a species of blood fluke that infects the superior mesenteric venules and veins (more frequently in the large intestine) of humans and animals. Adult male and female worms copulate and produce about 3,000 eggs per worm per day [5]. Some of these eggs are excreted in the feces; however, some others become free in the general circulation and can be filtered in the liver, lungs, and even the central nervous system [5]. Egg granuloma in the liver parenchyme (portal triads), fibrosis, portal hypertension, cirrhosis, abdominal distension, and esophageal varices are the main clinical manifestations. Its correlationship with colorectal cancer and liver cancer has been documented [5]. *S. mekongi* is biologically similar to *S. japonicum* except in a few different points. *S. mekongi* tends to be more pathogenic than *S. japonicum* [1].

8.2.1.1 Intermediate Hosts and Mode of Human Infections

The snail intermediate host of *S. japonicum* is several species of *Oncomelania*, namely, *O. nosophora* (south and southwestern China), *O. hupensis* (Yangtze River basin and south and southeastern China), *O. formosana* (Taiwan), and *O. quadrasi* (the Philippines) [1]. They are operculate with conical or turriculate shells, amphibious, freshwater species, and spend some parts of their time out of water, preferring moist soil in marshy habitats, at the edge of slow-flowing streams or irrigation canals [6]. The cercariae are discharged only when the snails are at or below the water level [1]. Usually less than 0.5 % of the snails are infected with the cercariae at any one time point [6]. The snail intermediate host of *S. mekongi* is the gamma strain of *Neotricula aperta* (syn. *Lithoglyphyopsis aperta* or *Tricula aperta*) which is distributed along the Mekong River mainly from Khong island of Laos to Kratie Province, Cambodia [7]. The cercarial infection rate of snails was 0.3 % in Khong Island [7].

The cercariae are furcocercous (fork-tailed) and actively swim in water. They are directly infectious to the skin of humans or animals, and thus contact with water can cause infection. Farmers and canal boatmen are principally infected but all ages, including children, can be infected while bathing or wading shallow waters [1]. Using contaminated water for laundry purposes is also a risk for infection [1]. On contact with the skin of the definitive host, the cercariae cast off their tails and

penetrate down to the cutaneous capillary beds, and enter the venous circulation [1]. The adult flukes, male and female, can live indefinitely in the human body as well as in animal reservoir hosts; survival of worms for 47 years has been documented [1].

8.2.1.2 Reservoir Hosts

The natural definitive hosts of *S. japonicum* include mammals including humans, dogs, cats, rats, mice, field mice, cattle, water buffalo, pigs, horses, sheep, and goats [1]. For the purpose of control, attention to domestic cattle and buffalo in agriculture, and that to stray dogs and rats in nature, is essential [1]. With regard to *S. mekongi*, the reservoir hosts are wild and domestic mammals that include dogs, cats, and pigs [7]. In the laboratory, mice and hamsters could be successfully infected with *S. mekongi* [7].

8.2.1.3 Geographical Distribution

The current endemic areas of *S. japonicum* include some limited areas of East Asia, particularly Yangtze River basin, Boyang and other lakes, and mountainous areas of Sichuan and Yunnan Province, China, and Mindanao, Leyte, and other small sites in the Philippines [6]. *S. japonicum* was prevalent until the 1960s–1970s in several foci of Japan [1], but there were no new infection cases since 1976, and a declaration was made on elimination of schistosomiasis in Japan [7]. The Paloe District of Celebes, Indonesia, was endemic with *S. japonicum*; however, it is now unclear whether transmission still occurs in this area [1, 6]. In Taiwan, enzootic cycle was reported; however, no human schistosomiasis has been documented [5]. In 2000, the number of infected people with *S. japonicum* was estimated to be about 1.1 million, exclusively in Asia [8]. The number of people at risk of *S. mekongi* infection is estimated to be about 60,000 (likely infected cases being 11,000) in Laos and 80,000 in Cambodia [9, 10].

8.2.1.4 Population Epidemiology

The prevalence of *S. japonicum* is highest in children, and the greatest exposure to cercariae occurs in boys aged 5–10 years because of recreational activities in water [5]. Older children may have less recreational exposure but are likely to be exposed while performing agricultural activities, washing dishes and cloths, and bathing younger siblings in the streams and lakes [5]. Most infected individuals have low worm burdens, but a few may have very heavy burdens who probably make the greatest contribution to the dissemination of the infection [5]. In the case of *S. mekongi*, the highest rate of infection has also been found in children 7–15 years of age [1].

8.2.1.5 Environmental Factors Related to Transmission

Transmission is closely related to seasons. The cercarial shedding occurs only in rainy seasons when the snails are at or below the water level [1]. The vector snails, *Oncomelania* spp., are extremely resistant to a long-time desiccation, and on contact with water, the snails become active again and shed infective cercariae [5]. Heavy rains and flood, and construction of dams and other big environmental changes, will affect the transmission of *S. japonicum* infection [1, 5]. The newly constructed Three Gorges Dam area of China may appear to be an endemic area of *S. japonicum* [6]. As to *S. mekongi*, transmission occurs in rocky banks of the Mekong River basin, because the natural habitat of the snail host, *N. aperta*, is constituted by the small crevices in the partially submerged rocks [9]. The period of active transmission in *S. mekongi* is during the dry season, from February and April in Cambodia, and March to June in Laos [9].

8.2.2 Schistosoma mansoni

Schistosoma mansoni is parasitic in the inferior mesenteric venules and veins (mostly in the large bowel) of humans and rarely some animals like primates. Adult male and female worms copulate and produce 100–300 eggs per worm per day [5]. Some of these eggs are excreted in the feces; however, many others are swept away and become lodged in the microvasculature of the liver and other organs [5]. Eggs infiltrated in the intestinal wall may cause inflammation, thickening, and fibrosis which lead to intestinal symptoms including abdominal pain, bloody mucous stools, diarrhea, and dysentery [1]. Eggs deposited in the portal triads of the liver stimulate a granulomatous response, leading to continuous fibrosis of the periportal tissue and finally liver cirrhosis [5].

8.2.2.1 Intermediate Hosts and Mode of Human Infections

Aquatic freshwater snails of the genus *Biomphalaria*, including *B. glabrata*, *B. straminea*, *B. tenagophila* (Western Hemisphere), *B. alexandrina*, *B. sudanica*, *B. rüppellii*, *B. pfeifferi*, *B. choanomphala. B. smithi*, and *B. stanleyi* (Africa), have been found to be the intermediate host of *S. mansoni* [1, 11]. They are non-operculate without cover or lid on the shell and characterized by disk- or lens-shaped shells [6]. They can survive long protracted droughts, hiding in moist mud until the next rains come and rivers swell again [6].

The furcocercous cercariae swim in water and are infectious to the skin of humans and animals. Direct contact with water containing cercariae can cause infection [1]. Farmers are exposed to infection in irrigation ditches, and infection can also occur from bathing and washing dishes and clothes in contaminated water [1, 5].

8.2.2.2 Reservoir Hosts

The role of reservoir hosts in maintaining the endemicity of *S. mansoni* is negligible, although natural infections were found in monkeys, baboons, and gerbils [1]. The source of human infection is almost exclusively derived from human sources [1].

8.2.2.3 Geographical Distribution

The geographical distribution of *S. mansoni* depends on the distribution of the snail intermediate host and the opportunity to infect humans [5]. The endemic areas of *S. mansoni* are in 54 countries that are scattered in Africa, the Arabian Peninsula, and the Western Hemisphere including South America and the West Indies [8, 12]. Egypt, Sudan, Ethiopia, Cameroon, Congo, Uganda, Kenya, Tanzania, Zimbabwe, Zambia, South Africa, Madagascar, Mozambique, Malawi, Gambia, Guinea, Sierra Leone, Liberia, Mali, Mauritania, Senegal, Ghana, and Nigeria are the countries in Africa having endemic areas [1, 5, 6]. Saudi Arabia and Yemen are the countries where endemic areas of *S. mansoni* were reported [1, 6, 12]. The endemic countries in the Western Hemisphere include Brazil, Puerto Rico, Surinam, Venezuela, Vieques, Dutch Guiana, and Sergipe [1, 6, 8]. However, in Puerto Rico, there was little transmission during the first half of the 1990s and has been disappearing thereafter [5]. The estimated number of people infected with *S. mansoni* and/or *S. haematobium* is over 190 million [8].

8.2.2.4 Population Epidemiology

Schistosomiasis, due to *S. mansoni*, is transmitted mainly from infected persons who defecate in or near water where the appropriate snail host resides [5]. The overall prevalence in communities living under endemic conditions is usually between 30 and 100 % [6]. Its prevalence is highest in children, particularly in boys aged 5–10 years [5]. Most infected individuals have low worm burdens, but a few may have very heavy burdens who probably make the greatest contribution to the dissemination of the infection [5].

8.2.2.5 Environmental Factors Related to Transmission

Snail populations, cercarial density, and patterns of human water contact show strong temporal and spatial variations, resulting in a focal distribution of the infection within countries, regions, and villages [12]. Whereas *S. haematobium* mostly occurs in warm plains, *S. mansoni* can be transmitted in a variety of ecotypes, from savannah to rain forest and high lands of up to 2,500 m [6]. Transmission of both *S. mansoni* and *S. haematobium* takes place in the great lakes of Central and East Africa, and also in many other small and large, natural or artificial, lakes [6].

8.2.3 *Schistosoma haematobium and S. intercalatum*

Schistosoma haematobium is a blood fluke that infects the vesical and pelvic plexuses (in the urogenital system) of humans and a few species of nonhuman primates. Adult females can contain 20–100 eggs in the uterus at one time [5]. The eggs are deposited in the venous plexuses of the urinary bladder and excreted in the urine. However, eggs may also be deposited in the rectal venules and excreted in the feces. In light infections, there may be no clinical symptoms. However, in moderate to severe infections, dysuria, frequency, and hematuria due to cystitis [5], and in chronic cases, obstructive uropathy, bladder calcification, and even bladder carcinoma may occur [5]. *S. intercalatum* is morphologically and biologically similar to *S. haematobium* but clinically to *S. mansoni* [5]. *S. intercalatum* can cause an intestinal form of schistosomiasis like *S. mansoni*, although characterized by the lesions, mainly situated in the rectum and sigmoid level [5].

8.2.3.1 Intermediate Hosts and Mode of Human Infections

The snail intermediate host of *S. haematobium* is *Bulinus truncatus* in the Mediterranean and Arabian countries, whereas that is *B. truncatus rohlfsi*, *B. guernei*, *B. senegalensis*, *B. globusus*, or *B. africanus* in Africa [1]. In India, the snail host is a limpet, *Ferrisia tenuis*, and in Portugal, it is *Planorbis metidjensis* [1]. They are aquatic, non-operculate without cover or lid on the shell, and characterized by having conic shells with a left-twisted spiral [6]. They can survive in the mud when the water dries up, and retain their infectivity and resume cercarial shedding when the rainy season comes again [5, 6]. The snail host of *S. intercalatum*, which morphologically resembles *S. haematobium* but is biologically similar to *S. mansoni* (their habitat is mesenteric veins, and eggs are detected in the feces), is *Bulinus forkskalis* and *B. africanus* [5].

The eggs of *S. haematobium* soon hatch on dilution of the urine or stool with ten or more parts of water, and the miracidia become free and swim actively in water [1]. The cercariae are infectious to the skin of humans and animals, and direct contact with water containing cercariae can cause infection [1]. Farmers, women washing clothes in the streams, and children bathing or wading in the water are all subject to exposure [1]. In endemic foci of certain countries, religious practices tend to increase pollution of the water and encourage exposure to infections [1]. The mode of infection of *S. intercalatum* to humans is similar to that of *S. haematobium* and *S. mansoni*.

8.2.3.2 Reservoir Hosts

Humans appear to be the only important reservoir host of *S. haematobium*, although naturally infected monkeys, baboons, and chimpanzees were found in endemic areas [5]. The source of human infection is almost exclusively

derived from human sources [1]. This is also true for *S. intercalatum*, although natural infection was found also in nonhuman primates, insectivores, marsupials, and rodents [5].

8.2.3.3 Geographical Distribution

The geographical distribution of *S. haematobium* depends on the distribution of the snail intermediate host and the opportunity to infect humans [5]. The endemic areas, 53 countries [8], are scattered in Africa, Asia Minor, Cyprus, the islands off the African east coast, and southern Portugal [5]. Sudan, Ethiopia, east coast from Somaliland to Cape, vast areas in Central Africa, West Africa from Lake Chad and Nigeria as far south as Angola, North Africa from Egypt to Morocco, and east coast countries (Madagascar, Mauritius, and Réunion) are well-known endemic areas in Africa [1, 8]. Lebanon, northern Syria, Arabia, Iraq, and Iran are west Asian countries having endemic areas [1]. In India, an endemic focus has been found in an area south of Bombay [1]. The estimated number of people infected with *S. mansoni* and/or *S. haematobium* is over 190 million [8].

S. intercalatum has been found in ten countries of Africa (Cameroon, Equatorial Guinea, Gabon, Chad, Nigeria, Central African Republic, Democratic Republic of Congo, and Sao Tome, Egypt, and Madagascar), and a few countries in the Arabian Peninsula [5, 8]. However, transmission in Chad, Nigeria, Central African Republic, and Democratic Republic of Congo should be confirmed [8]. In endemic areas, prevalences of 5–25 % have been found [5].

8.2.3.4 Population Epidemiology

Schistosomiasis, due to *S. haematobium*, is transmitted mainly from infected persons who urinate in or near water where the appropriate snail host resides [5]. The overall prevalence in communities living under endemic conditions is usually between 30 and 100 % [6]. In most endemic foci, children are more frequently and more heavily exposed and infected than adults [1]. Most infected individuals have low worm burdens, but a few may have very heavy burdens and can probably make the greatest contribution to the dissemination of the infection [5]. Human *S. intercalatum* infection is all derived from human sources [5].

8.2.3.5 Environmental Factors Related to Transmission

S. haematobium mostly occurs in warm plains, whereas *S. mansoni* can be transmitted in a variety of ecotypes [6]. Transmission of both *S. mansoni* and *S. haematobium* takes place in the great lakes of Central and East Africa, and also in many other small and large, natural or artificial, lakes in Africa [6]. The infected snails can

be carried from infected foci into new irrigation projects [1]. The environmental factors related to the epidemiology of *S. intercalatum* infection are similar to those of *S. mansoni* [5].

8.3 Live Flukes

Human-infecting liver flukes are at least ten species; the major three being *Clonorchis sinensis*, *Opisthorchis viverrini*, and *O. felineus*. Infections with *Metorchis* species, i.e., *M. conjunctus*, *M. bilis*, or *M. orientalis*, appear to be emerging zoonotic infections. About 50 million people are infected with the liver flukes with 800 million people at risk of infection; 601 million for *C. sinensis*, 80 million for *O. viverrini* and *O. felineus*, and 91 million for *Fasciola* spp. [5].

8.3.1 Clonorchis sinensis

Clonorchis sinensis, the Chinese liver fluke, infects the bile duct of humans and animals in the Far East and Southeast Asia. It can cause inflammation of the bile duct (= cholangitis) and gall bladder (= cholecystitis) and obstruction of the biliary tract. The early stage clinical symptoms include jaundice, low grade fever, anorexia, easy fatigue, and gastrointestinal disturbances such as epigastric discomfort, indigestion, and diarrhea [13–15]. In chronic stages, various complications may occur that include biliary stones, fibrosis and cirrhosis of the liver, and even cholangiocarcinoma. It needs first and second intermediate hosts and a definitive host for completion of its life cycle.

8.3.1.1 Intermediate Hosts and Mode of Human Infection

The first intermediate host of *C. sinensis* is the freshwater snail, *Parafossarulus manchouricus* (syn. *P. striatulus*) in China, the Republic of Korea (hereafter Korea), Taiwan, and Russia, and also several other species of the Hydrobiidae (including *P. anomalospiralis*, *Bithynia fuchsiana*, *B. misella*, and *Alocinma longicornis*), Melaniidae (*Melanoides tuberculata* and *Semisulcospira libertina*), Assimineidae (*Assiminea lutea*), and Thiaridae (*Thiara granifera*) in China and Taiwan [16].

The second intermediate host, i.e., the source of human and animal infections, is at least 113 species of freshwater fish, with the majority being cyprinoid fish including *Pseudorasbora parva* [15–17]. Three species of freshwater shrimps were also reported to be the second intermediate host in China [15]. The presence of the snail and fish, together with reservoir hosts, is essential to transmission of *C. sinensis* in endemic areas, and this combination must be sustained for the parasite to remain endemic in a region [17]. The prevalence of infection in the snail host can be as low as 0.08 % even in highly endemic areas, but this is sufficient to maintain the life

cycle because the infected snails may release an average of 788 cercariae per snail per day, with a maximum of 5,840 cercariae per snail [13]. Cercarial shedding can occur from May to October in Korea [13]. The infection rate of freshwater fish with *C. sinensis* metacercariae is up to 100 % in endemic areas [18, 19].

The prevalence of *C. sinensis* in endemic areas is, of course, related to the human custom of eating raw fish or shrimps [17]. In southern China and Hong Kong, the morning congee (rice gruel) with slices of raw freshwater fish is an example of major dietary source of infection [16]. In Guangdong Province, half roasted or undercooked fish is another type of food involved, and in Fujian Province, eating raw shrimps is an important mode of human infection [16]. In Korea, slices of raw freshwater fish with red pepper sauce are the major type of fish dish responsible for *C. sinensis* infection [17].

In endemic areas of *C. sinensis* infection, small sized-fish, such as *P. parva*, *Acanthorhodeus* sp., *Rodeus* sp., and *Hemiculter* sp., are more frequently and more intensively infected with the metacercariae of *C. sinensis* than large fish [15]. However, large fish such as carps, for example, *Cyprinus carpio*, *C. carpio nudus*, and *Cyprinus auratus*, are practically more important in inducing human infections in endemic areas [15, 17]. The metacercarial burdens of these large carp are generally very low; however, they are preferred as a source of raw fish [15, 17]. In contrast, small fish such as *P. parva* generally having high metacercarial burdens are much less preferred to be eaten raw, particularly in Korea [13, 17]. This contributes to accumulation of infections from large fish with small numbers of metacercariae over a period of 20–30 years [13].

8.3.1.2 Reservoir Hosts

The natural definitive hosts of *C. sinensis* are mammals including humans, dogs, cats, rats, pigs, badgers, weasels, camels, and buffaloes [17]. However, the role of the reservoir hosts, especially cats, dogs, and pigs, in maintaining endemicity has not been well established [16, 17]. In some areas of China and Vietnam, infection may be high among people and low among domestic animals and the reverse situation is also found; however, in other endemic areas, the prevalence among the reservoir hosts may be the same as that in humans [16, 17, 20]. This is not a trivial issue because the role of reservoir hosts may have an important bearing on the outcome of mass drug treatment control programs [17].

8.3.1.3 Geographical Distribution

The major endemic areas of *C. sinensis* include East Asia, particularly China, Taiwan, Korea, Vietnam, and Russia [14, 17, 21]. The number of infected people worldwide is currently estimated at least 20 million [21]. The geographical distribution is determined predominantly by the distribution of the snail intermediate host [21].

It is distributed between E 100° (Hanoi area, Vietnam) and E 140° (Amur river territory, Russia) and N 20° (Hanoi area, Vietnam) and N 50° (Amur river territory, Russia).

In Korea, a national survey in 2004 reported 2.9 % egg positive rate [22], and the number of infected people estimated in Korea was about 1.5 million. Major endemic areas are scattered along the five major rivers, including the Nakdong, Seomjin, Yongsan, Keum, and Han rivers. In China, a total of 24 endemic localities (provinces, municipalities, and autonomous regions) were reported [16]. Among them, Guangdong Province (including Hong Kong) and Guangxi Zhuang Autonomous Region, Heilongjiang, Jilin and Liaoning provinces were the most important regions showing significantly high infection rates of the people [23]. The number of infected people in nationwide China is estimated at about 12.5 million [21, 24]. In Hong Kong, the prevalence was high before but it has decreased remarkably owing to control measures [16]. In Taiwan, clonorchiasis was formerly endemic in three localities, Mei-Nung in the south, Sun-Moon Lake in the center, and Miao-Li in the north [25]. However, the current status is unknown. In Vietnam, clonorchiasis has been endemic mainly in the north, especially along the Red River Delta including Haiphong and Hanoi [13, 17, 20]. The number of population infected in Vietnam was estimated at about one million [26]. In Russia, human cases infected with *C. sinensis* were reported in the Amur River territory, the far eastern part of the country [16]. In a southern part of Khabarovsk region, along the Amur River, domestic cats revealed a high (74.6 %) infection rate with *C. sinensis* [16].

8.3.1.4 Population Epidemiology

Characteristic patterns of age and sex prevalence are known in endemic areas of *C. sinensis* [17]. The prevalence is generally higher in men than in women, and higher in adults than in children [13, 17, 20]. For example, men of 25–55 years old and women over 45 years are the most highly affected groups [16]. The prevalence begins to increase from the age of 20 years and reaches the peak at the age of 40–50 years, particularly in man [14]. This reflects most likely the behavior pattern of men, who more often gather together for dinners with raw or pickled fish usually accompanied by alcohol [17].

8.3.1.5 Environmental Factors Related to Transmission

Water contamination with human or animal feces containing eggs plays an important role in the transmission of *C. sinensis* [16]. Pigpens, cowsheds, and even toilets nearby water drainages, streams, and ponds are the major sources for the eggs [16]. As reported in Hubei Province, the prevalence is higher in plain areas than in mountainous areas because the houses are always near the streams and rivers in plain areas and water is easily contaminated, resulting in high infection rates of the snails and fish [16].

With regard to the regulation of snail development as well as parasite development in the snails, temperature and season are the primary determinants and

seasonal variation is evident in the infection prevalence [13, 16]. The cercarial shedding occurs from May to October in Korea, and between March to October in Taiwan, which has a more southerly latitude [17, 27].

Seasonal fluctuation was also recognized in the infection rate of fish hosts [16]. In Shandong Province, China, the peak prevalence of *C. sinensis* metacercariae in fish occurred in October-November, and the metacercarial density peaked in November [16]. Thus, it was suggested that, in southern parts of China, the peak risk period for human infection is September to November [16]. In Korea, seasonal tendency of metacercarial density in fish seems less prominent.

8.3.2 Opisthorchis viverrini

Opisthorchis viverrini, the cat liver fluke, infects the bile duct of humans and animals in the Indochina Peninsula. The pathogenesis and pathology are similar to those observed in *C. sinensis* infection. Chronic complications may include pyogenic cholangitis, biliary calculi, cholecystitis, cirrhosis of the liver, pancreatitis, and cholangiocarcinoma. Its potential for inducing cholangiocarcinoma may be higher than that of *C. sinensis* [17]. It needs first and second intermediate hosts and a definitive host for completion of its life cycle.

8.3.2.1 Intermediate Hosts and Mode of Human Infection

The first intermediate host of *O. viverrini* is the freshwater snail, *Bithynia* (*siamensis*) *goniomphalos*, *B.* (*siamensis*) *funiculata*, and *B.* (*siamensis*) *siamensis* in northeast, north, and central Thailand, respectively [28]. In Laos, *B.* (*siamensis*) *goniomphalos* has been shown to play the role of the first intermediate host [29, 30]. In southern parts of Vietnam, cercariae of *O. viverrini* were found from *Melanoides tuberculata* [20]. The cercarial prevalence in the snail host may be quite low, 0.08–1.6 %, but this level is sufficient to maintain the life cycle [28]. However, a recent survey reported that the cercarial prevalence was up to 6.93 % (average 0.73 %) in 25 wetland localities of northeastern Thailand and up to 8.37 % (average 1.08 %) in 23 wetland areas of Laos [31].

Species of freshwater fish reported to be susceptible for *O. viverrini* infection were 18 cyprinoid species which include *Cyclocheilichthys siaja*, *Hampala dispar*, *Puntius orphoides*, *P. gonionotus*, *P. proctozysron*, *P. viehoever*, *Labiobarbus lineatus*, *Esomus metallicus*, and *Osteochilus* sp. [17, 28, 32]. The infection rate of freshwater fish with *O. viverrini* metacercariae is variable according to locality but it can be up to 90–95 % in high endemic areas [33]. The presence of the snail and fish hosts, together with the natural definitive host, is essential for transmission of *O. viverrini* in endemic areas [17].

In northeast Thailand and Laos, it has been well known that *koi pla* is an important food source for *O. viverrini* infection, particularly among Thai of Lao descent

and Laotians [17]. The *koi pla* dish consists of raw fish flesh chopped with garlic, lemon juice, fish sauce, chili, roasted ground rice, and local vegetables [32]. However, the frequency of *koi pla* consumption has declined and generally confined to special social occasions [33]. Instead, the other two types of fish food, called *pla som* (moderately fermented fish for a few days to weeks) and *pla ra* or *jaewbhong* (extensively fermented for at least 2–3 months), has become more important as the infection source of *O. viverrini* [33]. Preserved fish dish like *pla ra* or *jaewbhong* is an important staple consumed daily by 60–98 % of northeastern Thai people and lowland Laotians [33]. However, as the viability of metacercariae depends on the concentration of salt and degree of fermentation, *koi pla* is the most highly infective, followed by *pla som*, and then *pla ra* and *jaewbhong* in which viable metacercariae are rare [33]. *O. viverrini* adult flukes probably survive less than 10 years in the human host [33].

8.3.2.2 Reservoir Hosts

The natural definitive hosts of *O. viverrini* include humans, dogs, cats, rats, and pigs [33]. However, the infection of the animal reservoir hosts is not closely associated with human infections [33]. In areas of high endemicity with *O. viverrini*, humans are the most common definitive host responsible for continuation of the parasite life cycle [32].

8.3.2.3 Geographical Distribution

O. viverrini is distributed widely along the Mekong River basin, particularly in Thailand, Laos, Vietnam, and Cambodia [17, 34, 35]. The estimated number of infected people in total is 9–10 million [36, 37]. The geographical distribution of *O. viverrini* is determined in close relationship with the distribution of the snail (*Bithynia* sp.) intermediate host. The parasite is distributed actually between E 97° (Chiang Mai area, Thailand) and E 107° (Kratie area, Cambodia) and N 10° (Cantho area, Vietnam) and N 20° (Vientiane area, Laos).

In Thailand, this liver fluke is distributed mainly in the north (19.3 % prevalence; likely to include minute intestinal flukes) and northeast (15.7 % prevalence) regions [33], although the prevalence has shown a substantial decline after the 1990s [37]. On the other hand, in Laos, the infection is rather increasingly reported after the 1990s, particularly in those areas along the Mekong River; high prevalences of 53–67 % were documented [17, 38–41]. Even among schoolchildren, the prevalence was considerably high (10.9 %) among 29,846 subjects examined nationwide, with the highest prevalence (25.9 %) found in Savannakhet Province [38]. In Vietnam, there are endemic areas in southern parts, including Phu Yen, Da Nang, Dak Lak, and Binh Dinh [20, 42]. The highest prevalence was found among people in Phu Yen (15.2–36.9 %) [20].

8.3.2.4 Population Epidemiology

The population epidemiology of *O. viverrini* infection is closely related to eating habit of the source food such as "koi pla," "pla som," and "pla ra" in endemic communities. The youngest group (0–5 years) generally shows the lowest prevalence and intensity, while infections in adolescents (15–19 years) often plateaus followed by a decline thereafter [32, 33]. In some areas, the worm burden declines with increase of age, possibly because of a late-developing immune response, lower parasite survival in heavily fibrosed bile ducts, death of parasites in heavily infected persons, or reduced exposure in the elderly [33]. The incidence and worm burdens tend to be greater in males than in females [33].

8.3.2.5 Environmental Factors Related to Transmission

An important factor responsible for the propagation of *O. viverrini* infection is unsanitary latrine system in rural areas [32]. Latrines are often unavailable in remote rural areas, and people have the habit of defecating on the ground in the bush not far from their houses, many of which are situated around the lakes, water beds, or on the bank of streams [32]. The transmission is often seasonal particularly where changes in rainfall and temperature are marked [17]. In endemic countries like Thailand, water pollution by the feces containing *O. viverrini* eggs takes place mainly in the rainy season, and in the last portion of this season and the first third of dry season the largest number of human infections occur [32].

The possible effects of global climate change on *O. viverrini* distribution are debated [37]. Increased temperature is likely to reduce the developmental time of immature stages and might potentially reduce cercarial host-searching time [43]. An increase in rainfall could lead to the extension of wetlands and, therefore, suitable new habitat, with an increased likelihood of parasitic gene flow between them through the expansion of snail and fish host distributions [37].

8.3.3 Opisthorchis felineus

Opisthorchis felineus, the cat liver fluke, infects the bile duct of humans and animals in Eastern and Southen Europe and mid- and western parts of Russia. The pathogenesis and pathology are similar to those observed in *C. sinensis* and *O. viverrini* infection. In chronic infections, anorexia, dyspepsia, dryness of mouth, bitter taste, fatigue, nausea, intolerability for greasy food, and pain in the hypochondrium are the major symptoms [44]. In severe cases, acute pancreatitis, bile peritonitis, hepatic abscess, obstruction of bile ducts, jaundice, and recurrent cholangitis may occur [44]. Its potential for inducing cholangiocarcinoma has been underestimated. In Russia, the highest incidence of cholangiocarcinoma in humans was documented in the same area with the highest incidence of *O. felineus* infection [45].

8.3.3.1 Intermediate Hosts and Mode of Human Infection

The first intermediate host of *O. felineus* is *Bithynia leachi* species complex which include *B. leachi*, *B. troscheli*, and *B. inflata* distributed in East Europe [45]. Various species of freshwater fish take the role of the second intermediate host for *O. felineus*, which include 23 cyprinoid fish species [45]. The chub (*Idus melanotus*), tench (*Tinca tinca* and *T. vulgaris*), bream (*Abramis brama* and *A. sapa*), barbel (*Barbus barbus*), carp (*Cyprinus carpio*), *Blicca bjoerkna*, *Leuciscus idus*, *Alburnus lucidus*, *Aspius aspius*, and *Scardinius erythrophthalmus* are the fish species involved, with the first two being the most commonly infected [32].

In the past decade, humans in European Union got infected with *O. felineus* via ingestion of raw marinated fillets of fish [44]. However, recently in Italy, there was an outbreak of *O. felineus* infection in 211 people from 2003 to 2011 through consumption of the raw fillet of the tench (*T. tinca*) fished from two lakes named Bolsena and Bracciano [44]. Consumption of raw fish fillets occurs at home, in small restaurants along the shores of lakes, and at gastronomic events, and furthermore, raw fillets of the tench are served at restaurants because of their low cost and great availability during the summer as well as their particular taste [44]. Along the tributaries of the Ob river, West Siberia, riparian people eat the freshwater fish, *Rutilus rutilus lacustris* (Siberian chebak roach), freshly salted, dried in the sun, or pickled in garlic juice [32].

8.3.3.2 Reservoir Hosts

The natural definitive hosts of *O. felineus* include humans, carnivorous mammals (dogs, cats, and foxes), chipmunks, beavers, Caspian seals, wild pigs, and domestic pigs [32, 45]. Experimental definitive hosts include the hedgehog, rabbit, guinea pig, house mouse, golden hamster, and black-belled hamster [45]. Stray cats in central Italy revealed 36.6, 73.3, and 40.0 % infection rates in the lake coasts of Bolsena, Bracciano, and Trasimeno, respectively [44].

8.3.3.3 Geographical Distribution

O. felineus is widely distributed from the Iberian Peninsula (Portugal and Spain) to Eastern Europe and West Siberia (north of Kazakhstan) [45]. With regard to human infections, patients were recorded previously in Lithuania (before 1901), Poland (before 1937), Romania (before 1957), and Spain (before 1932) but recently no cases seem to occur in these countries [44]. However, in the last 50 years, many human cases have been reported in European Union (Germany, Greece, and Italy), Eastern Europe (Balarus, Russia, and Ukraine), and West Siberia and Kazakhstan [44, 45]. Recently in Italy, there were eight small outbreaks of human *O. felineus* infection involving a total of 211 people from 2003 to 2011 around two central lakes named Bolsena and Bracciano [44]. The estimated number of infected people in

worldwide total is 1.6 million [36]. Infections in snails, fish, or reservoir hosts were reported in Germany, Italy, Poland, Portugal, Spain, Belarus, Russia, Ukraine, and Siberia [44]. The parasite is distributed between W 5° (Portugal) and E 95° (Kazakhstan and West Siberia) and N 38–40° (Greece) and N 55–70° (Western Siberia) [44].

8.3.3.4 Population Epidemiology

The population epidemiology of *O. felineus* infection is closely related to eating habit of the source food such as raw fish fillets of the tench or marinated fish fillets of other kinds of freshwater fish [44]. In recent outbreaks in Italy, almost all patients were adults aged 16–84 years and only one was a child aged 9 years; 61.4 % were males and 38.6 % were females [44]. In West Siberia, along the Op river, the greatest incidence was found among housewives, who frequently have a chance to consume pickled, salted, or dried freshwater fish [32].

8.3.3.5 Environmental Factors Related to Transmission

The seasonal dynamics of *Bithynia* snail infection with *O. felineus* cercariae is characterized by a one-peak curve, with the peak observed in July [32]. Related with this, the spring season until the late May presents a great epidemiological danger for the snails to be infected with miracidia [32]. Increased temperature and rainfall may adversely contribute to spreading of this infection, although no proper documents related to this have been published.

8.3.4 Metorchis conjunctus, Metorchis bilis, and Metorchis orientalis

Metorchis conjunctus, the Canadian liver fluke, is a parasite of carnivorous mammals in Canada and the USA [46]. Only sporadic and asymptomatic human infections have been found in Canada since 1946, particularly in aboriginal populations from Quebec to Saskatchewan and the eastern coast of Greenland [17, 46, 47]. However, an outbreak of acute infection was reported in 1996 from 19 Korean immigrants in Canada; the victims ate wild-caught fish (*Catostomus commersoni*) prepared in insufficiently cooked but traditional dishes [46]. Fatigue, upper abdominal tenderness, fever, abdominal pain, headache, weight loss, and anorexia were principal clinical presentations of the patients [46]. The first intermediate host is an aquatic snail *Amnicola limosa limosa*, and the second host is several species of freshwater fish (*C. commersoni*, *C. catostomus*, *Salvelinus fontinalis*, and *Perca flavescens*) of which the most important one is the white sucker

C. commersoni [46]. The reservoir hosts include the wolf, fox, coyote, racoon, muskrat, mink, fisher, dog, and cat [46]. Infections have caused death of sled dogs in Central Canada; necropsies indicated that the cause of death was liver damage associated with this fluke infection [46]. This parasite is an emerging fish-borne parasitic zoonosis.

Metorchis bilis is an opisthorchiid trematode infecting carnivorous mammals including humans in Central and Eastern Europe and Western Siberia of Russia [45]. The detailed geographical range of *M. bilis* is thought to considerably overlap with that of *O. felineus* [45]. The detailed status of human infections and clinical manifestations in human patients are yet unknown. The first intermediate host is freshwater snails *Bithynia tentaculata* in European Territory of Russia and Kazakhstan and *Bithynia inflata*, *B. troscheli*, and *B. tentaculata* in Western Siberia [45]. The fish host is the same as those of *O. felineus*; the ide, roach, dace, tench, minnow, gudgeon, verkhavka, and silver carp [45]. The reservoir hosts include the wolf, white tailed eagle, muskrat, otter, and mink [45].

Metorchis orientalis is a liver fluke species infecting piscivorous birds and mammals including humans in East Asia [48–50]. The first report on human infections was made in 2001 which involved 4 (4.2 %) of 95 residents examined in Ping Yuan county of Guangdong Province, China; 12 adult flukes were recovered in two purged patients [49]. However, the detailed status of human infections and potential damage to humans are yet unknown. The geographical range of *M. orientalis* seems to be overlapped with that of *C. sinensis*. The first intermediate host is a freshwater snail *P. manchouricus* (syn. *P. striatulus*), and the fish hosts are various species of cyprinoid fish that include *P. parva*, *Pseudogobio esocinus*, *C. carrassius*, *C. carpio*, and *Zacco platypus* [51]. The reservoir hosts are ducks, dogs, and cats [48].

8.3.5 Fasciola hepatica and F. gigantica

Fasciola hepatica and *F. gigantica*, the sheep liver fluke and the giant cattle liver fluke, respectively, infects the bile duct of domestic animals including cattle and sheep all around the world [1, 52, 53]. The parasite life cycle, pathogenesis and pathology, epidemiology, and clinical symptoms are similar between *F. hepatica* and *F. gigantica*. The juvenile worms migrate through tissues within the abdominal cavity and move to the liver parenchyme and then finally the bile duct or to other ectopic organs [53]. They can cause inflammatory reactions and mechanical damage to the migratory tracks of the flukes. Humans are an accidental host infected through consumption of raw aquatic vegetables or raw liver of infected livestock animals [53]. The acute stage clinical symptoms include fever, abdominal pain, vomiting, loss of appetite, flatulence, diarrhea, urticaria, jaundice, ascites, and anemia [53]. Chronic symptoms may include biliary colic, abdominal tenderness, hepatomegaly, fatty acid intolerance, pruritus, pancreatitis, cholangitis, cholecystitis, severe anemia, and portal cirrhosis [53]. In ectopic infections, the clinical

manifestations vary according to the involved site. The pathogenicity of *F. hepatica* and *F. gigantica* to humans is not recognizably different, although in sheep *F. gigantica* is more pathogenic than *F. hepatica* [27].

8.3.5.1 Intermediate Hosts and Mode of Human Infection

The first intermediate host of *F. hepatica* is a wide variety of freshwater snails of the Lymnaeidae [54]. In Europe, *Galba truncatula* (syn. *Lymnaea truncatula*) is the preferred host snail, and in Africa, *G. truncatula* and *Pseudosuccinea columella* are important ones; in Asia, *G. truncatula* and *Austropeplea ollula* (syn. *A. viridis*) are involved, and in Australia and New Zealand, *Austropeplea tomentosa* (syn. *Lymnaea tomentosa*), *P. columella*, and *G. truncatula* have been recorded [54]. In Hawaii, Papua New Guinea, the Philippines, and Japan, the snail host involved is *A. ollula*, and in North and Central America, *Fossaria humilis*, *F. bulimoides*, *F. cubensis*, and *P. columella* are transmitting *F. hepatica* [54]. In South America, *Fossara viatrix* (syn. *F. viator*), *F. cubensis*, *G. truncatula*, and *Lymnaea diaphana* snails are involved [54].

As for *F. gigantica*, the most important snail species is *Radix auricularia* followed by *Radix rubiginosa*, *R. natalensis*, *Lymnaea rufescens*, *L. acuminata*, and *A. tomentosa* [53]. These snails live in deeper water and are in close to being true aquatic snails in their behavior [53].

Metacercariae of *F. hepatica* are found encysted on leaves of aquatic vegetables (watercress, alfalfa, and water lettuce) or green vegetation, bark, or other smooth surfaces above or below the water line [1, 27, 53]. They are resistant and remain viable for a long period on the plants when moist, but are killed by excessive heat or dryness [27]. In Latin America, France, and Algeria, people frequently acquire the disease as a result of eating raw watercress (*Nasturtium officinale*) on which the metacercariae have encysted [1]. Raw liver of an infected animal can also cause human infections; in these cases, young worms attach to the pharyngeal mucosa and cause pain, bleeding, edema, and dyspnea, of which condition is called “halzoun” [1]. Possibility of liver infection in humans through consumption of raw animal liver remains to be determined.

8.3.5.2 Reservoir Hosts

Mainly sheep, goats, and cattle are natural definitive hosts for *F. hepatica* [55]. Whereas most of the flukes in cattle are eliminated within 9–12 months and they are resistant against challenge infections, sheep and goats are susceptible to reinfection without conferring acquired immunity [55]. A wide variety of other domestic and wild animals as well as laboratory animals can be infected with *F. hepatica*, but they are usually not very important for transmission of human infections [55]. Reservoir hosts of *F. gigantica* include sheep, goat, cattle, buffalo, camel, pig, horse, and other domestic and wild animals [52].

8.3.5.3 Geographical Distribution

The geographical distribution of *F. hepatica* is almost worldwide especially where there is extensive sheep and cattle raising [53]. Approximately 2.4 million people in more than 60 countries are estimated to be infected and the number of people at risk is more than 180 million globally [53]. Human fascioliasis is a serious public health concern in the Andean countries (Ecuador, Bolivia, Chile, and Peru), the Caribbean area (Cuba), northern Africa (Egypt), western Europe (Portugal, France, and Spain), and the Caspian areas (Iran, Turkey, and neighboring countries) [52]. Sporadic cases have been reported in Korea, Japan, China, Thailand, and Vietnam [52, 55]. The highest prevalence and intensity ever reported have been found in the Northern Bolivian Altiplano; in this area, prevalences detected in some communities were up to 72 and 100 % by coprological and serological surveys, respectively [52]. In this area, intensities reached up to 5,000 eggs per gram of feces in children [56]. The geographical distribution of *F. gigantica* is a little different from that of *F. hepatica*, and human infections have been reported mainly in Asia (Japan, Korea, China, Russia, Vietnam, Thailand, Malaysia, India, Iran, and many others), Africa (Sudan, Senegal, Chad, Ghana, Niger, Central African Republic, Tanzania, Kenya, and many others), and Hawaii [27].

8.3.5.4 Population Epidemiology

The prevalence and intensity of *F. hepatica* infection is significantly higher in females than in males in Bolivia and Egypt [56, 57]. This gender role may be related to cultural, hygienic, and behavioral factors, females being occupied in washing household items in large canals where transmitting lymnaeiid snails are present, and agricultural tasks in irrigated plantations such as rice fields [52]. Females are also central to meal preparation in houses including management of freshwater plants potentially carrying attached metacercariae [52]. However, in Spain, the prevalence was similar in males and females [55]. Family clustering of the infected cases is a typical feature because the family shares the same food [55]. Fascioliasis is predominantly a rural diseases, and sheep- and cattle-herders are more frequently affected than other professions [55]. All age groups can be infected; however, those less than 5 years of age had the lowest chance of infection [55].

8.3.5.5 Environmental Factors Related to Transmission

Fascioliasis is unique in its capability of giving rise to human endemic areas from below sea level (on the shores of Caspian Sea) up to very high altitude (in Bolivia, Peru, Ecuador, and Venezuela) [52]. In endemic areas, climatic conditions are critical and decisive for the development of both the *Lymnaea* snails and the flukes and transmission [52, 55]. The snails of *F. hepatica* are more resistant to low temperature compared with high temperature and desiccation; they can survive

through the winter but become weak at low humidity and temperatures over 25 °C [55]. In France, human infections were more frequently observed in the years with heavy rain fall [55]. Human fascioliasis can occur in any seasons but more frequently in cooler seasons of the year [55]. The metacercariae of *F. gigantica* survive longer at high temperatures and are more susceptible to desiccation than those of *F. hepatica* [52].

8.3.6 Dicrocoelium dendriticum and D. hospes

Although *Dicrocoelium dendriticum* and *D. hospes* are liver flukes of mainly sheep, goat, and cattle, it can accidentally infect humans in rare instances [58]. Mere egg positive results in human fecal examinations may represent a spurious infection due to ingestion of infected livers of animals, and genuine infection can be verified through parasite finding in surgical operations, evidence of permanent egg shedding through time, egg recovery in duodenal aspirates, or the existence of related clinical symptoms [27]. The pathology depends on the number of flukes infected and the duration of infection and in many instance without notable symptoms; however, a prolonged period of constipation or diarrhea, nausea, vomiting, abdominal discomfort, and epigastric pain may occur [27]. Mechanical and toxic damages to the host are much less than in fascioliasis and opisthorchiasis, because of the small worm size and its smooth and spineless surface [27].

8.3.6.1 Intermediate Hosts and Mode of Human Infection

More than 90 land snail species have been found to act as competent vectors of *D. dendriticum*, and several of them, in particular, *Cochlicopa lubrica*, are diffused worldwide [58]. *Helicella corderoi* is distributed in Spain, *Zebrina hohenackeri* is in the Caucasus, *Helicella obvia* is in Germany, and *Cernuella virgata* is in Italy [58]. The cercariae are extruded from the snails in clusters of a thousand, enveloped in a mucilaginous substance, commonly known as "slime ball" and are ingested by various (more than 14) species of ants (*Formica fusca*, *F. pratensis*, and *F. rufibarbis*), the second intermediate host [58]. Humans acquire the infection accidentally by swallowing an infected ant together with the food, such as vegetables, fruits, and others while staying in endemic areas [27]. Both *D. dendriticum* and *D. hospes* are zoonotic and are able to establish in the bile ducts of humans [58].

8.3.6.2 Reservoir Hosts

D. dendriticum infection is common in small ruminants in many European and Asian countries, for example, 100 % of sheep flocks having individual burdens of 1,650–2,837 worms [27]. Cattle are also an important reservoir and the prevalence increases

with age [27]. Goats, deer, elk, rabbits, and pigs are less important reservoir hosts [27]. *D. dendriticum* also affects species of wild ruminants, such as camelids (lamas and alpacas) in South America and yaks and buffaloes in India [58].

8.3.6.3 Geographical Distribution

D. dendriticum has a cosmopolitan distribution in herbivorous mammals, mainly ruminants, of the Holartic region [27]. It is distributed almost every country in the European continent and adjacent islands and is found along the northern coast of Africa [27]. In Asia, it is found in Russia, Turkey, Syria, Iran, India, China, the Philippines, and Japan; in the Americas, it is found in the USA, Canada, Cuba, Colombia, and Brazil [27]. *D. hospes* is found in Africa [58].

8.3.6.4 Population Epidemiology

Up to 1982, approximately 300 human cases were reported in the literature based on recovery of eggs in the feces [27]; however, population epidemiology of human dicroceoliasis is unknown.

8.3.6.5 Environmental Factors Related to Transmission

Cercariae may be shed from the snails intermittently at short intervals dictated by sudden climatological changes, such as decrease in atmospheric pressure and temperature, and increase in relative humidity notably during thunderstorm [27]. In sheep pastures in Germany, it was found that the majority of *H. obvia* snails become infected in the autumn of their second year of life, when their shell diameter was of medium size [27]. The formation of the slime balls is associated with a drop in the temperature in the snail environment, and the slime ball output could be provoked only in May and June [27].

8.4 Lung Flukes

Lung flukes infecting humans are eight species around the world, namely, *Paragonimus westermani*, *P. heterotremus*, *P. skryabini*, *P. miyazakii*, *P. africanus*, *P. uterobilateralis*, *P. kellicotti*, and *P. mexicanus* [2, 59]. Human infection with *P. siamensis*, which was originally described from experimental cats in Thailand, was recently documented [60]. However, the species identification of the responsible specimen needs further verification. The estimated number of *Paragonimus*-infected patients globally is 21 million and the number of people at risk is 293 million [61].

8.4.1 Paragonimus westermani

Paragonimus westermani (syn. *P. pulmonalis*, *P. philippinensis*), first discovered in the lungs of a Bengal tiger [62], is the representative species of the lung flukes infecting humans and animals. It is distributed mainly in Asia but recently has also been found in Papua New Guinea and North America [63, 64]. Its pulmonary infection can cause fatigue, chest pain, cough, pleural effusion, dyspnea, bronchiectasis, hemoptysis with rusty-colored sputum, pneumothorax, interstitial pneumonitis, and bronchopneumonia [65]. However, it can also frequently invade other visceral organs, eliciting extrapulmonary paragonimiasis; the brain, spinal cord, and abdominal organs [62, 66]. It needs snails as the first intermediate host and crabs and crayfish as the second intermediate host and a wild animal definitive host for completion of its life cycle in nature. A triploid form of *P. westermani*, once called *P. pulmonalis* [62], was later regarded as a variation within a species, with both being a single origin [67].

8.4.1.1 Intermediate Hosts and Mode of Human Infection

The snail intermediate host includes various species of *Semisulcospira* (*S. libertina*, *S. calculus*, *S. amurensis*, *S. extensa*, *S. multicincta*, and *S. nodiperda*) and *Melanoides tuberculata* [67]. The reported second hosts are numerous species of freshwater crayfish (*Cambaroides similis*, *C. schrenki*, *C. dauricus*, *Procambarus clarki*, and *Macrobrachium nipponensis*) and crabs (*Eriocheir japonicus*, *E. sinensis*, *Parathelphusa maculata*, *P. malaysiana*, *Geothelphusa candidiensis*, *G. miyazakii*, *Hunanopotamon angulatum*, *H. obtusum*, *Potamon hispidum*, *P. hokuoensis*, and *Sinopotamon* spp.) [67]. Human infection is caused by consumption of raw or undercooked freshwater crabs or crayfish. In China, the famous dishes causing human paragonimiasis is "drunken crab" and in Korea, a most common source is "Kejang (= sauced crab)" [68–70]. In Japan, an important infection source is "oboro-kiro (= crab juice soup)" and in Thailand and the Philippines, "goong ten (= raw crayfish salad)" and "kinuolao (= raw crab)," respectively, are responsible for inducing human paragonimiasis [69, 70]. In Korea, the juice of crushed crayfish had been traditionally used as a remedy for measles and other febrile illnesses in children and thus became an important source of infection [68]; however, this tradition has almost disappeared now. Another important mode of infection in Japan is ingestion of raw wild boar meat containing metacercariae or juvenile worms [70–72]. Paragonimiasis patients sometimes claim that they had never eaten freshwater crabs or crayfish. Finger contamination with metacercariae might occur while cooking the crabs or crayfish, and the chopping board or other cooking utensils might also be contaminated [70].

8.4.1.2 Reservoir Hosts

The definitive hosts for *P. westermani* are humans and wild animals that include monkeys, dogs, foxes, wolves, cats, tigers, lions, leopards, pigs, and rats [67]. Wild boars are a paratenic host [70], in which worms do not mature and stay in

muscles and tissues; when these immature worms were eaten by humans, they could develop into adult worms. Experimentally hamsters, mice, rats, guinea pigs, and rabbits can be infected with *P. westermani*; however, worms seldom mature in the lungs of these hosts and thus these animals are also regarded as a paratenic host [67].

8.4.1.3 Geographical Distribution

P. westermani has been reported from various parts of Asia, including China, Taiwan, Korea, Japan, Southeast Siberia, the Philippines, Malaysia, Thailand, Cambodia, Laos, Vietnam, Sri Lanka, India, Nepal, and Pakistan [2, 59, 67]. Recently, the distribution of this species appears to extend to Papua New Guinea [63] and North America, i.e., the USA [64, 73]. More than ten million people are estimated to be infected with *P. westermani*.

8.4.1.4 Population Epidemiology

Because the diagnosis of *P. westermani* is usually difficult to perform by sputum examination to detect eggs (only less than a half of the patients reveal sputum eggs), the prevalence among people has seldom been studied even in highly endemic areas. In the past, intradermal tests to detect antibody reactions were popularly used; however, cross-reactions frequently occurred and its specificity was not sufficiently good. ELISA is currently the most useful tool for the diagnosis of paragonimiasis, and 2.8 % in 1993 (out of 3,973 cases examined) and 1.1 % in 2006 (out of 1,869 cases) seroprevalences were reported among hospital-referred general patients in Korea [74]. However, no much data are available on the seroepidemiological status of infection using ELISA. The prevalence, as estimated by sputum examination, was generally higher in men than in women and higher in aged people than in young children [75, 76]. The higher prevalence in males is probably due to their greater use of crab meat as a stimulant during drinking [75, 76]. However, no substantial difference in the male-to-female ratio was also reported by several workers [65]. Higher prevalence in children than in adults was also reported in Vietnam [77].

8.4.1.5 Environmental Factors Related to Transmission

The snail intermediate host of *P. westermani* is most frequently found in swift-moving mountain streams which may be at some distance from human habitations; such streams are also the preferred habitats for the freshwater crabs or crayfish which serve as the second intermediate hosts [75, 76]. It seems probable that the miracidia which infect the snails come more commonly from eggs passed by wild or domesticated animals harboring the adult flukes than human patients [75].

Therefore, mountainous areas rather than plain areas tend more likely to become endemic areas of *P. westermani*. The optimum temperature for the development of *P. westermani* eggs into miracidia was reported to be from 28 to 32 °C; it took 10–16 days [75]. In temperatures lower than this, 16–25 °C for example, it took longer days (25 days) for the eggs to develop into miracidia [75].

8.4.2 Paragonimus heterotremus

Paragonimus heterotremus (syn. *P. tuanshanensis*) was first found in China in 1964, and now known to be distributed in southern and western parts of Asia [2, 67, 78]. Clinical manifestations are similar to those seen in *P. westermani* infection [62]. In a central area of Thailand, there was an outbreak of infection involving at least 33 (sputum egg-positive cases) persons out of 503 examined [62]. In northern mountainous regions of Vietnam, among 2,216 sputum samples examined, 142 (6.4 %) revealed eggs of *P. heterotremus* [77]. An endemic focus has been found in Nagaland area of India [78].

8.4.2.1 Intermediate Hosts and Mode of Human Infection

The snail intermediate host includes *Assiminea* sp., *Neotricular aperta*, *Oncomelania hupensis chiui*, *O. hupensis formosana*, *O. hupensis hupensis*, *O. hupensis nosophora*, *O. hupensis quadrasi*, *Paludomus* sp., and *Tricula gregoriana* [67, 79]. The second host is the freshwater crab, *Esanthelphusa dugasti* and *Siamthelphusa paviei* in Thailand, and *Potamon flexum* and *Potamiscus smithianus* in China, *Potamiscus tannanti* in Vietnam, and *Potamiscus manipurensis* in India [67, 77, 78]. In Vietnam, people liked to eat raw crab; 68.1 % of people surveyed answered that they had consumed crabs raw [77]. In Nagaland area of India, villagers believed that raw crabs or its extract and soup provided them strength and nutrition, and some believed that ingestion of raw crab extract can cure fever and allergy [78].

8.4.2.2 Reservoir Hosts

P. heterotremus has been found to infect humans, dogs, cats, gerbils, and monkeys in the nature, and mice, rats, and rabbits can be infected experimentally [67]. In Vietnam, the infection rate of dogs in northern mountainous areas was 12.1 % out of 222 dogs examined [77].

8.4.2.3 Geographical Distribution

This species has been found in China, Vietnam, Laos, Thailand, and India [2, 78]. Human and/or animal infections have been found in southern parts of China (Guangxi Province), northern mountainous areas of Vietnam, mountainous areas of

Laos, central Thailand, and northeastern parts of India [62, 77, 78]. This lung fluke may potentially be distributed in Cambodia [62].

8.4.2.4 Population Epidemiology

In an area of northeastern India, the egg positive rate in the sputum was 20.9 % in children (55 of 263 examined) and 4.1 % in adult population (17 of 412 examined); the egg positivity was not significantly different by sex [80]. Little information has been available in other countries.

8.4.2.5 Environmental Factors Related to Transmission

Environmental factors, similar to those of *P. westermani*, may also affect the transmission of *P. heterotremus* infection; however, no much information is available.

8.4.3 Paragonimus skrjabini and P. miyazakii

The Chinese lung fluke, *Paragonimus skrjabini* (syn. *P. szechuanensis*, *P. hueitungensis*), was first described from viverrid cats purchased in a market of Guangzhou, China [67, 81], and is now known to distribute in several Asian countries [62, 82, 83]. Human infections were first found in Sichuan, China [81]. *P. skrjabini* is a zoonotic parasite primarily the lung fluke of animals which is less adapted to humans [70]. Thus, subcutaneous, cerebral, and eye involvements are more frequent than pulmonary lesions [62, 70]. *P. miyazakii*, which morphologically resembled *P. kellicotti*, was first described from dogs fed the metacercariae from *Geothelphusa dehaani* (syn. *Potamon dehaani*) in Japan [84], and later human infections were found in Kanto District, Japan [85]. Pleural infections are more common in *P. miyazakii*-infected patients (a less suitable host) compared with *P. westermani-infected* patients in whom lung parenchymatous lesions are more common [70].

8.4.3.1 Intermediate Hosts and Mode of Human Infection

The snail intermediate host of *P. skrjabini* is *Akiyoshia orientalis*, *Assiminea lutea*, *Tricula* spp. (*T. gushuiensis*), and *Neotricula* spp. [62, 67]. The second host is freshwater crabs, *Sinopotamon denticulatum*, *S. yaanense*, *Aprapotamon grahami*, *Isolapotamon* spp., *Tenuilapotamon* spp., and *Potamiscus manipurensis* [67, 83]. As for *P. miyazakii*, the snail host is *Bythinella kubotai*, *Bythinella nipponica*, and *Oncomelania hupensis nosophora*, and the second host is the freshwater crab

Geothelphusa dehaani (= *Potamon dehaani*) [67]. In China, consumption of raw, undercooked, or picked crab is very common [86], and this may be an important source of infection with *P. skrjabini*. Human infections with *P. miyazakii* occurred by eating raw or improperly cooked potamonid crabs or drinking crab juice, a folk remedy for whooping cough in Japan [62]. Wild pig or boar meat is a significant alternative source of human infections with *P. miyazakii* in Japan [70].

8.4.3.2 Reservoir Hosts

Natural definitive hosts for *P. skrjabini* other than humans include monkeys, dogs, cats, and weasels [67]. Mice, rats, cats, dogs, and monkeys can serve as the experimental definitive hosts [67]. As for *P. miyazakii*, humans, dogs, cats, martens, weasels, and wild boars are natural definitive hosts [67]. Experimental hosts include hamsters, mice, guinea pigs, and rabbit [67].

8.4.3.3 Geographical Distribution

P. skrjabini is known to distribute in 16 provinces of China and some parts of northeastern India [62, 82, 83]. Miyazaki [62] found a worm in Thailand; however, no further reports have been available from Thailand. High prevalences of *P. skrjabini* infection in humans and crabs were recently reported in seven townships surrounding the Three Gorges Reservoir in Yangtze River, China [87]. The distribution of *P. miyazakii* has been reported in Japan; Kyushu, Shikoku, and Honshu Prefectures are the main areas [62]. In Kyushu, Japan, a total of 104 paragonimiasis patients were diagnosed during 1986–1998, among which six were caused by *P. miyazakii* [88].

8.4.3.4 Population Epidemiology

Slightly higher prevalence was noted in *P. skrjabini* infection among the age group of 7–17 years than other age groups in Sichuan and Chongqing Province, China [87]. On the other hand, all of the ten *P. miyazakii* patients first reported in Japan were adults whose age ranged 27–50 years; nine were males and one was female [85].

8.4.3.5 Environmental Factors Related to Transmission

The construction of Three Gorges Dam in Yangtze River, China, beginning in 1994 changed the depth and the flow pattern of the Yangtze River, and environmental conditions for *P. skrjabini* infection have been changed to reveal increased

prevalences of human and crab infections [87]. Abundance of wild animals at surroundings may also facilitate the transmission of *P. skrjabini* and *P. miyazakii*; however, no much information on this point has been available.

8.4.4 Paragonimus kellicotti and P. mexicanus

Paragonimus kellicotti was first found from a cat and a dog in Ohio, the USA [65]. Its autochthonous human infection was first identified in 1986 in Illinois, the USA [89] and total 20 human cases (the majority of cases occurring in Missouri) have been documented [64, 90, 91]. The clinical manifestations are similar to those seen in *P. westermani* infection, and ectopic infections in the brain and the skin were possible [92]. *P. mexicanus* (syn. *P. peruvianus*, *P. ecuadoriensis*, *P. amazonicus*, *P. caliensis*, *P. inca*), according to Tongu [93], was first collected from two opossums in Mexico [94] and is now known to be distributed in many Latin American countries. It has been argued that *P. ecuadoriensis* should be resurrected considering its genetic distinctness from *P. mexicanus* [95]. The clinical manifestations of *P. mexicanus* infection seem not different from those caused by *P. westermani*.

8.4.4.1 Intermediate Hosts and Mode of Human Infection

The first intermediate host of *P. kellicotti* is amphibious snails *Pomatiopsis lapidaria*, *P. cicinnatiensis*, and *Oncomelania hupensis nosophora* [67]. The second hosts include various species of crayfish, i.e., *Cambarus bartoni*, *C. robustus*, *C. virilis*, *Orconectes propinquus*, *O. rusticus*, *Procambarus blandingi*, and *P. clarkii* [67]. The snail intermediate host for *P. mexicanus* is *Aroapyrgus allei*, *A. colombiensis*, and *A. costaricensis* [67]. The second host is the freshwater crabs, *Hypolobocera aequadorialis*, *H. chilensis*, *H. graciliognathus*, *Pseudothelphusa americana*, *P. dilatata*, *P. nayaritae*, *P. propinqua*, *P. terrestris*, and *Ptychophallus* spp. [67]. Some patients of *P. kellicotti* infection ate undercooked crayfish caught from rivers in Missouri, the USA [92]. Mexicans favor cerviche that contain uncooked crustaceans that may contain viable *P. mexicanus* metacercariae [65]. Peruvians eat raw crab with vegetables and lemon juice [70].

8.4.4.2 Reservoir Hosts

P. kellicotti has been found in mammals, including humans, skunks, red foxes, coyotes, minks, dogs, pigs, cats, and bobcats in central and eastern parts of the USA and adjacent areas of Canada [65]. The natural definitive hosts for *P. mexicanus* are humans, opossums, dogs, and cats [67].

8.4.4.3 Geographical Distribution

The geographical distribution of *P. kellicotti* is confined to North America, including mid-west areas and Missisipi Basin of the USA and Atlantic coast, Ontario, and Quebec in Canada [67]. *P. mexicanus* is now known to be distributed in Peru, Ecuador, Costa Rica, Panama, Guatemala, and possibly in Colombia and Brazil, although the species occurring in Colombia and Brazil have not been definitely identified [67, 96, 97].

8.4.4.4 Population Epidemiology

The majority of *P. kellicotti* cases were males and all but one were adults [91]. Little information is available on the population epidemiology of *P. mexicanus*.

8.4.4.5 Environmental Factors Related to Transmission

Transmission of *P. kellicotti* to natural definitive hosts is closely correlated with the ecological condition and availability of the crayfish host. In the case of *P. mexicanus*, environmental conditions favorable for survival of the crab hosts can facilitate its transmission to the wild mammalian hosts.

8.4.5 Paragonimus africanus and P. uterobilateralis

Paragonimus africanus was first found in the mongoose from Cameroon in 1965, and now human infections are known in Cameroon and Nigeria [2, 67, 98]. *P. uterobilateralis* was first described from mongooses in Cameroon in 1965, and its human infection has been found in Cameroon, Nigeria, Gabon, Liberia, and Guinea [2, 67, 98]. The clinical features of *P. africanus* and *P. uterobilateralis* are similar to those caused by *P. westermani*.

8.4.5.1 Intermediate Hosts and Mode of Human Infection

The snail host of *P. africanus* is an aquatic snail *Potadoma freethii* or *Melania* spp., and the second host is the freshwater crabs, *Sudanonautes africanus* and *S. granulatus* (syn. *S. pelii*) [67, 98]. The snail intermediate host of *P. uterobilateralis* is unknown, and the second host is the freshwater crab *Liberonautes chaperi*, *L. latidactylus*, *L. nanoides*, *L. paludicolis*, *Sudanonautes africanus*, *S. granulatus*, *S. aubryi*, and *S. fowleri* [67]. Eating uncooked crabs is the principal mode of infection with *P. africa* and *P. uterobilateralis* [98].

8.4.5.2 Reservoir Hosts

The natural definitive host for *P. africanus* includes humans, black mongooses, civets, drills, and dogs [67, 98]. The natural definitive hosts for *P. uterobilateralis* include humans, dogs, cats, otters, mongooses, swamps, shrews, and other rodents [67].

8.4.5.3 Geographical Distribution

P. africanus is distributed in Cameroon and Nigeria and human infections were found also in these countries [67]. *P. uterobilateralis* is distributed in Liberia, Nigeria, Cameroon, and Gabon, and human infections have been identified in Liberia, Nigeria, Gabon, and possibly in Cameroon [67, 98, 99]. Some lung flukes found in Cameroon and some others discovered in Equatorial Guinea, Benin, and Ivory Coast have not been determined at a specific level [67, 98].

8.4.5.4 Population Epidemiology

In Cameroon, 454 (4.0 %) of 11,236 persons or biological products (intermediate hosts) examined since 1932 have been found infected with *Paragonimus* spp., almost exceptionally *P. africanus* [98, 100]. In a peri-urban area of Cameroon, 2.56 % of 1,482 schoolchildren were sputum/stool egg-positive for *P. africanus*; among symptomatic children, 25 (17.0 %) of 147 boys and 13 (8.0 %) of 162 girls were positive for eggs [101]. In Nigeria, 1,778 (25.0 %) of 7,105 persons or biological products examined since 1939 were found infected with *Paragonimus* spp., particularly, *P. uterobilateralis* [98]. In an endemic village in Nigeria, the prevalence and intensity was higher in children than in adults and males showed higher prevalence and intensity than females [102].

8.4.5.5 Environmental Factors Related to Transmission

No special environmental factors related to transmission of *P. africanus* and *P. uterobilateralis* have not been documented.

8.5 Intestinal Flukes

At least 59 different species have been reported as intestinal flukes infecting humans and animals around the world [103]. Among them, *Metagonimus yokogawai*, *Heterophyes nocens*, *Haplorchis taichui*, *Echinostoma hortense*, *Echinochasmus japonicus*, *Fasciolopsis buski*, *Neodiplostomum seoulense*, *Prosthodendrium*

molenkampi, *Phaneropsolus bonnei*, and *Gymnophalloides seoi* are important in public health points of view [103]. The estimated number of infected people is at least 40–50 million [103].

8.5.1 Metagonimus yokogawai, M. takahashii, and M. miyatai

Metagonimus yokogawai, *M. takahashii*, and *M. miyatai* are minute intestinal flukes which belong to the family Heterophyidae (= heterophyids) [103]. They are distributed mainly in Asia, particularly in the Far East [104]. *M. yokogawai* was originally described in 1912 in Taiwan and then reported from Asian and European countries [104]. *M. takahashii* was originally described in 1930 in Japan and now known to exist also in Korea [104]. *M. miyatai* was originally found in Japan in 1941 but reported as a distinct species in 1993 based on specimens from Japan and Korea [104]. These flukes can cause mild to severe gastrointestinal troubles and indigestion which lead to malabsorption and weight loss. In immunocompromised hosts, the flukes may cause extraintestinal invasion [103].

8.5.1.1 Intermediate Hosts and Mode of Human Infection

The snail hosts for *M. yokogawai* are *Semisulcospira libertina* and *S. coreana*, and those for *M. takahashii* are *S. libertina*, *S. coreana*, and *Koreanomelania nodifila* [104]. The snail hosts for *M. miyatai* are *S. libertina*, *S. dolorosa*, and *S. globus* [104]. The cercariae are of the ophthalmo-pleuro-lophocercous type [104]. After they are shed in water, they freely swim and infect freshwater fish.

The fish hosts for *M. yokogawai* include the sweetfish *Plecoglossus altivelis*, dace *Tribolodon hokonensis* or *T. taczanowskii*, and perch *Lateolabrax japonicus* [104]. On the other hand, the fish hosts for *M. takahashii* include the crussian carp *C. carassius*, carp *C. carpio*, dace *T. taczanowskii*, and perch *L. japonicus* [104]. The fish hosts of *M. miyatai* include *Z. platypus*, *Zacco temminckii*, *P. altivelis*, *T. hakonensis*, *T. taczanowskii*, *Opsariichthys bidens*, *Morocco steindachneri*, and *Phoxinus lagowskii steindachneri* [104]. The metacercariae encyst in the fish muscle but rarely under the scale or in the fin of the fish [104]. The metacercariae can live in the fish hosts for at least 2.5 years [104].

The usual mode of human infection with *M. yokogawai*, *M. takahashii*, and *M. miyatai* is ingestion of raw or improperly cooked flesh of the freshwater fish [103]. Endemic areas are scattered along riparian villages, where local people traditionally eat these raw fish [104].

8.5.1.2 Reservoir Hosts

The definitive hosts of *Metagonimus* flukes are fish-eating birds and mammals, including humans. In case of *M. yokogawai*, dogs, rats, cats, foxes, and kites (bird) harbor the adult flukes [104]. However, the significance of each animal host as the

source of human infections (i.e., as reservoir hosts) has not been verified [104]. Mice, rats, cats, dogs, gerbils, hamsters, and ducks are experimental definitive hosts for *M. yokogawai* [104]. Four (9.8 %) of 41 cats purchased from a market in Seoul had *M. yokogawai* infection [105], and 78 (17.8 %) of 438 feral cats from a market in Busan were found infected with *M. yokogawai* worms (under the name *Metagonimus* sp.) [106]. Pelicans, kites, and other species of birds, and dogs, cats, and other mammals were reported to be the natural and experimental definitive hosts for *M. takahashii* [104]. Rats and various strains of mice were used as experimental definitive hosts for *M. takahashii* [104]. Natural definitive hosts for *M. miyatai* include the dog, red fox, raccoon dog, and black-eared kite, and mice, rats, hamsters, and dogs are experimental definitive hosts for *M. miyatai* [104].

8.5.1.3 Geographical Distribution

The geographical distribution of *M. yokogawai* is wide, from Far Eastern Asia to Middle Europe, although its epidemiological significance in causing human diseases is much less in Europe than in Asia [104]. In Korea, almost all large and small rivers and streams in eastern and southern coastal areas are endemic areas of *M. yokogawai* [103, 107]. The Sumjin, Tamjin, and Boseong Rivers, Geoje Island, and Osip Stream (Samchok-shi, Gangwon-do) are the highest endemic areas with 20–70 % egg positive rates of the riparian residents [104, 108]. The national average prevalence of heterophyid eggs (mostly *M. yokogawai*) in 2004 was 0.5 % and the estimated number of infected people in Korea is about 260,000 [109]. In Japan, the reported prevalence in humans in Japan had been 0.5–35.1 % until the 1960s depending on the locality, which was generally lower than that in Korea. However, in some areas, for example, those along the Takatsu River, Shimane Prefecture, the prevalence among residents in 1965 was high up to 71.9 % among 798 examined [110]. In addition, between 1982 and 1988, an epidemiological survey was performed around the Hamamatsu Lake, Shizuoka Prefecture, in which a 13.2 % egg positive rate among 4,524 lakeside people examined was reported [111]. In Taiwan, where *M. yokogawai* was originally described, the current status of human infections is unknown, although metacercarial infections in fish have been recently documented [112]. In mainland China, little information is available on human *M. yokogawai* infection, although it was mentioned that human infections exsit in Guangdong, Anhui, Hubei, and Zhejiang Province [113]. In Russia, *M. yokogawai* infection is highly endemic in the Amur and Ussuri valleys of the Khabarovsk territory, with the prevalence among the ethnic minority group people of between 20 and 70 % [113]. In the north of Sakhalin Island, the infection rate was 1.5 % among Russians and 10 % among ethnic minorities [113]. In Europe, no human infections are known so far.

The geographical distribution of *Metagonimus takahashii* is rather narrow, only in Japan and Korea [104]. In Japan, this fluke was reported in Okayama, Hiroshima, and around the Lake Biwa [114, 115]. In Korea, the presence of human *M. takahashii* infection was first demonstrated in 1988 from riparian people along the Hongcheon River, Gangwon-do by recovery of adult flukes [116]. Subsequently, an

endemic focus was later discovered in 1993 from Umsong-gun, Chunchungnam-do, along the upper reaches of the Namhan River [117].

M. miyatai was described as *Metagonimus* Miyata type in 1980 in Korea by detecting eggs in the feces, which was slightly larger in size than those of *M. yokogawai* [104]. Later, adult flukes were recovered from 32 people living along the Namhan river in Umsong-gun and Yongwol-gun [117]. In Japan, epidemiological studies particularly on human infections are scarce. Saito et al. [118] enlisted dogs, foxes, racoon dogs, black-eared kites for animal definitive hosts in Shimane, Kochi, and Yamagata Prefectures. Cercariae, rediae, and metacercariae were detected in the Hiroi River basin in Nagano Prefecture [119]. Small rivers in Shizuoka Prefecture were found to have *M. miyatai* infection in fish [120].

8.5.1.4 Population Epidemiology

In a survey performed in Tamjin River basin of Korea in 1977, the egg positive rate of *M. yokogawai* was 26.4 % on average and that of males and females was 35.3 % and 18.6 %, respectively [121]. The prevalence became higher as the age of people was increased [121]. This general pattern of high prevalence in male adult population seems to be consistent. In an endemic area of Samchok-shi, Korea, the egg positive rate was remarkably higher in males (46.6 %) than in females (16.3 %) and the highest in adults aged 61–70 years followed by 41–50 and 51–60 years [108]. Among the young people under 20 years of age, there were no infected cases [108]. Age- and sex-prevalences of *M. takahashii* and *M. miyatai* are essentially the same as those of *M. yokogawai*.

8.5.1.5 Environmental Factors Related to Transmission

Environmental factors related to transmission of *C. sinensis* and *O. viverini* are also applicable to *M. yokogawa*, *M. takahashii*, and *M. miyatai*.

8.5.2 Heterophyes nocens, H. heterophyes, and H. dispar

Heterophyes nocens was first reported in 1916 from experimental dogs and cats fed mullets (*Mugil cephalus*) in Japan [3]. Human infections with this fluke occur in Japan, China, and Korea [103]. Cercariae are shed from brackish water snails, *Cerithidea cingulata* (= *Tympanotonus microptera*) [114]. The second intermediate hosts are brackish water fish, including the mullet or goby *Acanthogobius flavimanus* [122]. Humans become infected by eating raw or inadequately cooked fish hosts. Natural definitive hosts other than humans include domestic or feral cats [103, 123]. Prevalences ranging from 10 to 70 % were detected in residents of southwestern coastal areas, including many islands, of Korea [103, 124].

In Japan, human infections were reported from Kochi, Chiba, Yamaguchi, Chugoku, Hiroshima, and Shizuoka Prefectures [3, 125].

Heterophyes heterophyes was first discovered in 1851 at autopsy of an Egyptian, and is now known to cause human infections along the Nile Delta of Egypt and Sudan [3, 113]. It is also present in Greece, Iran, Turkey, Italy, and Tunisia [3, 113]. The snail host is *Pirenella conica* in Egypt, and the second intermediate hosts are brackish water fish that include *M. cephalus*, *Tilapia nilotica*, *Aphanius fasciatus*, and *Acanthogobius* sp. [3]. The metacercariae of *H. heterophyes* can survive up to 7 days in salted fish [3]. Humans are contracted by eating raw or inadequately cooked brackish water fish. A variety of mammals other than humans takes the role of the reservoir host; dogs, foxes, and jackals [3]. In Egypt, human infections are prevalent on northern parts of the Nile Delta, particularly around the Lakes Manzala, Borollos, and Edco where fishermen and domestic animals frequently consume fish [113]. In Khuzestan, Islamic Republic, the mean prevalence of heterophyid fluke infections was 8 % (2–24 % in range) [113].

Heterophyes dispar was first discovered in 1902 in the intestines of dogs and cats in Egypt, and then from mammals including the fox and wolf in the northern Africa and eastern Mediterranean [113]. Human infections were reported from two Koreans who returned from Saudi Arabia [126] and in Thailand [113]. Brackish water fish are the infection source harboring the metacercariae [103].

8.5.3 Haplorchis taichui, H. pumilio, and H. yokogawai

Haplorchis taichui was described in 1924 from birds and mammals caught in Taiwan [3]. Natural human infections were first reported in the Philippines [127]. This fluke is currently known to be distributed widely in Asia (Taiwan, the Philippines, Malaysia, Thailand, Laos, Vietnam, South China, Bangladesh, India, and Sri Lanka) and the Middle East (Palestine, Iraq, and Egypt) [103]. Hyperendemic areas of *H. taichui* infections, with average individual worm burdens of 21,565 and 12,079 worms in each patient were recently reported in Saravane and Champasak Province, respectively, in Lao PDR [128]. The snail hosts are freshwater dwellers, *Melania obliquegranosa*, *Melania juncea*, or *Melanoides tuberculata* [103]. A wide variety of freshwater fish serve as the second intermediate host; *Cyclocheilichthys repasson*, *Cyprinus auratus*, *Cyprinus carpio*, *Gambusia affinis*, *Hampala dispar*, *Labiobarbus leptocheila*, *Puntius binotatus*, *Puntius brevis*, *Puntius gonionotus*, *Puntius leicanthus*, *Puntius orphoides*, *Puntius palata*, *Pseudorasbora parva*, *Rhodeus ocellatus*, *Zacco platypus*, *Raiamas guttatus*, *Mystacoleucus marginatus*, and *Henichoryhnchus siamensis* [103]. Dogs, cats, and birds are the natural definitive hosts [48].

Haplorchis pumilio was first recorded from birds and mammals in 1886 in Egypt and then subsequently found in Taiwan in 1924 [103]. A successful experimental human infection was reported in 1924 [103], and natural human infections (12 cases) were described for the first time in Thailand in 1983 [129]. The geographical

distribution of this fluke is essentially the same as that of *H. taichui* and is now known to be distributed in the Philippines, Thailand, Laos, Vietnam, South China, Taiwan, Malaysia, India, Sri Lanka, Iraq, Egypt, and Korea [103, 130]. The freshwater snail, *Melania reiniana* var. *hitachiens*, is the first intermediate host, and various freshwater fish species, which belong to the Cyprinidae, Siluridae, and Cobitidae, serve as the second intermediate hosts [103]. The natural definitive hosts include dogs and cats [103].

Haplorchis yokogawai was originally described from dogs and cats experimentally fed the mullet *Mugil cephalus* in Taiwan in 1932 [103]. An experimental human infection with this fluke was successful in Taiwan; however, natural human infections were first reported in the Philippines [127]. Currently this fluke is distributed in the Philippines, South China, Malaysia, Indonesia, Thailand, Laos, Vietnam, India, Australia, and Egypt as human or animal infections [103]. Cercariae are shed from freshwater snails, *Melanoides tuberculata* or *Stenomelania newcombi* [103]. *Cyclocheilichthys armatus*, *Hampala dispar*, *Labiobarbus leptocheila*, *Misgurnus* sp., *Mugil* spp., *Onychostoma elongatum*, *Ophicephalus striatus*, and *Puntius* spp. are the fish intermediate hosts [103]. The natural definitive hosts are dogs, cats, cattle, and other mammals [48].

8.5.4 Centrocestus formosanus and Other Centrocestus species

Centrocestus formosanus (syn. *C. caninus*) was originally described from an experimental dog fed freshwater fish infected with the metacercariae in Taiwan in 1924, with a successful experimental infection in a human volunteer and a natural infection in a fox [103]. Possible occurrence of natural human infections was mentioned in Taiwan and Japan [114, 131], and actual human natural infections were found in China and Vietnam but without detailed description of the worms [103]. Two human infections under the name of *C. caninus* were reported in Thailand [132]. However, seven human infections with a detailed worm description were reported recently in Lao PDR [133]. These cases were with no exceptions mixed-infected with other trematode species including *O. viverrini* and *H. taichui* [133]. The distribution of this fluke is currently almost all over the world, including Asia (Taiwan, China, Japan, the Philippines, Thailand, Lao PDR, Vietnam, and India), Europe (Turkey), and North and South Americas (the USA, Mexico, and Brazil) [103, 133]. The snail, *Stenomelania newcombi*, is the first intermediate host, and the freshwater fish, including *Cyclocheilichthys repasson*, *Puntius brevis*, and *Osteochilus hasseltii*, play the role of the second intermediate host [103]. Natural definitive hosts include dogs, foxes, chickens, and ducks [133].

C. armatus and *C. kurokawai* are the two species for which a human case each has been documented in Korea and Japan, respectively [103, 134]. *C. armatus* was described from experimental dogs, cats, rabbits, rats, and mice fed cyprinoid fish infected with the metacercariae in 1922 [103]. The first intermediate host of

C. armatus is the fresh water snail, *Semisulcospira* sp., and the second hosts are freshwater fish, including *Zacco platypus*, *Zacco temminckii*, *Rhodeus ocellatus*, *Gobius similis*, *Pseudorasbora parva*, and *Pelteobagrus fulvidraco* [103]. Its natural definitive hosts are cats and fish-eating birds that include the large egret *Egretta alba modesta* [103, 123]. There is no information on the life cycle of *C. kurokawai*.

8.5.5 *Pygidiopsis summa*

Pygidiopsis summa was first found in dogs fed brackish water fish infected with the metacercariae in Japan and thereafter reported from Korea [103]. Human infections were found by recovery of eggs in the feces in 1929 and of adult flukes in 1965 in Japan [103]. In Korea, eight human infections were first discovered from a salt-farm village in a western costal area of Okku-gun, Chollabuk-do [135]. It is now known to be distributed also on many western and southern coastal islands in Korea [124]. The snail intermediate host is a brackish water species, *Cerithidea* sp. or *Tympanotonus* sp., and the second host is brackish water fish, including the mullet *Mugil cephalus* and goby *Acanthogobius flavimanus* [103]. Natural definitive hosts other than humans are domestic or feral cats [103, 106].

8.5.6 *Stellantchasmus falcatus and S. pseudocirratus*

Stellantchasmus falcatus was first described from cats experimentally fed the mullet harboring the metacercariae in Japan [103]. Human infections were first known in Japan and thereafter in many Asian-Pacific countries, which include the Philippines, Hawaii, Japan, Palestine, Thailand, Vietnam, and Korea [103]. The first intermediate host is brackish water snails *Stenomelania newcombi* or *Thiara granifera* , and the second host is the mullet and the half-beaked fish *Dermogenus pusillus* [103]. Natural definitive hosts are cats, and experimental hosts include rats and mice [103, 123].

Stellantchasmus pseudocirratus was first described from naturally infected dogs and cats in Palestine, and then in cats, dogs, and mice fed mullet in Taiwan [103]. Human infections were reported in the Philippines and Hawaii [48, 127]. The fish host is the mullet [103].

8.5.7 *Stictodora fuscata and S. lari*

Stictodora fuscata was originally described from cats experimentally fed infected mullets in Japan [3]. The first human infection was found in a young Korean man who regularly consumed raw mullets and gobies [136]. Thirteen additional cases were detected in a seashore village of a southwestern coastal area [3, 107].

The fish hosts include the goby *Acanthogobius flavimanus* and the carp *Pseudorasbora parva* [48, 103]. Natural definitive hosts are feral cats and dogs [48, 106, 123]. Experimental definitive hosts include cats (*Felis catus*) were used as an experimental definitive host [137].

Stictodora lari was first found in the small intestine of the sea gull *Larus crassirostris* in Japan [103, 138]. The first human cases were reported in six people who resided in two southern coastal villages of Korea [139]. The snail host reported is a brackish water gastropod *Velacumantus australis* in Australia [140]. The fish hosts include a number of estuarine fish, including the goby *Acanthogobius flavimanus* [103, 140]. Natural definitive hosts include feral cats [123]. Experimental definitive hosts are cats and dogs [137].

8.5.8 *Echinostoma revolutum* Group (37-Collar Spined Group)

Echinostoma revolutum, together with *E. echinatum* and *E. cinetorchis*, are the three major so-called 37-collar spined, "revolutum" group, *Echinostoma* spp. flukes infecting humans [4]. *E. revolutum* is the oldest species of all echinostomes ever recorded in the literature [4]. It was first found in 1798 from a naturally infected wild duck *Anas boschas fereae* in Germany [141] and is now found widely in Asia, Europe, Africa, Australia, New Zealand, and North and South America [103]. Its human infection was first reported in 1929 in Taiwan, and the prevalence among people in Taiwan was estimated to be between 2.8 and 6.5 % [113]. Human infections have also been known in China, Indonesia, Thailand, Russia, Cambodia, and Lao PDR [103, 142, 143]. In Pursat Province, Cambodia, 7.5–22.4 % of schoolchildren were infected with *E. revolutum* [142]. The fluke infects the intestine of humans and animals (birds and mammals) and can cause gastrointestinal troubles, mucosal ulcerations, and mucosal bleeding [4]. Its first intermediate host includes freshwater snails, *Lymnaea* sp., *Physa* sp., *Paludina* sp., *Segmentina* sp., and *Heliosoma* sp. [1]. The second host includes tadpoles, snails *Physa occidentalis*, *Lymnaea* sp., and *Filopaludina* sp., and clams *Corbicula producta* [1, 113, 144, 145]. The source of infection among schoolchildren in Pursat, Cambodia was undercooked snails and clams of unidentified species sold on the road to their homes after school [142]. Natural definitive hosts are ducks, gooses, rats, muskrats, dogs, and cats [48, 103, 123].

Echinostoma echinatum (syn. *Echinostoma lindoense* Sandground & Bonne, 1940) was originally described from the intestine of mammals in Germany in 1803 [141]. High prevalences (24–96 %) of human infections were reported in Celebes, Indonesia in 1940, under the name of *E. lindoense* [103]. Now this species is known to be distributed widely in Europe, Asia, and South America [103]. The snail hosts include *Lymnaea*, *Planorbarius*, *Planorbis*, *Anisus*, *Gyraulus*, *Biomphalaria*, and *Viviparus* [146]. The second hosts are mussels, *Corbicula lindoensis*, *Corbicula*

sucplanta, and *Idiopoma javanica*, and snails, *Biomphalaria glabrata* [103]. Natural definitive hosts include birds and mammals [146]. Rats, mice, ducks, and pigeons can be experimental definitive hosts [1, 147].

Echinostoma cinetorchis was first discovered in 1923 from rats in Japan [103]. Human infections were first reported in Japan, and then in Korea and China [103]. The snail hosts are *Hippeutis cantori* and *Segmentina hemisphaerula* [103]. The second hosts include a wide range of freshwater snails (*H. cantori*, *S. hemisphaerula*, *Radix auricularia coreana*, *Physa acuta*, *Cipangopaludina chinensis malleata*, and *Cipangopaludina* sp.), tadpoles (*Rana nigromaculata*, *R. rugosa*, and *R. japonica*), and freshwater fish (especially the loach *Misgurnus anguillicaudatus*) [103]. Natural definitive hosts include rats [103]. Experimental definitive hosts include mice and rats [148].

8.5.9 Echinostoma hortense and E. ilocanum

Echinostoma hortense was originally described in 1926 from rats in Japan, and then from rats in Korea and China [103]. Human infections were first reported in Japan in 1976 and thereafter in Korea and China [103, 107]. In Liaoning province, China, six patients who had eaten raw loach were found infected, and in Cheongsong-gun, Korea, 22.4 % prevalence was reported among the residents living along a small stream where various species of freshwater fish are available [103]. Occasionally, cinical cases with significant abdominal symptoms are diagnosed by extracting worms through gastroduodenal endoscopy in Korea [103]. The snail host includes *Lymnaea pervia* and *Radix auricularia coreana*, and the second hosts include loaches, *Misgurnus anguillicaudatus* and *M. mizolepis*, and several other species of freshwater fish, including *Odontobutis obscura interrupta*, *Moroco oxycephalus*, *Coreoperca kawamebari*, and *Squalidus coreanus* [103]. In Liaoning province, China, 69.7 % of the loach *M. anguillicaudatus* from a market was infected with *E. hortense* [113]. Natural definitive hosts include rats, dogs, and cats [103, 123]. Experimental infection was successful in mice, rats, and humans [149].

Echinostoma ilocanum was first found in 1907 from five prisoners in Manila, the Philippines [103]. Subsequently, human infections have been found in Celebes, Java, Indonesia, China, Thailand, the Philippines, India, and Cambodia [103, 150]. In northen Luzon, the Philippines, the prevalence among the Ilocano population ranged from 7 % to 17 % [103], and in Oddar Meanchey Province, Cambodia, the prevalence in students and general population was 0.7 % and 1.8 %, respectively [150]. The snails host includes *Gyraulus* or *Hippeutis*, and the second hosts are large snails, *Pila conica* (the Philippines) and *Viviparus javanicus* (Java); these large snails are the source of human infections [1, 113]. Natural definitive hosts are rats and dogs [1].

8.5.10 *Echinochasmus japonicus, E. lilliputanus, and Other Echinochasmus spp.*

Echinochasmus japonicus was first described in 1926 from experimental dogs, cats, rats, mice, and birds fed the metacercariae encysted in freshwater fish in Japan, and is now known to occur in the Far Eastern countries [103, 107]. A successful experimental human infection was reported in Japan, and natural human infections were found in China and Korea [103]. In Fujian and Guangdong Province, China, *E. japonicus* is prevalent among people (4.9 %) and animals (39.7 % in dogs and 9.5 % in cats) [113]. The snail hosts include *Parafossarulus manchouricus*, and the reported second hosts are 18 species of freshwater fish, including *Pseudorasbora parva*, *Hypomesus olidus*, and *Gnathopogon strigatus* [151, 152]. Natural definitive hosts are ducks, egrets, cats, and insectivores [103].

Echinochasmus liliputanus was originally described from dogs, cats, and birds in Egypt, Syria, and Palestine [48]. Human infections were first discovered in Anhui Province, China in 1991, the prevalence being 13.4 % among 2,426 people examined [113]. Thereafter, more than 2,500 human cases have been reported in Anhui Province, China [153]. The snail host is *Parafossarulus striatulus*, and the fish host is *Pseudorasbora parva* and goldfish [153]. Humans may be infected with this parasite through consumption of raw or improperly cooked fish or drinking untreated water containing the cercariae [103]. Natural definitive hosts include badgers, foxes, raccoons, dogs, and cats [103].

Echinochasmus perfoliatus was first described from dogs in Romania in 1902, and then found from dogs and cats in Hungary in 1908 [103]. It is now a common intestinal fluke of dogs and cats in Hungary, Italy, Romania, Russia, Japan, China, and Taiwan, and of red foxes in Denmark [103]. An experimental and a natural human infection were reported in Japan [103]. After then, 1.8 % prevalence was reported among people in Guangdong, Fujian, Anhui, and Hubei Provinces of China [113]. The snail host includes *Parafossarulus manchouricus*, *Bithynia leachi*, and *Lymnaea stagnalis* [48]. The fish hosts are *Carassius* sp., *Zacco platypus*, *Zacco temminckii*, and *Pseudorasbora parva*, and the metacercariae are encysted only on the gills [113, 147]. Natural definitive hosts are rats, cats, dogs, foxes, fowls, and wild boars [103].

Echinochasmus fujianensis was first described in 1992 from humans, dogs, cats, pigs, and rats in Fujian Province, China [113]. In five areas of Fujian province, China, the prevalence among residents ranged 1.6–7.8 %, and about two-thirds of the infected people were children of 3–15 years [113]. The snail host is *Bellamya aeruginosa*, and the fish hosts are *Pseudorasbora parva* and *Cyprinus carpio* [113]. Natural definitive hosts include dogs, cats, pigs, and rats [113].

Echinochasmus jiufoensis was described as a new species in 1988 found at an autopsy of a 6-month-old girl who died from pneumonia and dehydration in Guangzhou, China [154]. Its life cycle is unknown.

8.5.11 *Artyfechinostomum malayanum and A. oraoni*

Artyfechinostomum malayanum (syn. *Echinostoma malayanum*; *Artyfechinostomum sufrartyfex*, *Artyfechinostomum mehrai*) was first described from a human in Malaysia under the name of *Echinostoma malayanum* [1], and then found in Malaysia, Singapore, Thailand, Indonesia, India, the Philippines, and Lao PDR [103, 143]. The first intermediate host is a freshwater snail, *Indoplanorbis exustus* and *Gyraulus convexiusculus*, and the cercariae encyst in various species of snails, i.e., *Pila scutata*, *Lymnaea* (*Bullastra*) *cumingiana*, and *Digoniostoma pulchella* [113]. Natural definitive hosts include humans, pigs, rats, cats, dogs, mice, hamsters, and house-shrews [1, 103].

Artyfechinostomum oraoni was first reported in 1989 from 20 human infections in a tribal community near Calcutta, India [155, 156]. The snail host is *Lymnaea* sp. [157], and the second host is unknown. *A. oraoni* may provoke fatal diarrhea in pigs [156].

8.5.12 *Acanthoparyphium tyosenense and Echinoparyphium recurvatum*

Acanthoparyphium tyosenense was first described in 1939 based on worms recovered from the small intestines of the duck *Melanitta fusca stejnegeri* and *M. nigra americana* caught in Korea [4]. It is now known to be distributed in Korea and Japan [158]. Human infections were first discovered in two coastal villages of Chollabuk-do (Province), Korea [159]. The snail host is marine megagastropods *Lunatia fortuni* and *Glassaulax didyma* [158] or marine gastropods *Tympanotonus microptera*, *Cerithidea cingulata*, and *Cerithidea largillierti* [103]. The second hosts include brackish water bivalves, i.e., *Mactra veneriformis*, *Solen grandis*, *Solen strictus*, and *Ruditapes philippinarum*, and brackish water gastropod, *Neverita bicolor* [158, 159]. The patients in Korea used to eat improperly cooked marine bivalves and gastropods [159]. Natural definitive hosts include ducks, and experimental infection of chicks and sea gulls *Larus crassiostris* was successful [103].

Echinoparyphium recurvatum was first found in 1873 from various avian species, and now recognized as a parasite of also mammals [103]. The first human infection was identified in Taiwan in 1929 and then in Indonesia and Egypt [1, 113]. The snail hosts include *Physa alexandrina*, *P. fontinalis*, *Planorbis planorbis*, *Lymnaea pervia*, *L. peregra*, *Valvata piscinalis*, and *Radix auricularia coreana* [103, 160]. The second hosts are tadpoles and frogs of *Rana temporaria* and snails, *P. planorbis*, *Lymnaea* sp., *R. auricularia coreana*, and *Lymnaea stagnalis* [103]. Natural definitive hosts are house rats, wild rats (*Arvicanthis niloticus*), and various species of birds [103].

8.5.13 *Hypoderaeum conoideum and Isthmiophora melis*

Hypoderaeum conoideum was discovered in 1872 from various species of birds in Europe and is now known to be an intestinal fluke of the duck, goose, and fowl in Europe, Japan, and Siberia [1, 48, 147]. Human infections were first reported from northeastern Thailand, where the prevalence of *H. conoideum* was 55 % among 254 residents examined [161]. The snail host includes *Planorbis corneus*, *Indoplanorbis exustus*, *Lymnaea stagnalis*, *L. limosa*, *L. ovata*, *L. tumida*, *L. pegrera*, *L. corvus*, and *L. rubiginosa*, and the second hosts are snails and tadpoles [103].

Isthmiophora melis was first described in 1788 from rodents and carnivores in Europe and North America [48, 147]. Human infection with *I. melis* was first confirmed in 1916 in a diarrheic patient in Romania and then in an autopsy case of a Chinese patient [1]. Further human cases were reported in China and Taiwan [113]. The snail host is *Stagnicola emarginata angulata*, and the second host includes tadpoles and presumably the loach, a kind of freshwater fish [113]. Natural definitive hosts include various domestic and wild animals [103].

8.5.14 *Fasciolopsis buski*

Fasciolopsis buski, the largest fluke among those parasitizing the human host, was first discovered in 1843 in the duodenum of an Indian sailor [1, 52, 162]. This fluke is now known to be a common intestinal parasite of humans and pigs in China, Taiwan, Thailand, Vietnam, Laos, Cambodia, Bangladesh, India, Indonesia, Myanmar, the Philippines, Singapore, and Malaysia [103, 162]. The number of population infected with *F. buski* is estimated to be at least ten million in southern Asia [162]. The prevalence among human population ranged from 0.04 % in Cambodia to 8.6–50 % in Bangladesh, 25–61 % in Taiwan, and up to 85 % in some areas of China [162]. The snail host is *Segmentina* sp., *Hippeutis* sp., and *Gyraulus* sp. [52]. Metacercariae may float in the water or they can attach to the body surface of aquatic plants, such as water chestnut *Eliocharis tuberose*, water caltrop *Trapa natans*, water hyacinth *Eichhornia* sp., roots of the lotus, water bamboo *Zizania* sp., and other aquatic vegetations *Salvinia*, *Valisneria*, and *Eichornia* spp. [1, 113, 162]. Consumption of raw or improperly cooked aquatic plants, or peeling off the hull or skin of the plants by mouth before eating the raw nut, is the principal mode of human infection [113]. Natural definitive hosts include pigs, dogs, and rabbits [162].

8.5.15 *Gymnophalloides seoi*

Gymnophallloides seoi was first discovered in 1988 from a Korean woman suffering from acute pancreatitis and gastrointestinal discomfort [163, 164]. The home village of the patient, a southwestern coastal island (Aphaedo Island, Shinan-gun,

Korea), was subsequently found to be a highly endemic area with 49 % prevalence and heavy worm loads of 106–26,373 worms per person [164]. Now *G. seoi* is known to be distributed on 25 seashore villages of western and southern coastal islands and three coastal villages (land) of Korea [103]. The first intermediate host has not been determined. The second host is oysters *Crassostrea gigas* [164]. People in endemic areas are infected though consumption of raw oysters. Aged people tend to show higher infection rate than children but there is no sex difference in the prevalence. Natural definitive host other than humans is the Palearctic oystercatchers *Haematopus ostralegus* and feral cats [165, 166]. Experimental definitive hosts are wading birds, such as the Kentish plover *Charadrius alexandrinus*, Mongolian plover *Charadrius mongolus*, grey plover *Pluvialis squatarola* and mammals including gerbils, hamsters, cats, and several strains of mice [167, 168].

8.5.16 *Spelotrema brevicaeca and Gynaecotyla squatarolae*

Spelotrema brevicaecae was originally reported in 1935 from birds and accidentally also from a human autopsy case and subsequently from 11 additional cases in the Philippines [127]. Eggs of this fluke caused acute cardiac dilatation and egg granuloma in the heart, brain, and spinal cord [127]. The snail host is unknown. The second host is a brackish water crab *Carcinus maenas* and shrimp *Macrobrachium* sp. [1]. Natural definitive hosts include birds and mammals including monkeys [169].

Gynaecotyla squatarolae was originally reported in 1934 from birds in Japan under the name *Levinseniella squatarolae* [169]. Human infection was first documented in 2011 from a Korean female who habitually consumed brackish water crabs in soy sauce [130]. The clinical significance of this fluke is unknown. The snail host is not yet determined. The second host is backish water crabs *Macrophthalmus dilatatus*, *M. japonicus*, and *Helice depressa* [169, 170]. Natural definitive hosts are avian species that include *Squatarola hypomelaena*, *Erolia alpina sakhalina*, and *Arenaria interpres interpres* [169, 171].

8.5.17 *Neodiplostomum seoulense*

Neodiplostomum seoulense was originally described in 1964 from house rats in Korea [172]. This parasite is now known to be distributed countrywide among rodents in Korea, predominantly in mountainous areas [172], and also in a northeastern part of China [103]. Human infection was first reported in 1982 from a young man who suffered from acute abdominal pain and fever and had a history of consuming improperly cooked snakes 7 days prior to hospital admission [172]. Twenty-six additional human cases were found from soldiers who had eaten raw snakes during their survival training [103, 173]. The snail host is *Hippeutis cantori* and *Segmentina* (*Polypylis*) *hemisphaerula* [103]. The second host includes

tadpoles and frogs of *Rana* sp.; the snake *Rhabdophis tigrina* is regarded a paratenic host [172]. Natural definitive hosts are rats and mice [172]. Experimental definitive hosts include mice, rats, and guinea pigs [172].

8.5.18 *Prosthodendrium molenkampi and Phaneropsolus bonnei*

Phaneropsolus bonnei was first described in 1951 from a human autopsy in Indonesia, and then found in monkeys in Malaysia and India in 1962 [174]. Subsequently, this fluke was reported from 15 human autopsies in northeast Thailand [174]. Thereafter, high prevalences were found in various localities of northeast Thailand and Lao PDR [103]. The snail host is presumed to be *Bithynia goniomphalos* but needs confirmation [175]. The second host is insects, particularly the naiad and adult stages of dragon- and damselflies [175]. Local people in northeast Thailand and Laos occasionally eat naiads of these insects [103]. Natural definitive hosts other than humans include monkeys [175].

Prosthodendrium molenkampi was first described in 1951 from two human autopsies in Indonesia [175]. Subsequently, this fluke was found in 14 human autopsies in northeast Thailand [174, 175]. Since then, high prevalences were reported in different areas of northeast Thailand and Lao PDR [103]. The snail host is presumed to be *Bithynia goniomphalos* but needs confirmation [175]. The second host is the naiad and adult stages of dragon- and damselflies [175]. People in northeast Thailand and Laos occasionally eat naiads of these insects [103]. Natural definitive hosts are monkeys, rats, and bats [175].

8.5.19 *Plagiorchis muris and Other Plagiorchis spp.*

Plagiorchis muris was originally described in 1922 based on worms recovered from mice experimentally infected with the metacercariae in Japan [176]. An experimental human infection was reported in the USA, and natural human infections have been documented in Japan and Korea [103, 176]. The snail host is *Lymnea pervia* in Japan and *Stagnicola emarginata angulata* in the USA [103]. The second hosts include mosquito larvae, insect naiads, freshwater snails, and freshwater fish [176]. Natural definitive hosts are rats, mice, and cats [103]. Rats can be used as an experimental definitive host [176].

Plagiorchis harinasutai was reported as a new species in 1989 based on specimens recovered from four human cases in Thailand [177]. The life cycle, pathology, clinical manifestations, and public health importance are not known [177].

Plagiorchis javensis was originally described in 1940 from a human case in Indonesia, and two additional cases were reported in Indonesia [113]. The second host is presumed to be larval insects, and reservoir hosts are birds and bats [113].

Plagiorchis philippinensis was first discovered in 1937 at autopsy of a resident in Manila, the Philippines [113]. The second host is insect larvae, and reservoir hosts include birds and rats [113].

Plagiorchis vespertilionis was originally described in 1780 from the brown long-eared bat in Europe, and then found in many countries, including Korea [178]. Human infection was reported in a Korean patient who habitually consumed raw flesh of snakehead mullet and gobies [178]; however, whether these fish took the role for a source of infection is unclear. Its clinical significance is unknown. The snail host is *Lymnaea stagnalis*, and the second hosts include mosquito larvae, caddis-fly larvae, mayfly larvae, and dragonfly nymphs [179].

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Chapter 9
Diagnosis of Human Trematode Infections

J. Guillermo Esteban, Carla Muñoz-Antoli, Rafael Toledo, and Lawrence R. Ash

9.1 Introduction

Digenetic trematodes, or digeneans or flukes, are a major group of human parasites included within the phylum Platyhelminthes, commonly called "flatworms." There is a considerably large number of fluke species parasitizing humans, although some of them are only sporadically reported in humans or these may be isolated cases. However, this chapter focuses on the diagnosis of species that are of great medical importance as a large number of people are infected by them.

The human trematode species that are the subject of the present chapter are grouped according to the typical microhabitat in which the adult parasite usually resides. In this sense, four different groups will be considered: liver, lung, intestinal, and blood flukes. However, among the various parasite stages involved in the life cycle of each species, the egg and the adult specimen can be found in the definitive host and can be used for the diagnosis of the trematode infection. Nevertheless, this chapter focuses mainly on the parasitological diagnosis based on the finding and recognition of eggs as they can be obtained more easily. Thus, in basic diagnosis, the clinical samples usually required for finding eggs of the trematode species included in each of these groups are as follows: feces for liver, lung, intestinal, and blood flukes; urine for the only blood fluke species; and sputum for lung flukes. But the possibility that parasites may occur in sites other than their usual locations must always be considered.

J.G. Esteban (✉) • C. Muñoz-Antoli • R. Toledo
Departamento de Biología Celular y Parasitología, Facultad de Farmacia,
Universidad de Valencia, Av. Vicent Andrés Estellés s/n, Burjassot, 46100 Valencia, Spain
e-mail: Jguillermo.Esteban@uv.es; Carla.Munoz@uv.es; Rafael.Toledo@uv.es

L.R. Ash
Infectious & Tropical Diseases, Fielding School of Public Health, University of California,
650 Charles E. Young Drive South, Los Angeles, CA 90095-1772, USA
e-mail: larryash@ucla.edu

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In the first part of this chapter, the current status of knowledge on diagnostic techniques used when examining feces, urine, and sputum, which are the clinical samples used for direct or parasitological diagnosis based on the search, finding, and identification of trematode eggs, is reviewed. Moreover, some general comments are made on other diagnostic techniques.

The second part of the chapter is dedicated to expose the most significant characteristics used to identify eggs and also to other aspects concerning the patient's anamnestic data, as well as the analysis of the different morphological characteristics or particular features that have to be considered in the establishment of a precise diagnosis of the species included within each genus, for each of the trematode groups. Nevertheless, diagnosis at species level based on eggs might be complicated or even impossible given the morphological convergence and similarity of some trematode eggs. Thus, it might be convenient to carry out diagnosis at genus, or even family, level. Furthermore, some complementary information on other diagnostic techniques used, not exclusively based on the finding and recognition of eggs, is given.

9.2 Diagnostic Techniques for Human Trematodiases

9.2.1 Parasitological Techniques

Microscopic demonstration of trematode eggs in feces, urine, and sputum remains the most widespread tool in fluke diagnosis. For each sample, the main techniques that can be used in parasitological diagnosis are compiled, which are habitually applied in laboratories of clinical parasitology as they are fundamental, well known and can easily be reproduced. Nevertheless, it must be highlighted that there is no technique that may be considered definitive and unique and may fail when used in a single sample.

For fecal samples: flukes produce eggs, mostly passed in feces. As a consequence, fecal samples can be used for their diagnosis and the analysis of more than one sample per patient always increases the likelihood of a finding, particularly when keeping in mind the natural day-to-day fluctuations of fecal egg excretion.

Diagnosis can be carried out by wet mount, which is the simplest and most widely used technique for the examination of feces, and this method should be performed in all laboratories, especially in cases of heavy infections. However, if the number of eggs is low, as in cases of light infections, the examination of a small amount of feces used for a direct wet mount may be insufficient to reveal their presence. As the burden or intensity of parasitization of a patient cannot be known beforehand, the use of concentration techniques is always required.

Concentration techniques through the flotation of parasites in aqueous zinc sulfate, a saturated salt solution, or a magnesium sulfate solution, are probably less efficient and, therefore, appear to be inappropriate for the detection of some flukes,

basically those producing large eggs (i.e., *Fasciola, Fasciolopsis, Schistosoma*). Consequently, sedimentation procedures, achieved by simple gravity or through centrifugation, are probably most frequently used in diagnostic laboratories as the sediment will generally contain the eggs present in the stool sample. Among the different sedimentation techniques by centrifugation, also called "diphasic sedimentation," the formalin–ether (or formalin–ethyl acetate; or formalin–gasoline) sedimentation and the MIF concentration techniques, depending upon the type of preservative used for fixed fecal samples (SAF or Formalin 10 % and MIF, respectively), or some modifications of them, are the most highly effective ones [1–5]. A new technique for diagnosis has been proposed based on the differential sedimentation of eggs of *S. mansoni* when subjected to a slow continuous flux of 3 % saline solution through a porous plaque [6].

Quantitative coprological data analysis in epidemiological surveys is also possible using different quantitative methods. Stoll's dilution egg counting technique was one of the first techniques used [7].

Although not being habitually applied in hospital laboratories, the Kato–Katz technique is most widely used for diagnosing flukes in field surveys in numerous countries as it is simple, quantitative, inexpensive and provides reproducible results [8–10], although sensitivity and negative predictive values are highly dependent on the prevalence and intensity of infection among the population. In this sense, when multiple samples (two or three repeated stool examinations on three consecutive days) are microscopically examined, a significant increase in the sensitivity of this technique is observed. However, among the various drawbacks of this technique, it should be cited the fact that egg recognition between semipermeable cellophane and the microscope slide might often be difficult due to the deficient observation of some morphological details between slide and cover stands out.

The more recent FLOTAC technique [11, 12] is a multivalent fecal egg count technique originally developed in veterinary medicine which has shown to be highly efficient in the diagnosis of soil-transmitted helminth infections [13–15]. Although studies proving its wide applicability for the diagnosis of trematodiases are still scarce, this technique has shown to be useful, at least until now, for the diagnosis of schistosomiasis caused by *Schistosoma mansoni* [16], human dicrocoeliasis [17–19], and even experimental fascioliasis in rats [20]. However, it is a more complicated technique and, consequently, may require improvements in terms of cost and accessibility for basic laboratories [15], as well as an adequate training of laboratory workers.

For embryonated eggs containing a miracidium when passed in feces, i.e., schistosome eggs, the miracidial hatching technique [21], using a side-arm distillation flask, can be applied to help in the detection of miracidia that have hatched from the eggs. This long-established, yet tedious technique is a traditional approach for assessing trematode infection and has been used for more than five decades, and continues to be widely used, in some countries, e.g., China [22]. This method is simple, and its potential for high sensitivity has been recognized but not standardized for quantitative measurement [23]. A similar technique has more recently been proposed for diagnosing schistosomiasis in individual or large-scale investigations [24].

Finally, a new diagnostic technique, named by the authors "Helmintex," has been developed for the detection of schistosome eggs through their interaction with paramagnetic beads in a magnetic field [25]. Its high sensitivity may improve diagnosis in cases of light infections, e.g., in infected travelers with low burdens, although its cost-effectiveness must be assessed in relation to other techniques.

For urine samples: urine specimens are primarily examined for *S. haematobium* eggs, although it should also be noted that subjects with *S. mansoni* infection may pass eggs of this parasite in their urine. As the number of eggs in urine varies throughout the day, a single collection of terminal urine of at least 10 mL should be collected between 10 am and 2 pm, with prior physical exercise combined with the intake of liquids. Alternatively, a 24-h collection of terminal urine can be made. The entire sample must be examined as eggs may be very scarce. If urine is kept for an hour or longer, add 1 mL of undiluted formalin (37 % formaldehyde solution) to each 100 mL of urine. This will preserve any eggs that might be present, but if not available, 2 mL of ordinary household bleach can be added to each 100 mL of urine [5, 9].

The methods used for the detection of eggs are based on sedimentation and filtration. The sedimentation method for a 24-h terminal urine collection is less sensitive but cheaper and simpler to perform. The syringe filtration method, using a polycarbonate or nylon filter (pore size 12–20 μm), or alternatively a paper filter, placed in a filter holder (diameter 13 or 16 mm), is used in public health care mainly when quantitative information is required [9].

The miracidium hatching test, described above in fecal samples, may also be used for examining large quantities of urine.

For sputum samples: human paragonimiasis, the only lung fluke infection, may be diagnosed by finding characteristic eggs in sputum. In this infection, the sputum is often viscous and may be tinged with blood and brownish material (so called "iron filings"). When examined on a direct smear, it will show clusters of typical *Paragonimus* eggs. To ensure a good sample, the sputum must be collected in the early morning and the patient's mouth has to be rinsed with hydrogen peroxide beforehand. The sputum may be directly centrifuged and the sediment examined as a direct wet mount; if it is viscous, an equal volume of 3 % sodium hydroxide must be added, centrifuged and the sediment examined; or a formalin–ether concentration procedure may be performed on the sample before examination [5].

A recent study documents the usefulness and validity of the Ziehl–Neelsen staining technique for the detection of *Paragonimus* eggs in sputum slides, which appears to have superior sensitivity to the standard wet film direct sputum examination, the best cost-effectiveness, and, moreover, eliminates the risk of tuberculosis transmission [26].

Due to the difficulties in obtaining a good clinical sample containing material from the lower respiratory passages, rather than a superficial sample consisting primarily of saliva, it is more common to look for *Paragonimus* eggs in feces using any of the aforementioned techniques for fecal samples.

9.2.2 Other Diagnostic Techniques

When patients are in the invasive or acute phase of the disease (consequently without egg production), or with a low parasite burden (therefore, with low shedding or even the absence of egg shedding), in chronic, ectopic or even spurious infections, or when flukes are unable to reach maturity, parasitological techniques are inappropriate, and consequently other diagnostic techniques have to be applied for human fluke diagnosis. In this context, noninvasive (based on imaging diagnosis) and invasive (based of obtaining different aspirates and body fluids) techniques should be mentioned.

In the course of their normal life cycles, the adult specimens of trematodes settle in organs and tissues, in such a manner that if these are examined along that time, the adult flukes or even the eggs may be found. Some trematodes, however, may migrate to organs or tissue other than typical, so-called "ectopic sites." Consequently, biopsy material or tissue removed during surgery or at necropsy can be examined directly or can be fixed and examined as stained histological sections for diagnostic purposes. It is a sensitive and specific clinical diagnostic method in routine clinical practice, being neither simple nor convenient for population-based surveys. The recognition and proper identification of parasites, even their broad classification, in microscopic sections of human tissue may be impossible unless the microscopist has considerable experience. In this sense, the specimens should be submitted to parasitological experts. In addition, several texts and reviews that provide detailed information on the pathology of trematode infections and the morphology of parasites in tissue can also be consulted [27–29].

Immunodiagnostic tests provide the advantage of being applicable during all stages of the disease and consequently they are commonly used in the diagnosis of trematode infections in field situation. However, routine diagnosis in a laboratory of clinical parasitology is a very different matter.

Among the different immunodiagnostic techniques used, the enzyme linked immunosorbent assays (ELISA) is the most sensitive and most widely used routine method for diagnosing many trematode infections. Nevertheless, all these immunological methods present problems related to the methodology used in the process of antigen obtention as well as false positive results as a consequence of antigen and antibody detection even after parasitological cure. Moreover, they often cannot distinguish between current and past infection and may even present different levels of cross-reactivity. For these reasons, molecular tools should be considered despite their higher cost and the requirement for special laboratory equipment [30].

In recent years, attention has focused on teleparasitology as a diagnostic tool as it allows the rapid Web transfer of parasitological microscopic images from specimen preparation in the field or laboratory to a local o central server where an expert in the field in question is able to establish a fast and qualified diagnosis [31].

9.3 Parasitological Features

9.3.1 General Features

Each trematode species produces eggs that exhibit a natural biological variation in size, but are, for the most part, highly uniform in shape, color, and developmental stage. When an egg or an egg-like object is found, some features should be carefully observed in order to make a specific identification. According to Ash and Orihel [32], the most significant characteristics used to identify eggs are as follows:

Size: length and width constitute suitable criteria of great relevance in the case of many parasite species, as size variability can be considerable, from 18 to above 150 μm in length, and of a diameter between 12 and 14 μm or even above 90 μm.

Shape: egg shape also varies greatly, typically spheric to elongate or ovoid, being common in most species. Nonetheless, subtle differences in shape may be particularly relevant when separating species even within the same genus.

Developmental stage after shedding: in trematode eggs, the developmental stage of the ovum within freshly passed eggs is characteristic for each parasite species. Concretely, they are unembryonated eggs containing vitelline cells (usually corresponding to eggs of a larger size) and others that are embryonated containing a miracidium (schistosomes and eggs of a smaller size) when passed in feces.

Thickness of eggshell: usually, trematode eggs have a smooth shell that may vary considerably in thickness, depending on the species, although it is an important feature in separating eggs from vegetable cells or other plant material that may have irregular, limiting membranes. In some cases, modifications of the shell structure, i.e., operculum (present in all trematode eggs other than schistosome eggs), spines (only in schistosome eggs) and knobs (only in some eggs), may be important in the identification.

Besides the listed criteria, other aspects concerning the patient's anamnestic data have to be considered:

Geographical distribution: the trematode group includes species that are typically cosmopolitan, while others have a concrete and specific distribution. Thus, a trematodiasis may be endemic in one country, area or zone or may be present through isolated or sporadic human cases. Moreover, it has to be taken into account that several human fluke species may occasionally appear very far away from the original endemic area. These are either imported cases resulting from international travel, refugees, seasonal workers, expatriates of NGOs or soldiers on international missions, children adopted abroad, immigrants, and visiting friends and relatives, or they may be related to the consumption of certain foodstuffs, above all imported fish, as fish exporters tend to send shipments by air freight and without freezing in order to reach a competitive edge in the market with the ensuing increase in the risk of different fish-borne trematodiasis for the consumer.

The geographical distribution is related to the biological cycle of each species, which, from a general point of view, involves two or three different hosts: a vertebrate definitive host; an invertebrate first intermediate host (usually a gastropod mollusk), either terrestrial or aquatic according to the nature of the life cycle; and frequently, a second intermediate host (carrying an encysted metacercarial stage). Thus, it is the first intermediate host that, as a consequence of the marked specificity of the trematode species for their respective first intermediate snail host species, acts as a limiting factor of the geographical distribution of each trematode.

Sociocultural, environmental and hygienic factors: the assessment of these factors is crucial as they are related to the infection route. Among trematode species, two different routes are known: the great majority of species show an indirect or passive infection mechanism related to food habits [33–44]. The definitive host becomes infected when the encysted metacercarial stage is ingested, either with the intermediate host (numerous invertebrates and poikilothermal vertebrates—amphibians, annelids, arthropods, fish, mollusks) raw or undercooked, on vegetation or with water. Other species show a direct or active infection route related to contact with freshwater leading to the active penetration of the infective form (furcocercaria) through the skin.

Clinical manifestations: in certain trematodiases, the symptoms presented by the patients, basically in chronic phases of trematode diseases, may be useful in the establishment of the etiological diagnosis of the distomatosis. Nevertheless, patients often develop subclinical or only mild symptoms when the number of parasites is scarce, which may be the situation in numerous cases, so that infected subjects do not attend specialists. In other cases, no pathognomonic symptoms are involved and consequently may be confused with infections of a different etiology. Consequently, in these cases, symptomatology is of little importance when establishing the correct specific diagnosis.

Other general features: it is worth mentioning that atypical or distorted eggs may occasionally be seen, necessitating in these cases the search for more typical forms in order to make a reliable diagnosis. Also, the availability of trained and experienced personnel being able to visualize and recognize trematode eggs in different clinical samples is of utmost importance. In this context, along recent years, a Web-based virtual microscopy for parasitology is being adapted for education and quality assurance [45].

9.3.2 Particular Features

Liver flukes: Within this group, 11 species have been reported in humans [46, 47], and what the most relevant of them have in common is their hepatic location (bile ducts and gall bladder) of the adult stage and eggs expelled through the host's feces. According to medical importance, owing to the number of people infected, six

species of four genera (*Clonorchis, Opisthorchis*, *Dicrocoelium*, and *Fasciola*) belonging to three trematode families (Opisthorchiidae, Dicrocoeliidae, and Fasciolidae) are the subject of the present analysis.

Genus *Clonorchis*: The eggs of the so-called "Chinese or Oriental liver fluke," *C. sinensis*, are broadly ovoid, moderately thick-shelled, light yellowish-brown, with a large convex seated operculum, which fits into a rimmed extension of the eggshell, giving a "shoulder-like" appearance. At the abopercular end there is often a small protuberance or small knob (Fig. 9.1a). Mature eggs are embryonated when laid, containing a miracidium, and measure 26–35 × 12–19 μm (average 29 × 16 μm), which is the typical morphology of eggs of this species in feces or duodenal fluid using light microscopy [32]. Nevertheless, in patients with biliary obstruction, eggs are not passed in stools but may be found in bile sediments, appearing aggregated and adhered to or wrapped with bilirubinate particles or mucoid matter, as well as in gallstones, where they appear deformed with eggshell thickening, in which deposits of bilirubinate or mucoid matter may be observed, having lost the operculum, or without a miracidium [48].

As a consequence of the small size of eggs, and the need to differentiate them from eggs of the other two major opisthorchiids (mainly *O. viverrini*) and heterophyid (i.e., *Heterophyes, Metagonimus, Haplorchis*) flukes, which may be present in the same area, parasitological diagnosis must be carried out with caution [49]. In fact, in a comparative study carried out between eggs of *C. sinensis* and Heterophyidae in Korea, the results revealed that differential diagnosis of human infection by fecal examination is inconclusive, and thus isolation of adult worms is required to determine the exact species [50]. Nevertheless, the "shoulders" of eggs have been used for differentiation, as they are usually not as prominent in *O. viverrini*, and heterophyid eggs usually have an inconspicuous operculum flush with its shell surface. More recently, the surface ultrastructure of eggs by scanning electron microscopy in Korea revealed that *C. sinensis* eggs are covered with prominent and elevated ridges called "muskmelon-like structures," which were not observed in some heterophyid (i.e., *Metagonimus, Heterophyes, Stellantchasmus, Stictodora, Pygidiopsis*) and gymnophallid (*Gymnophalloides*) eggs [51].

Different types of cholangiography, mainly retrograde endoscopic cholangiopancreatography, as well as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI) are techniques which permit obtention of radiological images used for the clinical diagnosis of pathological effects and complications derived from clonorchiasis infection, particularly in subjects with a moderate or heavy infection [49, 52–54].

Various antigen proteins of *C. sinensis* have been identified in the crude extract and excretory–secretory products of adult worms or purified from soluble extracts of worm lysates, and also some recombinant *C. sinensis* proteins have been obtained [55–59]. They have either been used unblended or in cocktails for diagnostic purposes in various immunological tests, i.e., an intradermal test (IDT), an indirect hemagglutination test (IHA), counter-immunoelectrophoresis (CIEP), a complement fixation test (CFT), an indirect fluorescent antibody test (IFAT), and an ELISA

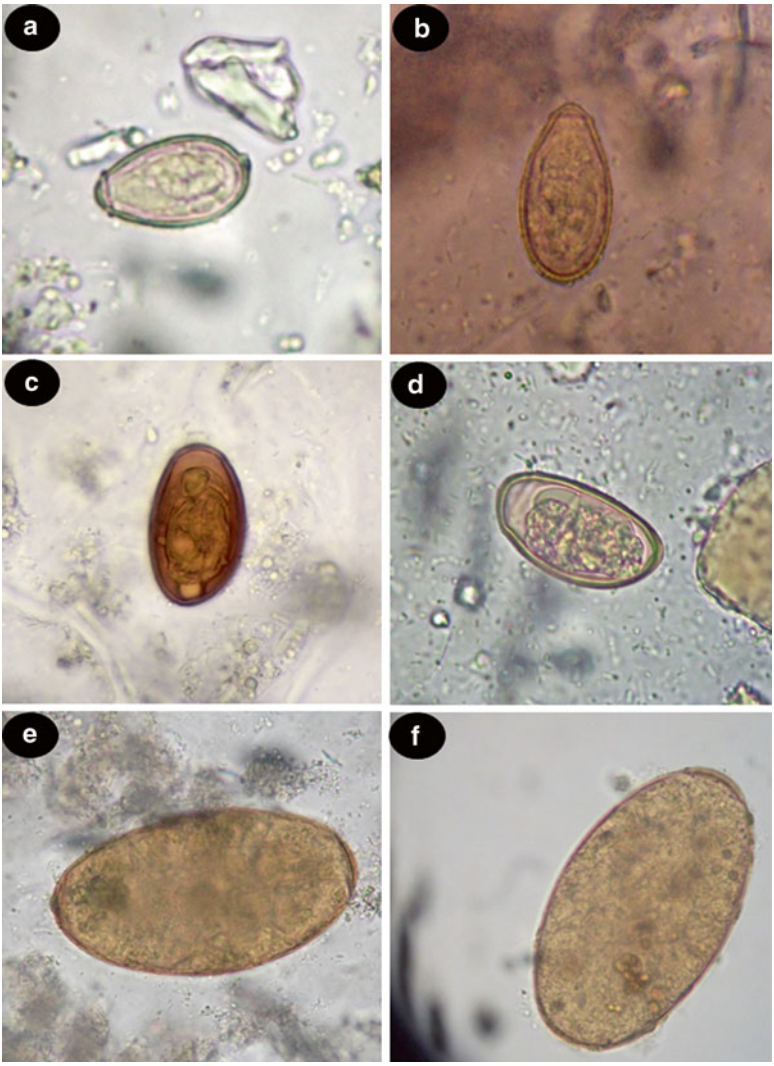

Fig. 9.1 Photomicrographs of various eggs of liver flukes detected in formalin-preserved human fecal material: (**a**) *Clonorchis sinensis* (size: 27.5×17.5 μm); (**b**) *Opisthorchis* sp. (30.0×15.0 μm); (**c**, **d**) *Dicrocoelium dendriticum* (42.5×22.5 μm and 41.3×22.5 μm, respectively); and (**e**, **f**) *Fasciola hepatica* (127.5×72.5 μm and 130.0×77.5 μm, respectively)

[40, 56, 60]. Among them, ELISA is the most commonly used technique for antibody and circulating antigen detection in serological tests, as well as for urinary antibody and stool antigen detections [60, 61]. However, the results obtained with this particular technique present different levels of sensitivity and specificity depending upon of high and low-risk patient groups [56, 62].

In the last few years, various PCR-based techniques have been developed for detecting different stages of *C. sinensis* in humans and animals in different clinical samples [48, 57, 63, 64] in endemic areas of this species is alone [65], but also for to discriminate this specie from the other two *Opisthorchis* and some heterophyid species [57, 66]. Recently, a duplex real-time fluorescence resonance energy transfer PCR followed by melting curve analysis has been developed for the detection and differentiation of eggs of *C. sinensis* and *O. viverrini* in human fecal samples in single or mixed infections [64].

Genus *Opisthorchis*: the species *O. viverrini* and *O. felineus* are included in this genus. The egg of either species is elongate ovoid, light yellowish-brown, 22–36×11–22 μm (mean 28×16 μm) in size, with an operculum that fits into the thickened rim of the shell proper ("shoulder"-like), a minute tubercular terminal thickening (not always visible), and contains a miracidium when passed in feces (Fig. 9.1b). Duodenal aspirates and bile examinations are suitable alternatives to stool samples for the parasitological confirmation of eggs of these species [32].

The dimensions of the eggs are 22–32×11–22 μm in *O. viverrini* and 21–36×10–17 μm in *O. felineus*, though the distinction between them is very difficult on the basis of the morphological characteristics owing to their overlapping intraspecific variability. However, worth mentioning are the differences found by different authors with regard to the ratio of the mean length over the mean breadth of the eggs (1.75 in *O. viverrini* vs. 2.75 in *O. felineus*) [67, 68]. Although both *Opisthorchis* species are distributed very far away from each other (Thailand, Laos, Vietnam, and Cambodia in *O. viverrini* vs. Russia, Ukraine, Belarus, Kazakhstan, and the Baltic countries in *O. felineus*) [69, 70], the export and import of fish and fish products worldwide may cause the presence of *Opisthorchis* in countries where it is a priori not expected [71–73].

In the case of *C. sinensis*, whose eggs closely resemble those of *O. viverrini* in size and shape and the geographical distribution overlaps with that of *O. viverrini* in several countries of southeastern Asia [69]. In this case, special attention should be paid to "the shoulder" which in the case of *O. viverrini* is usually not as prominent.

The distinction of *O. viverrini* eggs from various intestinal flukes such as heterophyids (*Haplorchis* and *Stellantchasmus)* and lecithodendriids (*Prosthodendrium* and *Phaneropsolus*) seems to be most problematic point. Nevertheless, some facts such as the presence of muskmelon-like structures on *O. viverrini* is very characteristic and distinctly different from small intestinal fluke eggs [74–76], or the absence of iodophilic bodies in *O. viverrini* eggs and its presence in *Prosthodendrium* and *Phaneropsolus* when stained with iodine, may be of diagnostic usefulness [74, 75]. The use of potassium permanganate solution (1 % w/v) in fecal examination can also be helpful for the differentiation of *O. viverrini* eggs (the muskmelon-like ridge was clearly observed on the egg surface) from *Haplorchis* (a light striae pattern is revealed) and *Phaneropsolus* (a smooth egg shell is observed, while the small knob and the shoulder are not as prominent as in those other genera) eggs [75, 77].

In the case of *O. felineus* diagnostic difficulties mainly occur with a minor opisthorchiid species, *Metorchis bilis*, since both species overlap in a considerable

territory of Central and Eastern Europe and Western Siberia [70], though the first is mainly recorded in Eurasia. The difficulties of discovering and differentiating eggs of both species in feces (average of 26×10 μm in *O. felineus* vs. 30×20 μm in *M. bilis*) does not make a specific diagnosis with the help of only light microscopy possible.

X-ray diagnosis (cholecystocholangiography), US, CT, and MRI are methods commonly employed for a preliminary human opisthorchiasis diagnosis, even in field surveys [42, 78, 79].

As in the case of *C. sinensis*, several immunological techniques have been developed either through antibody or circulating antigen detection, or through coproantigen detection, the majority being developed as ELISAs [80–85].

Some tumor markers (CA 125 and CA 19-9), measured by radioimmunoassay in serum samples, have been used in the early detection of *O. viverrini*-associated cholangiocarcinoma [86], which has more recently been the target of studies aiming at the discovery and verification of new biomarkers based on strategic samples collection [87].

In recent years, DNA hybridization and PCR-based techniques have been used for the identification of opisthorchiasis agents [80, 82]. Concretely, PCR methods for the detection of *Opisthorchis* eggs in human stool samples in areas where they are the only species [31, 81, 88–90], or coexist with another minor opisthorchiid—*M. bilis* [91, 92], and even in areas where they coexist with other liver (*C. sinensis*) and intestinal (*Haplorchis taichui*) flukes have been developed [64, 92–95]. More recently, two highly specific and sensitive modalities of loop-mediated isothermal amplification (LAMP) techniques have been used to differentiate DNA of *O. viverrini* from other liver (*Clonorchis, Fasciola*) and intestinal (*Haplorchis*) flukes [96, 97].

Genus *Dicrocoelium*: the most important species of commonly named "lancet flukes" or "small liver flukes" is *D. dendriticum*, whose eggs have an asymmetrical oval shape, a thick, dark brown shell, and an inconspicuous operculum (Fig. 9.1c). They are embryonated containing a miracidium when passed in feces, and measure 35–45×22–30 μm. As the result of the ingestion of infected livers from herbivorous animals, eggs in the feces of humans may be found. These cases are "spurious infections" or "pseudo-parasitisms," which have to be distinguished from genuine infections [98, 99]. Therefore, it might be interesting to study the egg content in detail, as different phases of embryonation in the egg can be observed (Fig. 9.1d), although in cases of genuine dicrocoeliosis all the eggs must be well embryonated and most would even have a deep golden brown color [100]. Nevertheless, either informing the patient about not ingesting animal liver for some time, together with the microscopic analysis of three fecal samples collected on consecutive or even alternating days, or the examination of duodenal or biliary aspirates should be the most reliable protocols to establish the distinction between false and genuine infections.

Genus *Fasciola*: the eggs of the two species included in this genus, *F. hepatica* and *F. gigantica*, are large, operculated—with a small and indistinct operculum—light yellowish-brown, broadly ellipsoidal, and unembryonated when excreted in feces (Fig. 9.1e, f).

In guides for clinicians and diagnostic analysts, as well as in books of medical parasitology and/or tropical medicine, it is common to find the size of *Fasciola* eggs within the specific range of 130–150×63–90 μm for *F. hepatica* and 160–196×70–90 μm for *F. gigantica*, being the most frequent criterion used for the differentiation between both species [32]. Moreover, the usual presence of a roughened or irregular area at the abopercular end of the *Fasciola* egg shell is used to distinguish them from *Fasciolopsis buski* eggs, an intestinal fluke with very similar eggs in size and morphology.

The results obtained in a recent study carried out to validate the identification of *Fasciola* species based on the shape and size of eggs shed by humans have made it necessary to reconsider the knowledge available so far at the moment of parasitological diagnosis, as the application of the classic egg size range and even the irregularity in the eggs shell may lead to erroneous conclusions [101]. Thus, for areas where *F. gigantica* is absent (as in the Americas and Europe), the values for *F. hepatica* eggs in human stools should be 100–162×66–105 μm, while for areas where both fasciolid species are present (as in many parts of Africa and Asia), the egg size values should be 107–172×64–95 μm for *F. hepatica* and 151–182×85–106 μm for *F. gigantica* [101]. Moreover, the results of that study showed that the roughened or irregular area present, according to the traditional criterion, at the abopercular end of the *Fasciola* egg shell is population-dependent (frequent or infrequent according to the geographic origin of the human samples analyzed) (Fig. 9.1e, f) and consequently should be not used as a pathognomonic criterion to separate the eggs of both genera, *Fasciola* and *Fasciolopsis* [101]. In such instances, clinical evaluation and the geographic zone of origin of the patient may be an important aid in diagnosis, especially when considering that range values now overlap with the size of eggs belonging to other trematode species also able to infect humans and presenting a similar morphology (i.e., species of the genera *Gastrodiscoides* and *Echinostoma*).

The possibility of spurious fascioliasis cases due to the ingestion of infected liver from ruminants and the resulting presence of eggs in feces cannot be discarded. Like in *D. dendriticum* infection, repetition of stool examination after a few days of a liver-free diet is most reliable when establishing the distinction between spurious and genuine infections.

Along the twentieth century, significant contributions to human fasciolosis diagnosis focusing on direct parasitological techniques and indirect immunological tests, as well as on other noninvasive diagnostic techniques (radiology, radioisotope scanning, US, CT, and MRI), were made [102, 103]. In the last two decades, however, some significant papers on the use of radiological imaging features for the diagnosis of human fasciolosis [104], and on different immunological techniques based on the determination of serum anti-*Fasciola* antibodies, circulating secretory antigens or testing of coproantigens have been reported. Several antigenic fractions of *Fasciola*, as well as purified and recombinant antigens, have been successfully used for the serodiagnosis of fascioliasis. Among the different proteins (cathepsin proteases, saposin like-proteins, fatty acid binding proteins, etc.) documented, cathepsins are most frequently used for detecting anti-*Fasciola* antibodies

employing a large number of enzyme-linked immunosorbent assay techniques, from in-house assays—with sensitivity and specificity ranging from 92.5 to 100 % and 83.6 to 100 %, respectively—[105–119], to a commercial test currently available. Indeed, the DRG *F. hepatica* IgG ELISA test is a qualitative microtiter strip-based enzyme-linked immunosorbent assay for the detection of IgG class antibodies in human serum against excretion/secretion antigens of *F. hepatica* predominantly containing fluke cysteine proteases, with sensitivity and specificity values of 95.3 % and 95.7 %, respectively [119]. Another commercial kit available is SeroFluke, which it is a lateral flow immunoassay or immunochromatographic test, based on the detection of recombinant cathepsin L1 from *F. hepatica* that can be used with serum or whole blood samples and can be employed in major hospitals in hypoendemic countries, as well as in endemic/hyperendemic regions where point-of-care testing is required [120].

The MM3-COPRO ELISA test has been applied recently for the detection of the *Fasciola* coproantigen in hospital patients, as well as in human surveys carried out in hyperendemic areas of Andean countries [121, 122]. The high sensitivity and specificity shown by this technique allow for fast large mass screening, detection in the chronic phase, early detection of treatment failure or reinfection in post-treatment subjects, and are even useful in surveillance programs [122]. Moreover, taking into account that this technique can detect animal infections by both *Fasciola* species may be of great value to ensure human diagnosis, above all, in areas where both fasciolids coexist [123, 124].

Finally, and for differential diagnosis among *Fasciola* species, particularly in areas of distributional and zonal overlap, several PCR-based approaches, including PCR-linked restriction fragment length polymorphism, PCR-linked single-strand conformation polymorphism, and specific PCR assays, have been developed [125, 126].

Lung flukes: various species belonging to the genus *Paragonimus* are included in this group, whose adult stage parasitizes the lungs of humans, among other definitive hosts; paragonimiasis is the disease caused by these flukes. These adult trematodes discharge operculated and unembryonated eggs, being subsequently found in sputum or swallowed and excreted in feces. Nevertheless, the sensitivity of a sputum sample is greater than that of a stool sample, although in the latter case, sensitivity reaches 25 % when three stool samples are examined [127]. It has been suggested that the examination of a stool sample may be superior to a sputum sample in young and the very old subjects [128]. In this sense, Procop [127] suggested that if paragonimiasis is suspected on a clinical or radiologic basis, a stool examination for parasite eggs should be performed in conjunction with an examination of respiratory secretions, even though the diagnosis yield of a stool examination may be low.

Genus *Paragonimus*: the eggs are moderately large, thick shelled, golden-brown, and broadly ovoid. At the abopercular end of the egg, the shell is somewhat thickened [32]. Although different species of *Paragonimus* have been described [129–131], the most relevant morphological characteristics, together with the geographic

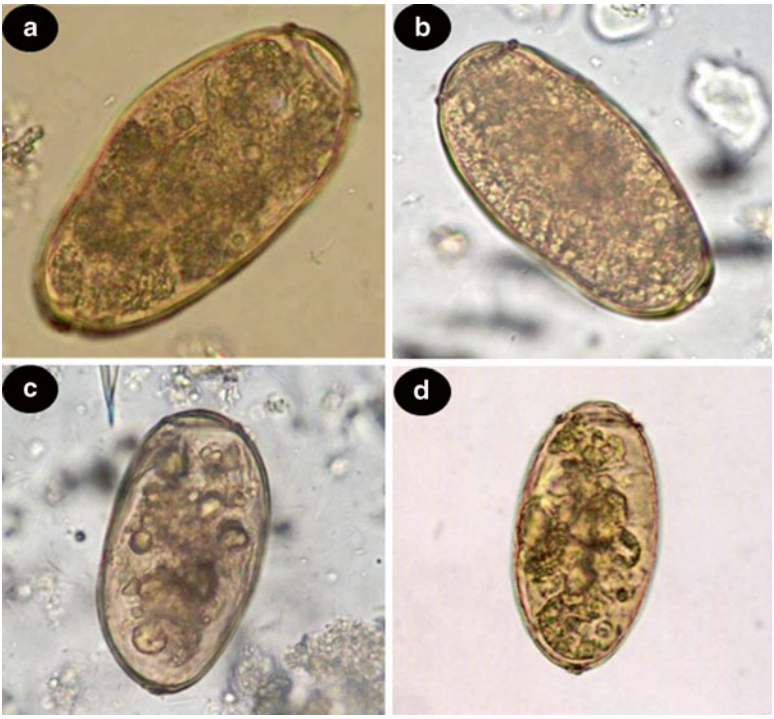

Fig. 9.2 Photomicrographs of various eggs of lung flukes of the genus *Paragonimus* detected (**a**–**c**) in formalin-preserved human fecal material: (**a**, **b**) *P. westermani* (size: 102.5×60.0 μm and 95.0×45.0 μm, respectively); (**c**) *P. africanus* (92.5×47.5 μm); and (**d**) *P. mexicanus* [from Ash and Orihel [32] with permission]

distribution, used to establish the diagnosis of the species most frequently found in humans [32, 127, 132, 133] are:

P. westermani: the eggs are large (80–120×45–70 μm), have a moderately thick, golden-brown shell that is thickened at the abopercular end, have a flattened, seated, and rimmed operculum, and it is broadest near the operculum (Fig. 9.2a, b); it is the most common species in Asia, but a number of other species, mainly *P. skrjabini* and *P. heterotremus*, have also been reported from China, Japan, Thailand, and countries along the Mekong River.

P. africanus: the eggs are similar (70–125×42–60 μm, mean: 91×49 μm) to *P. westermani* in size, although they tend to be narrower and the operculum is frequently more prominent; the thickening of its shell at the abopercular end is not as pronounced as in other species (Fig. 9.2c); it is an exclusively African species (West Africa: Equatorial Guinea, Cameroon, Nigeria, and possibly Ivory Coast).

P. uterobilateralis: the eggs are smaller (50–95×35–55 μm; mean: 69×42 μm) than the eggs of *P. westermani* and *P. africanus*, and its operculum is often not as

prominent as in other species, and the thickening of its shell at the abopercular end is not as obvious; it is the other African species (West Africa: Gabon, Cameroon, Nigeria, and Liberia).

P. mexicanus: the eggs are smaller in size (65–79 × 39–47 μm) when compared to *P. westermani*, and have a slightly thinner shell; its operculum and the thickening of its shell at the abopercular end (0.64–1.34 μm) are readily seen, being broadest at its equatorial part (Fig. 9.2d); and it is the most frequently species found in Latin America, although other related species (*P. caliensis*, *P. ecuadoriensis*, *P. peruvianus*, etc.) have also been described.

P. kellicotti: the eggs are similar (83–100 × 55–65 μm) to *P. westermani*, but smaller than *P. mexicanus* in size; broadest at its median part, its operculum is prominent and the thickening of its shell at the abopercular end (1.68–2.68 μm) is more pronounced than in *P. mexicanus*; it is a species rarely reported in humans in North America.

The differentiation of *Paragonimus* species on the basis of egg morphology is difficult. Thus, microscopic studies of many eggs in the samples to appreciate variations in size and shape, as well as in the appearance of operculum, are convenient combined with adequate parasitological training. In any case, the knowledge of other epidemiological features, mainly about how and where this trematodiasis has been contracted, may be helpful.

Eggs are also sometimes found in pleural effusion or pleural lesions removed surgically [134, 135], and occasionally the cytological examination of fluid obtained by thoracentesis, parecentesis, or fine-needle aspiration reveals eggs [136, 137]. Adult specimens have occasionally been coughed up and expectorated [138].

Biopsy of lung and other organs or tissues where the fluke may be ectopically located [127, 133, 136] may demonstrate the presence of eggs, whose size, shell thickness, and the presence of operculum usually allow at least an identification at genus level. However, adult or immature worms are very rarely found [28].

Diagnosis of this trematodiasis often has to be made using medical imaging methods (chest X-rays, CT, 18 F-fluorodeoxyglucose positron emission tomography, US, and MRI), above all, when the fluke is found at pulmonary and ectopic locations (mainly cerebral), or atypical manifestations of the disease are presented, being especially relevant in the fields of neurosurgery and neuroradiology [127, 139–143]. In geographic areas where pulmonary paragonimiasis overlaps with pulmonary tuberculosis, special attention must be paid to imaging findings in the diagnosis, as they are often clinically similar. The presence of a linear track to the pleura is a good criterion to separate paragonimiasis from other infectious or non-infectious lung diseases [140, 141, 144, 145].

Immunodiagnostic techniques are an alternative in lung fluke diagnosis. Major revisions [127, 129, 133, 135, 146, 147] of the most commonly used immunodiagnostic techniques, applied with varying success, have been compiled, including: an IDT, CFT, immunodiffusion methods (double immunodiffusion or Ouchterlony method, IEP and CIEP), IHA, IFAT, and, above all, different variants of tests based on ELISA and immunoblotting.

Crude adult worm extracts and partially purified antigens derived from trematode tissues or excretory–secretory products have been used in the diagnosis of this lung trematodiasis. Some components from adult worms such as 24-, 27-kDa *P. westermani*; 31.5-, 35-kDa *P. heterotremus*; and 34-, 21/23-kDa *P. kellicotti* have received special attention as antigenic targets [148–150]. Some proteins have been identified as cysteine proteases [151], and one of them (PwCP2) has been cloned and tested in sera from patients with paragonimiasis westermani, showing high sensitivity and specificity [148]. A recombinant major protein of a *P. westermani* egg antigen developed offers a highly sensitive and specific ELISA assay for the diagnosis of paragonimiasis [152]. All these antigens have been used for the detection, in blood, pleural effusion, or even cerebrospinal fluid, of parasite specific antibodies, mainly IgG, although IgE and IgM have also been suggested, through ELISA or Western blot techniques, which are now most widely used for serological diagnosis of paragonimiasis [149, 150, 153–156]. A Dot-immunogold filtration assay kit was also developed in China for the detection of antibody anti-*P. westermani*, using a *P. westermani* antigen and reporting a sensitivity and specificity of 99 % and 92 %, respectively [135]. To detect the presence of antigens from a *Paragonimus* species in some clinical samples, monoclonal and polyclonal antibodies have been used [127].

In the last decade, molecular tools have been commonly used as the genetic marker of species identification and phylogenetic studies in the genus *Paragonimus* [127, 157, 158]. Conventional PCR has been used to detect *P. kellicotti* DNA in clinical samples (lung biopsy and sputum), although its possible application to other clinical samples, such as bronchoalveolar lavage fluid, pleural fluid, or feces may be assumed [155]. Amplification of the nuclear ribosomal second internal transcribed spacer region (ITS2) and/or partial mitochondrial cytochrome oxidase subunit 1 gene (cox1) from *Paragonimus* DNA eggs collected from sputum or feces of patients infected with different species (*P. westermani, P. heterotremus, P. pseudoheterotremus, P. proliferus*) have been used for species identification [159–165]. A copro-DNA test for detection of *P. africanus* DNA from feces has been evaluated, and it has been found to be more sensitive than microscopic search for eggs in feces [154].

A LAMP assay for detection *of P. westermani* DNA in humans has also been developed recently [166], being highly specific, sensitive, rapid, simple, and cost-effective, as well as being approximately 100 times more sensitive than conventional specific PCR, in the detection of *P. westermani* infection [166]. The recent application of pyrosequencing technology to discriminate between different *Paragonimus* species coexisting in endemic areas is also worth mentioning. It is a real-time DNA sequencing technique whose applicability for the identification of *Paragonimus* species has been confirmed in field samples [167].

Intestinal flukes: about 70 species belonging to different families are reported in this group infecting humans around the world [168–170]. Among them, echinostomes and heterophyids are the two major groups in terms of the number of species involved, the number of people infected, and the distribution of endemic areas.

Fasciolid, gastrodiscid, and lecithodendriid species are also relevant, and there are numerous reports of other small fluke species of animals accidentally found in humans in Southeast Asia. All the species of this group are food-borne flukes transmitted by freshwater fish and snails, amphibians, terrestrial snakes, crustaceans, and aquatic insects as well as plants [169, 171], and consequently the endemicity of these flukes is associated with cultural and eating habits.

From an exclusively diagnostic point of view, species presenting large eggs (>50 μm) and others, the vast majority, presenting small eggs (<50 μm) are included in this group. Although accurate species identification is often difficult as the eggs of most of these flukes are similar in size and morphology, the most problematic distinction occurs within the group producing small eggs, called "minor intestinal flukes" (MIF) by some authors [50, 51, 74, 169, 170]; an abbreviation apparently rather inadequate as it coincides with a fecal preservative (merthiolate iodine formaldehyde—MIF) used in stool examinations.The term "tiny intestinal flukes" (TIF) is occasionally used as well [32]. In this context, it might seem more convenient to use the denomination "small intestinal flukes" (SIF), reserving the term "small trematode eggs" (STE) for the eggs of small liver and intestinal flukes [172, 173].

Echinostomatids: about 15 species of various genera have been reported causing human echinostomiasis in Asia and the western Pacific and probably also in Africa [168–170, 174]. The diagnosis of these intestinal flukes is based on the recovery of eggs in feces (Fig. 9.3a), usually being oval in shape, variable in size (but more than 50 μm), yellow, dark brown, or silver-white in color, with a thin and refractory shell, unembryonated when laid, and with a small, inconspicuous operculum and roughening or slight thickening of their shell at the abopercular end [32, 175]. Although specific diagnosis can be made through careful observations and measurements of the eggs, the recovery and identification of the adult fluke is strongly required for a definitive diagnosis, especially in areas where different species may cause human infection [170]. Nevertheless, the genus *Echinostoma* comprises the largest number of species producing eggs between 77–82×52–55 μm for *E. angustitestis* and 115–130×68–80 μm for *E. hortense* [170].

Echinostome eggs are similar those of fasciolids (*Fasciola* and *Fasciolopsis*), and even to those of gastrodiscids (*Gastrodiscoides*), making an adequate distinction rather difficult. However, the presence of wrinkles or a thickening at the abopercular end of the shell of echinostome eggs may facilitate their differentiation.

Immunological and molecular methods for the diagnosis of human echinostomiasis have not been developed.

Fasciolids: the largest fluke parasitizing humans, *Fasciolopsis buski*, which is largely confined to the oriental countries of the Far East and Southeast Asia is included in this group [37, 42, 174, 176]. Diagnosis is made by examining fecal specimens for the operculate, unembryonated, ellipsoidal, and yellow eggs, measuring 130–140×80–85 μm (Fig. 9.3b). The similarity in size and morphology of the eggs of this species and *F. hepatica* may lead to difficulties in establishing a correct diagnosis in areas of Southeast Asia where these species overlap in geographic distribution. In this case, certain facts—such as the difficult visualization of the

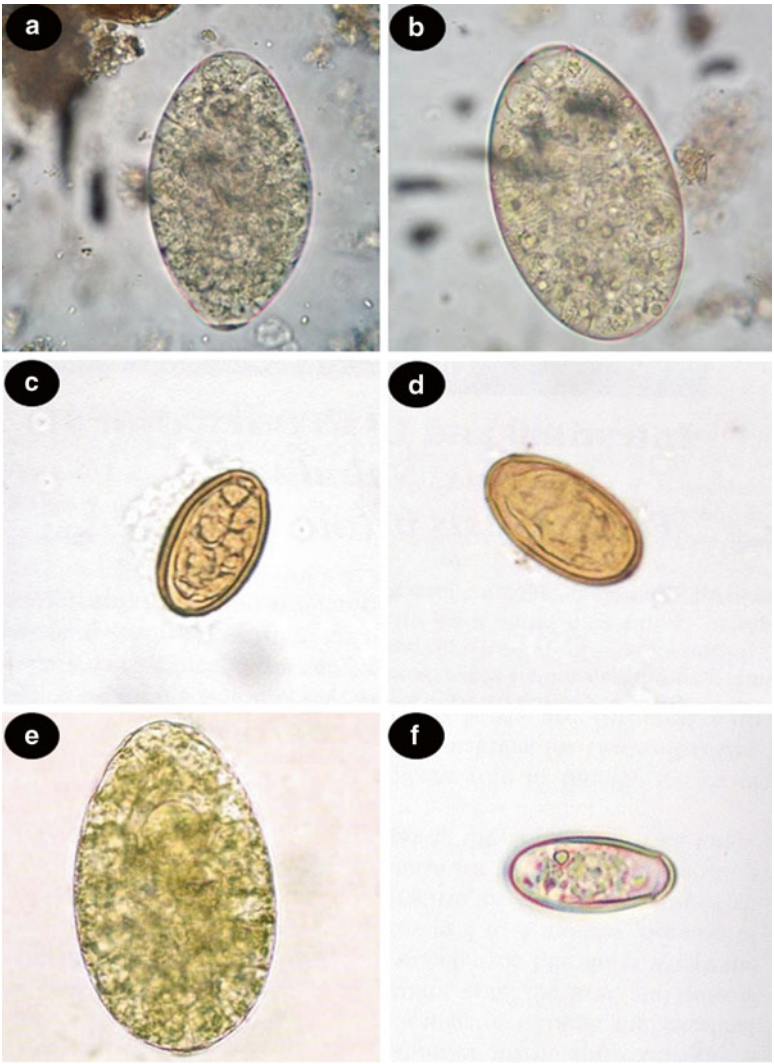

Fig. 9.3 Photomicrographs of various eggs of intestinal flukes detected (**a**, **b**) in formalin-preserved human fecal material: (**a**) *Echinostoma* sp. (size: 127.5×72.5 μm); (**b**) *Fasciolopsis buski* (140.0×80.0 μm); and *Heterophyes heterophyes* (**c**), *Metagonimus yokogawai* (**d**), *Gastrodiscoides hominis* (**e**) and *Prosthodendrium molenkampi* (**f**) [from Ash and Orihel [32] with permission]

operculum in *Fasciolopsis* eggs and that the shell at the abopercular end is not blemished as is often the case in *F. hepatica* eggs—should be mentioned, although the clinical evaluation and the geographic zone of origin of the patient may be decisive in the diagnosis [101]. Echinostome eggs have a slight thickening of their shell at the abopercular end that may facilitate their distinction from *Fasciolopsis* eggs [32]. However, the troublesome diagnosis concerning the group of large trematodes eggs

can easily be resolved when having adult specimens available, particularly considering that immunological or molecular methods for the diagnosis of human fasciolopsiasis have not been developed.

Heterophyids: a large number of species included in this family have been reported to parasitize humans, among which *Heterophyes heterophyes*, *Metagonimus yokogawai*, and *Haplorchis taichui* are generally considered the most important species implicated in human infections, commonly found throughout Asia [34, 174, 177].

Diagnosis is basically made by the recovery of the eggs in fecal examinations (Fig. 9.3c, d), which are small, ovoid, operculated, yellow–brown, and embryonated when passed in feces, measuring 20–30 μm in length and 15–17 μm in width (*M. yokogawai* 26–28 × 15–17 μm, *H. heterophyes* 28–30 × 15–17 μm, and *H. taichui* 20–30 × 14–17 μm) [32, 76, 77]. However, establishing a specific differentiation is very complicated, even at genus level, within this helminth group due to their similar morphology, and their reduced egg size [50, 51, 75–77]. Therefore, the term "*heterophyid fluke infection*" is recommended in their diagnosis [178]. As a consequence of the marked morphological similarity of these eggs to those of *Opisthorchis* and *Clonorchis*, or even those of lecithodendriid species, other terms such as "*opisthorchiid-like egg*" or "MIFs" are used in the definitive diagnosis especially in areas where these species coexist [169, 172].

However, several publications have helped to establish criteria for the differential diagnosis through parasitological techniques [50, 51, 75–77]. Surface morphology, the shape, and the size of eggs, in the latter case using different parameters, mainly the values of the length–width ratio and the Faust–Meleney index (= length of the egg by the square of the width of the egg), have been used by different authors to facilitate egg differentiation in light microscopy and scanning electron microscopy among various heterophyid species and even in relation to *Opisthorchis* and *Clonorchis* [50, 51, 76]. Thus, *Haplorchis* spp. eggs can be recognized by the presence of filamentous mesh-like structures or thread-like ridges on the shell surface distinctly different from the wrinkles or muskmelon-like structure on the shell surface of opisthorchiid eggs [75, 76]. When potassium permanganate temporary staining in fecal examinations is used, *H. taichui* eggs show a light striae pattern on the surface markedly different from the muskmelon-like prominent ridges of the surface of *O. viverrini* eggs as well as of the smooth egg shell of lecithodendriid *Phaneropsolus bonnei* eggs [77].

In spite of the different characteristics reported to be useful for the morphological differentation of heterophyid eggs in fecal samples, adequate specific and generic discrimination is very difficult and may require the recovery of adult worms after anthelmintic treatment and purgation with magnesium salt, or even surgery or autopsy [169].

From an immunological and molecular diagnostic point of view, several techniques have been developed. Serological tests such as ELISA are helpful in the human diagnosis of *M. yokogawai* and *H. taichui* [179–181]. Moreover, several PCR tests have also been applied to discriminate *Haplorchis* spp. from *O. viverrini*, with different sensitivity and specificity [93, 94, 182–185], and specific

primers have been successfully developed to identify *H. taichui* and to discriminate it from *O. viverrini* using high annealing temperature random amplified polymorphic DNA (HAT-RAPD) [186], which have been used for the recent development of a multiple PCR assay for the detection of *H. taichui* and *O. viverrini* in different hosts [95, 187].

Gastrodiscids: the only common amphistome of humans is *Gastrodiscoides hominis*, which causes a serious zoonosis throughout the Old World: India, Southeast Asia, Kazakhstan, the Volga Delta in Russia, and even in Africa [37]. Diagnosis of human infection is based on the finding of its characteristic eggs in feces (Fig. 9.3e), which are unembryonated and large, measuring 127–160×62–75 μm (mean 146×66 μm) [32]. The eggs of fasciolids (*Fasciola* and *Fasciolopsis*) and echinostomatids (i.e, *Echinostoma*) are similar to these eggs although gastrodiscid eggs are broad and tapered toward the opercular end, with a pale greenish-brown color, and do not present a thickening at the abopercular end. Diagnosis based on expelled adult worms is more accurate as no immunological and molecular methods have been developed for the diagnosis of this uncommon human trematodiasis.

Lecithodendriids: *Prosthodendrium molenkampi* and *Phaneropsolus bonnei* are the two species of this family mainly reported in humans from Thailand and Laos [169]. The eggs of both species are included in the SIF group, measuring 23–32 μm in length and 11–16 μm in width (*P. bonnei* eggs being thinner and bigger than *P. molenkampi* eggs), being morphologically similar to embryonated *O. viverrini* eggs [74, 75].

The differentiation between them under light microscope may be possible by the presence of an iodophilic body, a large mass at the posterior end of the miracidium that turns brown in 0.2 % iodine solution, in lecithodendriid eggs [74]. Furthermore, under light microscopy, even when using potassium permanganate staining for the surface morphology, as well as scanning electron microscopy, lecithodendriid eggs have smooth eggshells and indistinct or small shoulders and knobs (Fig. 9.3f) contrasting with the rough eggshells (so-called muskmelon pattern), the prominent aboperculum knobs, and the distinct operculum and shoulders presented by *O. viverrini* eggs [75, 77]. Nonetheless, morphological studies of adult worms obtained from expulsion chemotherapy, autopsy, or surgery are recommendable for the definitive diagnosis. Immunological and molecular techniques have not been developed for the diagnosis of these uncommon human intestinal flukes.

Blood flukes: the species of the genus *Schistosoma*, causative of human schistosomiasis, schistosomosis, or bilharziasis, concretely *S. mansoni, S. japonicum, S. mekongi*, *S. intercalatum*, and *S. guineensis*, responsible of intestinal schistosomiasis, and *S. haematobium,* responsible of vesical schistosomiasis, are included in this group.

Genus *Schistosoma*: common and conclusive means of diagnosing schistosome infection is egg detection. The most distinctive features of eggs used in the recognition are as follows: being non-operculate, containing a miracidium when passed in feces and/or urine, with a transparent shell and the presence of a spine. Within the genus, eggs can be distinguished from another on the basis of morphology, size, and location of the spine:

S. mansoni: eggs are large and elongate (114–175×45–68 μm), present in feces, and occasionally in urine, with a prominent subterminal spine (Fig. 9.4a). However, in some coverslip preparations, it will occasionally be hidden from view (Fig. 9.4b).

S. japonicum and *S. mekongi*: eggs are round, passed in feces, having a small and inconspicuous spine (Fig. 9.4c). Eggs of both species are similar, except that the former are bigger in size (70–100×50–65 μm vs. 51–78×39–66 μm). Fecal debris tends to adhere to the shells of these eggs that may be obscured and overlooked in fecal preparations, and the small spine may be inapparent and difficult to see, especially when the spine lies on the underside. Nevertheless, some strains of *S. japonicum* appear to have a more prominent spine than others.

S. intercalatum: eggs are large and elongate (140–240×50–85 μm), and are found exclusively in fecal specimens, not in urine. The equatorial bulge varies in prominence, and the terminal spine is usually longer, slightly curved, and more sharply pointed (Fig. 9.4d) than the terminal spine in *S. haematobium*. Eggs of *S. guineensis* a recently described species are anatomically indistinguishable from those of *S. intercalatum* [188]. The geographic origin of the infection should be considered in the differentiation of both species (Democratic Republic of Congo in *S. intercalatum* vs. Cameroon, Equatorial Guinea, Gabon, Nigeria, and Sao Tomé in *S. guineensis*) [189]. Moreover, sporadic cases of human parasitization by *S. bovis* are noteworthy, which have been correlated in some instances with spurious infections acquired by the ingestion of infected livers from cattle or other animals. However, these eggs are very large, 230–380×70–90 μm, with a terminal spine and an equatorial bulge invariable in prominence [32].

S. haematobium: eggs are large and elongate (112–170×40–70 μm), found in urine (Fig. 9.4e) and occasionally in feces (Fig. 9.4f), with a prominent terminal spine, variable in size and prominence. In some instances, cells in urine adhere to the eggs and obscure them, although the large size of these eggs usually makes them readily visible.

Schistosomiasis can also be proven through the detection of eggs in tissues obtained by rectal, intestinal, hepatic, prostatic, or vesical biopsies. In ectopic infections (in lungs, skin, central nervous system) or in light infections, a biopsy may provide the first clue of a schistosome infection. In all these cases, the tissue, preferably unfixed, is examined microscopically pressed between two glass slides. This is more sensitive than histological examination and allows a more accurate assessment of the species. This technique is also effective in the detection of eggs in fixed or unfixed surgical or autopsy specimens. Eggs may also be recovered by hydrolysis of tissues in 4 % KOH for 18 h at 37 °C for fresh tissues and 56 °C for fixed tissues [190].

Specific identification of eggs within the characteristic-appearing pseudotubercle (egg granulomas) in histological examination depends on morphological features, but also on epidemiological considerations. In geographic areas where *S. mansoni* and *S. haematobium* overlap in distribution, finding schistosome egg granulomas without the characteristic spines can make diagnosis difficult. When a modified Ziehl-Neelsen staining technique is used, the eggs of *S. haematobium* are not acid fast, whereas those of both *S. mansoni* and *S. japonicum* are [28]. Nevertheless, this

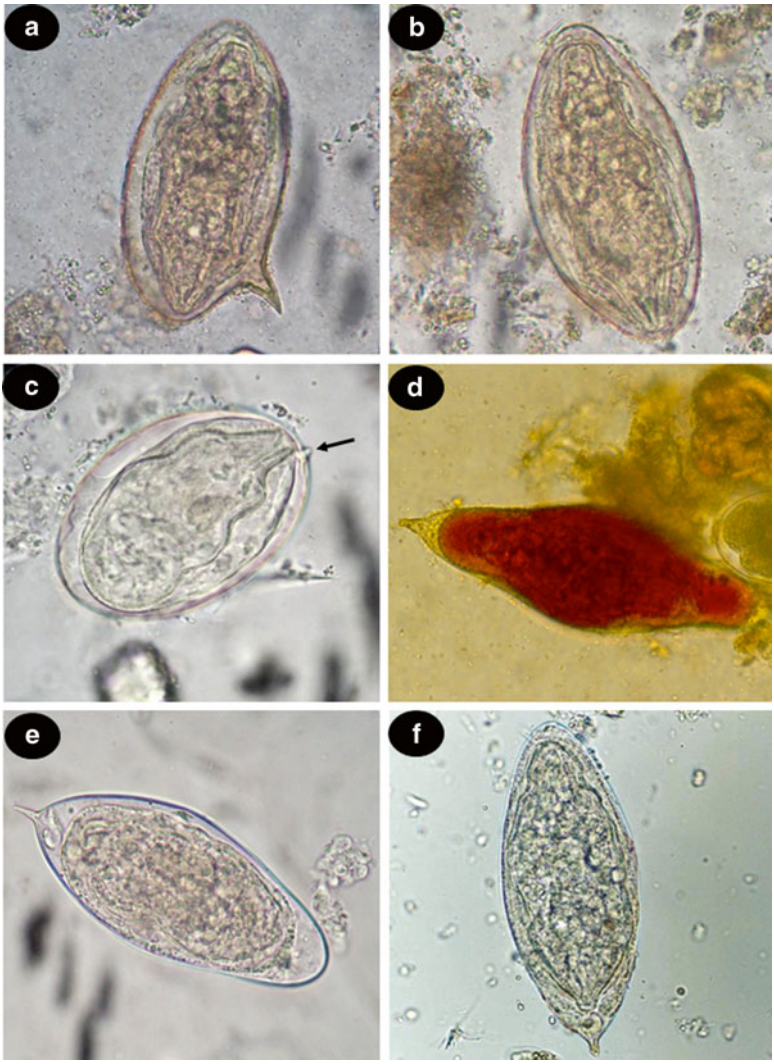

Fig. 9.4 Photomicrographs of various eggs of blood flukes of the genus *Schistosoma* detected in formalin- and MIF-preserved human fecal (**a**–**c**, **f** and **d**, respectively) and urine (**e**) samples: (**a**) *S. mansoni* showing the typical prominent lateral spine, which occasionally may be not visible (**b**) (size: 137.5×52.5 μm); (**c**) *S. japonicum* showing the small and inconspicuous spine (*arrow*) (87.5×62.5 μm); (**d**) *S. intercalatum* (140.0×67.5 μm); and (**e**, **f**) *S. haematobium* (135.0×62.5 μm and 125.0×52.5 μm, respectively)

differentation must be made with caution as the shells and spines sometimes fail to stain as expected [190]. *S. haematobium* eggs can also easily be confused with those of *S. intercalatum* in tissue sections, as both have a terminal spine and are similar in size. However, *S. intercalatum* eggs are usually longer (140–240 μm) and have a larger and more conspicuous spine. Terminal spines of *S. intercalatum* are 3–4 μm wide at the base, pointed at the tip, and 10–20 μm long [190].

Imaging techniques are other tools for the definition of the clinical manifestations and pathological outcomes of schistosomiasis, although they are restricted to hospitals. Indeed, various imaging techniques such as myelography, US, CT and MRI can be useful [191]. Cystoscopy, radiological, and endoscopy examinations are also applied [192], and more recently, confocal laser scanning microscopy has been used, in combination with standard cystoscopy, to detect *S. haematobium* eggs directly in the urothelium of a patient with urinary schistosomiasis [193]. In the last decade, portable ultrasonographic equipment has markedly facilitated schistosomiasis diagnosis [194, 195].

Additional supportive laboratory findings of schistosome infection might include evidence of peripheral-blood eosinophilia, anemia (iron-deficiency anemia, anemia of chronic disease, or macrocytic anemia), hypoalbuminemia, elevated urea and creatinine levels, and hypergammaglobulinemia [196]. Reagent strips for microhematuria and simple questionnaires for red urine are cheap, easy and effective tools for the screening and rapid epidemiological assessment of urinary schistosomiasis [197]. Measurement of soluble tumor necrosis factor receptors II and intracellular adhesive molecule 1 levels could serve as additional markers for assessment of schistosome-induced liver fibrosis [198].

During the last two decades, a great amount of literature on the immunological and molecular diagnosis of schistosomiasis has been published [199–201]. Serological methods have been extensively developed, being based on the detection of IgG, IgM, or IgE against soluble worm or egg antigens mainly by ELISA, IHA, or IF [201–203], and are important for the diagnosis in travelers, migrants and other occasionally exposed subjects returning home from disease-endemic areas [204–207]. A magnetic affinity ELISA based on soluble egg antigens, with a higher sensitivity, has been developed recently for the diagnosis of schistosomiasis japonicum in individuals with low-intensity infections [208].

Serological methods have also been implemented for antigen detection. Adult worm antigens, soluble egg antigen, and circulating anodic and cathodic antigens have been detected in serum, urine, or sputum of infected individuals with labelled monoclonal or polyclonal antibodies utilizing commercially available tests or mainly the ELISA approach [209–220]. An up-converting phosphor technology-based lateral flow assay for the detection of circulating anodic antigen in serum, with a higher sensitivity than ELISA, has been developed [221]. More recently, a rapid diagnostic test incorporating *S. mansoni* cercarial transformation fluid has been employed for the detection of antibodies in blood samples [222].

Mass spectrometry has been applied to identify various oligosaccharides originating from *S. mansoni* eggs and captured using specific monoclonal antibodies [203, 223]. However, further studies are required to validate its actual clinical applications.

The need to have a test available for diagnosing schistosomiasis in all phases of the clinical disease, including the capacity to diagnose Katayama syndrome and active disease, prompted several groups to develop molecular testing using DNA-based methods [224]. Different conventional PCR, PCR-coupled techniques, nuclear ribosomal RNA and mitochondrial genes, real-time PCR techniques, as well as a loop-mediated isothermal amplification, have been developed to detect schistosome DNA in clinical samples (feces, urine, sera, plasma, and vaginal lavage)

from humans [203, 207, 225–229]. A recent prospective European-wide multicenter study has demonstrated the applicability of a blood-based real-time PCR test for the detection of *S. mansoni* DNA in patients with acute schistosomiasis who acquired their infection in various endemic regions [230].

9.4 Concluding Remarks

The public health relevance of human trematodiases is unquestionable, and therefore, the correct diagnosis of these infections is crucial, mainly for assessing drug efficacy and monitoring the impact of control interventions.

The gold standard for the diagnosis of trematodiases is the detection of eggs through parasitological techniques, although some may have a certain lack of sensitivity, require an intensive effort, and can be time-consuming, necessitating, moreover, experienced technicians. Yet sometimes it is difficult to establish a diagnosis due to morphological similarities among various eggs. For all these reasons, specialists are concentrating efforts on the development of more sensitive and specific diagnostic methods focusing mainly on the immunology and molecular biology. The increased use of genomic, transcriptomic, and proteomic approaches offers a promising future for the diagnosis of these human fluke infections.

International travels, migratory flows, and other demographic changes in a globalized world imply a risk linked to the diagnosis of these human trematode infections mainly in non-endemic countries, and it is necessary to keep in mind that the direct microscopic examination of clinical samples and the identification of trematode eggs remains the gold standard in diagnosis. Consequently, it is indispensable to invest in the training of personnel to acquire the relevant skills and experience in parasitological diagnosis, which should go hand in hand with the quest for the development of more sensitive and specific techniques to be applied in the field and, above all, in clinical practice.

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Part III
Trematodes of Interest in Veterinary and Wildlife Disease

Chapter 10
Schistosomatoidea and Diplostomoidea

Petr Horák, Libuše Kolářová, and Libor Mikeš

10.1 Introduction

This chapter is focused on important nonhuman parasites of the order Diplostomida *sensu* Olson et al. [1]. Members of the superfamilies Schistosomatoidea (Schistosomatidae, Aporocotylidae, and Spirorchiidae) and Diplostomoidea (Diplostomidae and Strigeidae) will be characterized. All these flukes have indirect life cycles with cercariae having ability to penetrate body surfaces of vertebrate intermediate or definitive hosts. In some cases, invasions of accidental (noncompatible) vertebrate hosts (including humans) are also reported. Penetration of the host body and/or subsequent migration to the target tissues/organs frequently induce pathological changes in the tissues and, therefore, outbreaks of infections caused by these parasites in animal farming/breeding may lead to economical losses.

10.2 Schistosomatidae

Members of the family Schistosomatidae are exceptional organisms among digenean trematodes: they are gonochoristic, with males and females mating in the blood vessels of definitive hosts. As for other trematodes, only some members of Didymozoidae are

P. Horák (✉) • L. Mikeš
Department of Parasitology, Faculty of Science, Charles University in Prague,
Viničná 7, Prague 12844, Czech Republic
e-mail: petr.horak@natur.cuni.cz; mikes@natur.cuni.cz

L. Kolářová
Institute of Immunology and Microbiology, First Faculty of Medicine,
Charles University in Prague and General University Hospital in Prague,
Studničkova 7, Prague 12800, Czech Republic
e-mail: libuse.kolarova@lf1.cuni.cz

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gonochoristic, and only adults of Aporocotylidae and Spirorchiidae inhabit blood circulation of their hosts. The family Schistosomatidae comprises 14 genera parasitizing mammalian and avian hosts. Besides the genus *Schistosoma* that has medical and veterinary importance (human and mammalian parasites), three genera (*Bivitellobilharzia*, *Heterobilharzia*, *Schistosomatium*) infect mammals, and ten genera (*Allobilharzia*, *Anserobilharzia*, *Austrobilharzia*, *Bilharziella*, *Dendritobilharzia*, *Gigantobilharzia*, *Jilinobilharzia*, *Macrobilharzia*, *Ornithobilharzia*, *Trichobilharzia*) cause avian diseases.

10.2.1 Bird Schistosomes

10.2.1.1 Life Cycle

Bird schistosomes have a two-host life cycle; generally the eggs containing mature miracidia are released via feces from the infected host to the water environment where miracidia hatch in a while. In case of *Trichobilharzia regenti*, however, the miracidia hatch directly in the host nasal tissue, and leave the host upon contact with water. Miracidia are active swimmers and seek after appropriate intermediate snail host. In order to find the host, miracidia possess receptors to be able to respond to physical factors (gravity, light) as well as chemical cues. Concerning the latter stimuli, carbohydrates bound in miracidia-attracting glycoproteins (MAGs, miraxons) emitted by the snail hosts seem to serve for chemoorientation and host recognition by *Trichobilharzia szidati* (syn. *Trichobilharzia ocellata*) [2, 3].

Subsequent larval development of bird schistosomes, characterized by an asexual multiplication in intermediate hosts, proceeds in freshwater, brackish, or saltwater snails. The details of intramolluscan development are known for a limited number of bird schistosomes. With regard to morphogenesis of intramolluscan larval stages, and also their influence on snail physiology and immunity, *T. szidati* seems to be the most studied model [4]. The parasite inhibits certain immune functions, such as phagocytosis, efficiency of encapsulation and bacterial killing, and stimulates production of humoral defense molecules, e.g., molluscan defense molecule (MDM) and granularin. Moreover, the developing trematode larvae induce production of schistosomin, a molecule released probably from snail hemocytes and connective tissue cells, and competing with snail hormones for receptor binding sites on the target reproductive tissues (inhibition of oviposition) [5].

Several weeks (depending on temperature) after exposure to miracidia, the snails start to liberate cercariae. These freely swimming larvae (Fig. 10.1a) respond to some physical and chemical stimuli and exhibit specific behavior in the aquatic environment. *Trichobilharzia szidati* recognizes substrates being warmer than the ambient water and attaches to the surfaces containing ceramides and cholesterol. As the infection of a new host comprises several steps, the penetration itself is triggered by other chemical signals—unsaturated fatty acids for *T. szidati* and free sterols for *Austrobilharzia terrigalensis* [3]. Besides the action of cercarial muscles, secretions of cercarial penetration glands containing histolytic enzymes play a crucial role in skin penetration.

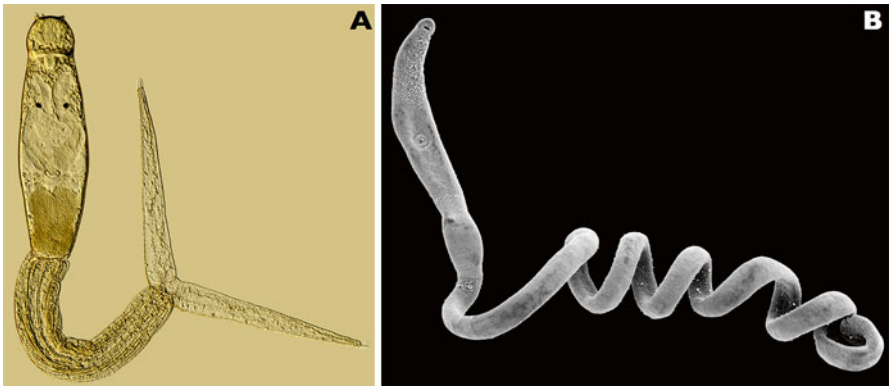

Fig. 10.1 (**a**) Ocellate furcocercaria of *Trichobilharzia regenti* (length of about 800 μm) from the intermediate snail host, *Radix labiata*. (Author: Dr. J. Bulantová); (**b**) Advanced schistosomulum of *Trichobilharzia regenti* isolated from the spinal cord of a duck 11 days post infection, and then cultivated 3 days *in vitro*. The estimated length of the fully mature worms is about 11 mm. (Author: Dr. J. Bulantová)

Whereas some human schistosomes use serine peptidases (e.g., cercarial elastase) as the main penetration enzymes, in *T. szidati* and *T. regenti* (and also in *Schistosoma japonicum*) cysteine peptidases (cathepsin B-like) from the penetration glands are supposed to play a role [6]. After the attachment, the body of cercariae enters the skin, whereas the tail is removed. Once in the host, cercarial body transforms to schistosomulum being able to evade host immune attack: (1) Thick glycocalyx on the surface is rapidly removed and a new surface double membrane of the tegument is formed. (2) The density of surface antigens drops to the level undetectable by specific antibodies and some lectin probes [7].

In birds, the adult schistosomes live either in visceral organs or nasal mucosa; therefore, we distinguish visceral and nasal schistosomes. These two groups also differ in migratory routes within the host body; this statement is however based on a few experimental models. After penetration of the host skin, visceral schistosomes enter blood vessels and use the blood circulation to reach the final location; during the migration schistosomula can therefore be found in the lungs, liver, etc. On the other hand, nasal schistosomes search for peripheral nerves and use them to crawl to the spinal cord and brain. This temporary location is typical in the phase before reaching the nasal area of their avian hosts. Of course, depending on the infection dose, some migrating schistosomula may be found in ectopic locations (e.g., nasal *T. regenti* in the lungs).

In addition to the infections of compatible definitive hosts, cercariae of some genera of bird schistosomes (e.g., *Austrobilharzia*, *Bilharziella*, *Gigantobilharzia*, *Trichobilharzia*) have been confirmed as the causative agent of human cercarial dermatitis. It means that the human skin possesses components that may be recognized by cercariae as signals for attachment and penetration of the accidental host. It is remarkable that the content of attractive fatty acids is even higher in the human skin when compared with the bird skin [3, 8].

10.2.1.2 Occurrence

Bird schistosomes can be found around the world [9]. Geographical latitude plays a minor role provided the intermediate snail and the definitive bird hosts are available for transmission. Therefore, bird schistosomes and cercarial dermatitis have been reported, e.g., from Iceland and Norway [10, 11].

The prevalence rate in birds may differ with regard to local conditions, but in some cases it can be high, reaching even 90–100 % (e.g., the USA and New Zealand) [12, 13]. Waterfowl (*sensu lato*) seems to serve as dominant definitive host; nevertheless, other groups of birds (e.g., passerines) have also been reported [9]. On the other hand, the infections of snail intermediate hosts are less frequent. In many localities, the prevalence rate does not exceed 1 %. However, outbreaks with prevalences up to 50 % have also been recorded (e.g., Denmark and Russia) [14, 15]. Predominantly, pulmonate snails of the families Lymnaeidae, Physidae, and Planorbidae are used for larval development of bird schistosomes. Nevertheless, other groups of snails may also be involved in the life cycles of some genera/species [9].

10.2.1.3 Damage to the Definitive and Accidental Hosts

As mentioned above, the infective larvae (cercariae) penetrate the skin of bird hosts; usually feet serve as the entry site, because the rest of the body covered by feathers with secretions of the uropygial (preen) glands is not attractive [3]. Penetration of the bird feet may lead to local inflammatory reactions and petechiae. Subsequent migration through the body and damage to the inner organs differ with regard to the two types of schistosomes.

Schistosomula of visceral schistosomes migrating via blood system can temporarily accumulate in the lungs, where hemorrhages, inflammation and subsequent tissue repair can be observed [16]. Later on, the target tissues (e.g., the vessels of liver and intestine) and adjacent organs can be affected by the presence of adult worms and eggs. During severe infections, more distant organs can also be impaired. Generally, tissue lesion, inflammatory reaction, formation of granuloma and local thickening occur. Blood circulation may be affected by thrombi, hemorrhages and perivascular cell infiltration [9, 17]. Death has also been ascribed to schistosome infections: in birds infected experimentally or in waterfowl [17].

Our knowledge of nasal schistosomes is based on one experimental model only—*T. regenti*. Schistosomula migrate through the peripheral nerves, spinal cord and brain in order to reach nasal mucosa [18]; in the central nervous system schistosomula (Fig. 10.1b) feed on the nervous tissue [18, 19]. The mode of migration of and the uptake of nervous tissue components by *T. regenti* are unique among schistosomes. Heavy infections may be accompanied by leg paralysis, balance/orientation disorders and death. In the nasal area, adult worms and eggs induce lesions, hemorrhages and granulomas [20, 21].

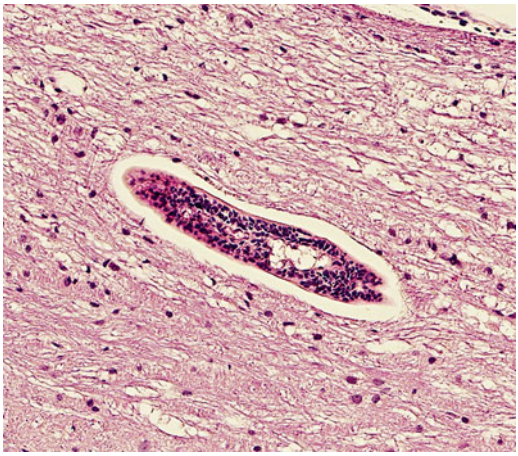

Fig. 10.2 Section of *Trichobilharzia regenti* schistosomulum migrating in the spinal cord of experimentally infected SCID mouse, 3 days after the fourth (repeated) infection. (Author: Dr. L. Lichtenbergová)

Cercariae of bird schistosomes may also attack accidental (incompatible) mammalian hosts, including humans. As far as human infections are concerned, an allergic skin reaction (cercarial dermatitis) develops in sensitized persons after repeated contacts with the agent. Cellular and humoral immune reactions, and clinical symptoms and signs have thoroughly been characterized [22]. These reactions represent an unpleasant event, but they provide immediate protection against the invading worms, because the worms are subsequently killed in the skin. On the other hand, there are numerous experiments with small laboratory mammals showing that the infection of primarily affected (i.e., non-sensitized) hosts is accompanied by transformation of some cercariae to schistosomula, and their subsequent migration to different inner organs. For example, schistosomula of visceral and nasal schistosomes can be found in the lungs and the spinal cord (Fig. 10.2) of experimental mammals, respectively [23, 24]. In the nervous tissue, immune cell infiltrates, activation of microglia and astrocytes, and damage to the nerve cells (axon injury and demyelination) are associated with mechanical damage caused by migrating worms [19]. After a few days/weeks, the migrating worms die in the incompatible host due to unknown reasons (probably due to immune attacks, lack of nutrients, etc.).

Due to various climate/environmental factors linked with changes in distribution and density of snails and birds, the number of reports on the occurrence of bird schistosomes and cercarial dermatitis is growing, and cercarial dermatitis is now regarded as a reemerging disease [9, 25].

10.2.1.4 Diagnosis and Control

In definitive hosts, patent infections by visceral schistosomes can be diagnosed directly, i.e., by finding schistosome eggs in feces. Nasal schistosomes lay their eggs in the nasal mucosa, and miracidia hatch already in the host tissue. Therefore, bill

lavage may be used to prove the presence of miracidia. Postmortem diagnosis is based on a careful examination of presumptive affected organs and tissues; adult worms (often slim and threadlike) and eggs can be found in this case [26]. Concerning indirect detection of schistosome infections, there are some data on the presence of specific antibody in sera of birds and mammals [4]. Nevertheless, routine and reliable serological tests are not available due to cross reactivity at the level of schistosome species and genera; search for a suitable antigen is in progress. In addition, molecular detection of schistosome species has successfully been applied in the field [27], and development of a tool for identification of parasite DNA in body fluids of experimental animals is in progress.

Control lies in preventive measures and chemotherapy. Concerning preventive measures, access of birds to schistosome-infested water reservoirs can be obstructed. Also, populations of snail intermediate hosts can be reduced in different ways: changes of biotopes (e.g., removal of aquatic vegetation), and mechanical, chemical (e.g., niclosamide), or biological (e.g., toxic bacterial products) intervention against the snails. Chemotherapy of birds (if applied) is based on praziquantel. Prevention of cercarial dermatitis consists in restricted access to schistosome-infested water reservoirs, protective clothing, or sun creams containing niclosamide [28]; the treatment is only symptomatic [17].

10.2.2 Mammalian Schistosomes

There is no exception in the life cycles within the family Schistosomatidae in terms of the number of hosts; all life cycles include an intermediate snail host and a definitive avian/mammalian host. Also mammalian schistosomes of the genera *Bivitellobilharzia*, *Heterobilharzia*, and *Schistosomatium*, and animal-parasitizing members of the genus *Schistosoma* follow this pattern, although the intermediate hosts are not always known. Some of these schistosomes belong to economically important parasites, causing pathology and sometimes death of livestock. In addition, cercariae of some representatives have been shown to penetrate the skin and inflict dermatitis on humans (e.g., *Heterobilharzia americana*, *Schistosoma turkestanicum*, *Schistosoma bovis*, *Schistosoma spindale*).

10.2.2.1 *Bivitellobilharzia*

Bivitellobilharzia spp. parasitize elephants in Africa (*B. loxodontae*), India and Sri Lanka (*B. nairi*); the latter species has recently been found in the greater one-horned rhinoceros (*Rhinoceros unicornis*) from Nepal [29]. The reported prevalence in south India is about 4 % [30], and in Africa (Republic of Congo and Central African Republic) about 33 % [31]. The intermediate hosts are unknown. Although the parasites may be responsible for death of the hosts [32], the information on the life cycle, biology, and pathogenicity is scarce.

10.2.2.2 *Schistosomatium*

Schistosomatium sp. is a parasite of rodents and lagomorphs in North America. Larval development occurs in lymnaeid snails. With regard to the host spectrum, economic importance of the parasite is low. On the other hand, the worms may be used as a suitable laboratory model. For example, cercarial peptidases [33], regulatory peptides [34], or impact on host physiology [35, 36] have been studied.

10.2.2.3 *Heterobilharzia*

Heterobilharzia americana is becoming an economically important parasite of carnivores, lagomorphs, rodents and some other mammals in the USA; lymnaeids (e.g., *Fossaria* spp. and *Pseudosuccinea* spp.) serve as the intermediate hosts. Raccoon (*Procyon lotor*) is the most important reservoir host in which the prevalence reaches up to 70 %. Dogs seem to be the most important domestic definitive hosts, although the number of reported infections is rather low. They suffer from lethargy, weight loss, anorexia, diarrhea, vomiting, etc. Adults of *H. americana* inhabit and lay eggs in the mesenteric venules. Some eggs leave the host body via feces, whereas the others spread hematogenously to other tissues, primarily the liver where granulomatous/eosinophilic inflammation and fibrosis can be found [37, 38]. Heterobilharziasis is likely to become a more important emerging disease of domestic dogs [39]. Recently, the species has been reported as a pathogen of horses, causing hepatic granulomas [40, 41]. Direct diagnosis is based on finding the eggs in feces; ELISA may also be used to detect circulating antigens. The animals can be treated with praziquantel [39] or benzimidazoles.

10.2.2.4 *Schistosoma* spp. in Animals

Currently, there are about 25 known species of the genus *Schistosoma*; eight of them have been confirmed as parasites of humans [42, 43]. Besides humans, members of the genus *Schistosoma* parasitize different groups of mammals. Recent estimates indicate that over 165 million cattle in Africa and Asia are infected with various species of *Schistosoma* [44]. In some cases they may cause significant economic losses to the livestock industry. At least three examples of these important species can be mentioned.

10.2.2.5 *Schistosoma turkestanicum*

Schistosoma turkestanicum (syn. *Orientobilharzia turkestanicum*) [43] occurs in Asia (predominantly in the middle and Far East) and recently in Europe (Hungary—see below). Adult worms are localized in portal and intestinal veins of a range of mammals, including cattle, sheep, goats, water buffaloes, horses, donkeys, mules,

and camels; infections of field rats from populations in Turkey have also been reported. Lymnaeid snails serve as intermediate hosts [45]. Prevalence in definitive hosts can reach high percentages; it can be as high as 100 % (e.g., in Iran) [46]. Recently, *S. turkestanicum* has appeared in southern Hungary, Europe. Here, the fluke has been found in the liver/portal veins of red deer (*Cervus elaphus*) in a prevalence of 31 %. *Radix auricularia* serves as the intermediate host [47]. Molecular data show that the Hungarian population of *S. turkestanicum* has been native since the Ice Age and probably established itself during the last interglacial period as red deer moved to Europe from North Africa and the Middle East [48].

In the endemic countries, intensity and seriousness of the infection may frequently be associated with the timing and quantity of rainfall; the highest intensity of infection has been reported from China where a sheep harbored over 40,000 worms, and a cattle animal hosted over 58,000 worms [45]. Goats seem to be more vulnerable to the infection than cattle and sheep. Wang et al. [45] referred to an unpublished outbreak that showed a 40 % fatality rate. Pathogenicity of *S. turkestanicum* towards their definitive hosts is similar to that of other *Schistosoma* species. In addition to the infections of animals, cercariae of *S. turkestanicum* may cause human cercarial dermatitis as reported from Iran [49], China [50], and probably Hungary [47]. This can happen because free fatty acids serve again as stimuli of cercarial penetration [51]. In definitive hosts, diagnosis is based on detection of the eggs in feces, postmortem examination of slaughtered animals, and use of serological tests. Praziquantel seems to be the drug of choice. Cercarial dermatitis requires measures similar to those against avian schistosomes.

10.2.2.6 Indian Schistosomes

A variety of domesticated animals in south and Southeast Asia can be infected by members of the *Schistosoma indicum* group. *Schistosoma indicum s. s*., *S. spindale*, and *S. nasale* represent species having veterinary importance. All the three species are transmitted by the same intermediate snail host, *Indoplanorbis exustus*. *Schistosoma indicum s. s*. can be found on the Indian subcontinent in equines, sheep, camel, goats, cattle, buffalos, etc. Infections affect liver and large intestine and can be accompanied by debility, diarrhea, and death. *Schistosoma spindale* lives in vessels of the small and large intestines of buffalos, cattle, goats, sheep, equines and rodents. It occurs on the Indian subcontinent and in Southeast Asia (Thailand, Indonesia, Malaysia, and Vietnam); for example, in south India, 68 % of cattle from Bangalore were infected [52]. Unlike *T. szidati* (see above), *S. spindale* cercariae use higher temperatures, but no chemical cues for host identification (attachment to the skin surface); penetration itself is triggered by free fatty acid fraction of bovine skin-surface lipids [53]. The infection affects liver where hepatic lesions with periportal cell infiltration and periportal epithelioid cell granulomas within perilobular zones can be found. Submucosal and mucosal granulomas develop in the small and large intestines [54]. Severe infections of cattle may lead to diarrhea, anemia, and death. The only nasal species among mammalian schistosomes is *S. nasale*

occurring in India, Sri Lanka, Myanmar, and Bangladesh. For example, in Sri Lanka the overall prevalence in cattle is about 12.6 % [55]. In south India, 72.7 % of cattle from Bangalore [52], and 11.1 % of cattle and 23.4 % of buffaloes from Wayanad were positive [56]. It causes snoring disease in buffalos, cattle, sheep, and goats. Histopathologically, granuloma formation around the eggs deposited in the nasal mucosa can be seen; in most severe cases cauliflower-like growths obstructing the nasal cavity can be observed [55]. It seems the animals differ in their susceptibility. In cattle, the infection leads to severe pathological lesions and pronounced symptoms, whereas in buffalos the course of infection is mild; buffalos show an innate tolerance to the parasite [55, 57]. Although both species, *S. nasale* and *T. regenti*, mate and lay eggs in the nasal mucosa of their mammalian and avian hosts, respectively, the mode of migration from the skin to the final location seems to be different: *T. regenti* follows peripheral nerves in order to enter the spinal cord and brain, and after that it immediately moves to the nasal area [18], whereas *S. nasale* seems to use blood circulation, because many immature/mature worms can be found in the lungs, liver, mesenteric veins, and heart of experimental animals [58, 59]. Diagnosis can routinely be performed by detection of eggs in feces (visceral schistosomes) or nasal mucous secretions (*S. nasale*). Also serological tests for detection of specific antibody or antigens in fecal samples (sandwich ELISA) have successfully been tested [44, 60, 61]. Chemotherapy of the infections is available, e.g., praziquantel [62] or triclabendazole [63] can be applied as drugs of choice.

10.2.2.7 *Schistosoma bovis*

Schistosoma bovis occurs in Europe (Spain and Mediterranean islands), southwest Asia, and Africa [64, 65]. In some areas it can represent a serious veterinary problem, e.g., in Sudan prevalence in cattle can reach nearly 90 % [66]. In other areas, lower prevalence rates have been reported, e.g., in Tanzanian cattle 34 % [67]. Cattle, horses, donkeys, sheep, goats, camels, pigs, antelopes, and rodents can serve as the definitive hosts. The intermediate hosts are represented by various species of the genus *Bulinus*, and in Spain by *Planorbarius metidjensis*. The adult worms parasitize intestinal mesenteric veins, and the disease can be linked with diarrhea and weight loss, although most infections in endemic countries are subclinical. Histopathological examination disclosed intestinal and liver lesions (granulomatous inflammatory reactions caused by the eggs and adults), and also other organs can be affected in heavy infections [68]. *Schistosoma bovis* is another suitable organism serving for molecular biology research. For example, it has been shown that *S. bovis* adult males express enolase on their tegumental surface; the protein is a receptor of plasminogen that plays a role in the activation of the host fibrinolytic system. In this way, the worms most probably avoid blood clot formation on their surface [69]. In addition, *S. bovis* annexin having fibrinolytic and anticoagulant properties has been demonstrated on the tegument of schistosomula and adult worms [70]. These essential molecules, together with other candidates (e.g., peroxiredoxin, paramyosin, cathepsin B) obtained by proteomic approaches, offer potential vaccine targets for

the control of schistosomiasis in ruminants [71]. In the past, vaccination of ruminants having a protective effect has been realized by irradiated schistosome larvae, and later on by recombinant *S. bovis* glutathione S-transferase [72]. Diagnosis lies in coprology as the most frequently used approach, but detection of specific antibodies against the parasite (recombinant) antigens has also been introduced; the latter method is more sensitive for detecting light infections and can be applied in large-scale examinations [65]. As for treatment, praziquantel has been shown to be highly effective [73, 74].

10.3 Aporocotylidae (syn. Sanguinicolidae)

Taxonomy of the family has a complicated history. For the blood flukes of fish, the family Sanguinicolidae was erected by Von Graff [75]. This family-group name has been widely accepted throughout the twentieth century. Nevertheless, some authors maintained the family name Aporocotylidae Odhner, 1912 for marine species of fish blood flukes [76], although it has been evident that the diagnostic characters of the two families are often uncertain and inconsistent, and there is little correlation between hosts and parasites [77]. Therefore, Overstreet and Køie [78] considered Aporocotylidae to be a junior synonym of Sanguinicolidae, and the latter taxon name has been accepted by Smith [79] in the *Keys to the Trematoda*. However, a deep and critical examination of the literature from the beginning of the twentieth century [80] revealed that "Aporocotylidae Odhner, 1912 is the earliest available family-group name for these flukes." Currently, the family consists of 33 genera (April 2014) parasitizing the circulatory system and body cavities of marine and freshwater teleost fish and chondrichthyans. Although the number of known genera is yet not high compared to some other trematode families, Aporocotylidae is apparently a highly radiated group of flukes, with numerous new genera and species being described recently, mainly from marine environments [81, 82]. Some species are of significant economic importance as pathogens of farm fish. Literature on blood flukes of cold-blooded vertebrates has extensively been reviewed by Smith [83–85].

10.3.1 Life Cycle

Relatively few complete life cycles of aporocotylids have been described, especially in marine species [86]; all of them are dixenous. There are still many associations between intermediate hosts and aporocotylid larval stages with unresolved species determination. Molecular approaches would help to solve these problems.

Contrary to schistosomes, aporocotylids are hermaphrodites. Eggs are mostly reported non-operculate, egg shell is thin and elastic, and reported by Smith [83] to contain elastin; however, McMichael-Phillips et al. [87] did not detect elastin in egg shells of *Sanguinicola inermis* by histochemical tests. Miracidia hatch directly in

the gill tissue and leave the host into the water environment. Mechanism of hatching was not studied in detail; the larvae leave the egg shell upon rupture, actively liberate themselves from gill filaments and seek for and invade an intermediate host, mostly by active penetration [83, 88]. Pigmented eyespot occurs in, e.g., *S. inermis*, suggesting the possibility of orientation based on phototaxis. Miracidia may be armed with a stylet used for mechanical disruption of host tissues; moreover, histolysis based on the action of proteolytic enzymes contained in the apical gland or paired lateral glands is highly probable [87].

The association of aporocotylids with a broad range of clades of marine/freshwater gastropods, marine bivalves and also marine polychaete annelids is intriguing and unique amongst trematodes; they are the only group of digeneans that may use a non-mollusc as the first intermediate host. However, it seems that the host spectrum of particular species is somewhat restricted, e.g., to a single family of intermediate hosts [89]. The development inside the intermediate host is not known for all genera. Most aporocotylids produce a few generations of sporocysts, usually one or two, which give rise to cercariae [83, 88]. A clear evidence of rediae was surprisingly found in *Aporocotyle simplex* from terebellid polychaetes [90, 91], while *Cardicola forsteri* reported from members of the same family of polychaetes produces sporocysts [86]. Parasitic castration of polychaete intermediate hosts was observed [90]. In molluscs, the development takes place in the digestive gland. The only record of an exceptional localization in the gill hemocoel has been published by Gilardoni et al. [92] for an unidentified aporocotylid species in the marine venerid clam *Amiantis purpurata*.

Cercariae are apharyngeate, non-ocellate, brevifurcate, without acetabulum, although exceptions occur, e.g., in *Cardicola* which possesses a simple tail [86]. Typically, a dorsal fin occurs on the body (lophocercaria type, Fig. 10.3a). The apical part is armed with a muscular conus called "head or cephalic organ," through which the ducts of penetration glands run to the openings at the apex. Four pairs of penetration glands have been reported for *S. inermis* [88] and five pairs for *Aporocotyle simplex* [90]. No studies have been done on the content of penetration glands, though proteolytic enzymes can be expected to play a role in histolysis. Periodicity of cercarial shedding from the infected snails was studied by, e.g., Martin and Vazquez [93]; light and temperature may affect the process [94]. Neither phototaxis nor response to shadow stimuli was observed in cercariae of *Sanguinicola lophophora* [94], whereas Meade [95] found phototropic response in *Sanguinicola klamathensis*. Response to cues, either chemical or physical, produced by hosts has not been studied. Cercariae penetrate their hosts mostly via gills and skin of the body or fins. Less scaled parts seem to be preferred [88, 90, 95, 96]. During penetration, transformation of cercariae takes place, which is characterized by tail loss, dissolving of dorsal fin, and formation of a double outer surface membrane common to all three families of blood flukes [97]. Qualitative and quantitative changes in surface glycocalyx composition of transforming larvae have been proved by altered reactivity with fluorescent lectin probes (Mikeš and Stiegeler, unpublished). Infection process, migration, and development in fish have been studied in greater detail in *S. inermis*, a parasite of cyprinid fishes. Maximum cercariae entered body

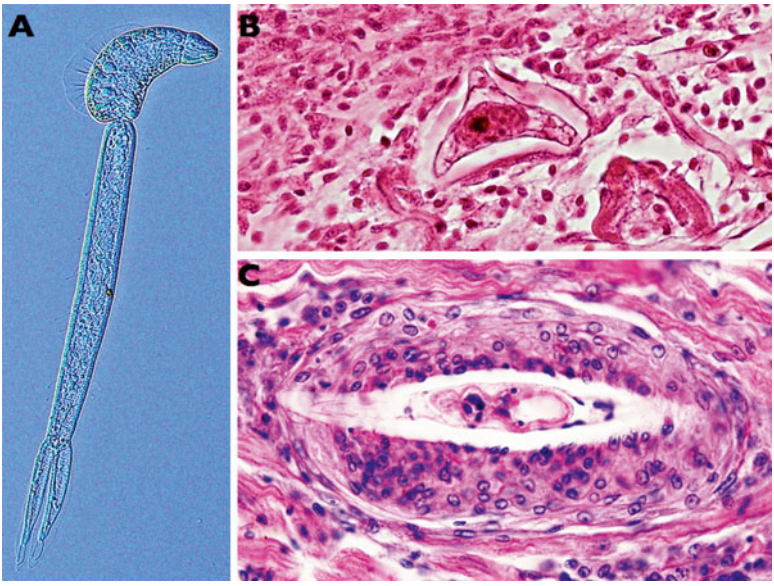

Fig. 10.3 (**a**) Lophocercaria of *Sanguinicola inermis* (length of about 350 μm) with a typical dorsal fin, released from the intermediate host, *Radix auricularia*. (Author: Dr. M. Soldánová); (**b**) Section showing the triangular egg of *Sanguinicola inermis* trapped in the gill tissue of young carp; miracidium with pigmented eye spot is located inside. (Author: Prof. P. Horák); (**c**) Spindle-shaped egg of *Cardicola laruei* in the cardiac muscle of the spotted sea trout, *Cynoscion nebulosus*. Granulomatous reaction around the egg is visible. (Author: Prof. I. Dyková)

of carp fingerlings within 30 min. The success rate of penetration was between 1 and 3 %. A sharp decline in the number of worms occurring in the skin was observed to start 60 days p.i.; 43 % of them were present in the skin even 90 days p.i.; the onset of egg production by adults in the heart/gills was 73 d.p.i. [96]. This is in contrast with the data of for example Kirk and Lewis [88, 98], who showed that around 1/3 of cercariae penetrated the fish, and faster migration occurred through the connective tissue and muscles towards the ventral and dorsal blood systems. The worms that penetrated the anterior part of fish were more successful in reaching the heart; localization in the gills and cardiac regions has been reported by the 21st–28th days post infection. Maximum life span of adults was 70 days, and the eggs contained fully developed miracidia within 7 days from being laid. The rate of migration seems to be strongly affected by temperature.

Adult aporocotylid flukes are usually localized in the heart, bulbus arteriosus, branchial vessels, or other large arteries. Few exceptions are represented by the occurrence in veins of the body cavity, e.g., in case of *Sasala nolani* that produces eggs conspicuously accumulating in the host gut wall [99]. Occasionally reported cases of extravascular localization of adult flukes may probably arise from coincidental or aberrant migration, or rupture of blood vessels. The range of fish species serving as hosts for aporocotylids is extremely broad. Both specialists and

generalists have been recognized within the family. As summarized by Cribb et al. [89], members of the family "are reported from at least 45 families of fishes and from a total of 18 orders of fishes including elasmobranchs, holocephalans and teleosts; they are one of only a handful of families to exploit all three of these taxa."

10.3.2 Occurrence

Fish blood flukes are cosmopolitan. Our knowledge of general distribution is incomplete as some areas of the world have not been explored by fish parasitologists. Research in the new regions is usually followed by descriptions of new species or genera. Particularly, marine environments are still grey areas and promise numerous discoveries in terms of diversity of Aporocotylidae, their biology, host associations, and ecology. Also, the information on distribution of particular species is often incomplete; again, better data are available on freshwater species, particularly those parasitizing fish of economic importance. In marine species, intermediate hosts are mostly unknown; as the life cycles of digeneans are obligatory dependent on appropriate intermediate hosts, the knowledge of their distribution could help to assess the expansion of parasites in fish. Nevertheless, the situation can be complicated by regular migration of some fish species, as depicted for *Cardicola forsteri* and *Cardicola* sp. in tuna fishes (*Thunnus* spp.) by molecular approaches [100]. Prevalence of the flukes in fish hosts varies greatly among various reports, and apparently, there is no common pattern. It can be near 100 %, especially in sites with high concentration of fish. Therefore, the prevalence and intensity of infections are often higher in extensively farmed fish [101, 102]. On the other hand, prevalence in wild populations of fish is commonly lower [103] as evidenced for *Cardicola laruei* in sea trouts *Cynoscion* spp. from the Gulf of Mexico and Atlantic Ocean (4–10 %). Some parasites migrate to a specific site within the host and start to lay eggs during a distinctive season, resulting in peaks of intense infections and related fish death [104].

The prevalence of larval stages in intermediate hosts used to be rather low, usually below 10 %. Faltýnková et al. [105] found 3.2 % prevalence of *Sanguinicola* sp. in *Valvata* snails in Lake Konnevesi, Finland, and Køie [90] referred to about 7 % of *Artacama proboscidea* (Polychaeta) from Oresund, Denmark, being infected by *Aporocotyle simplex*.

10.3.3 Damage to the Definitive Hosts

Some species of fish blood flukes act as serious pathogens causing mass mortalities and significant economic loss in fish industry. Pathological effects of aporocotylids on marine and freshwater fishes were thoroughly reviewed by Bullard and Overstreet [106] and Kirk [107], respectively. Similarly to other blood flukes (schistosomes

and spirorchiids), the main pathology arises from the presence of eggs in blood vessels and various organs/tissues. The eggs are laid by worms or passed by blood circulation from different sites of infection into the gill filaments of the host (Fig. 10.3b), from which miracidia can escape into the water. Additionally, a significant amount of eggs is sequestered in blood vessels or tissues of other organs including heart (Fig. 10.3c), kidney, liver, pancreas, spleen, etc. This may lead to embolism, hypoxia, infarction, and necrosis. Granulomatous inflammation is formed in the areas surrounding the eggs, and may finally result in irreversible tissue damage. The level of harm is obviously dose-dependent; heavy infections may be fatal, while in cases of nonlethal parasitemia various degrees of debilitation occur and decreased weight gain may be achieved.

Acute mortality in fish can be produced by a large number of miracidia escaping simultaneously from a heavily infected host, which is accompanied by severe hemorrhaging in the gills. Similarly, a high number of penetrating cercariae (and migrating successive stages) may kill the host, perhaps due to excessive amount of bioactive substances (e.g., peptidases) released from penetration glands into the host tissues. Experimental exposure of individual carp fingerlings to 2,500 cercariae of *S. inermis* was followed by severe edema, epidermal hemorrhage, and death within a few hours [108] (Mikeš, personal observation); however, such an extensive immediate exposure is not probable under natural conditions due to dilution of cercariae in the environment and mobility of the hosts, which can apparently perceive higher number of penetrating larvae (Mikeš, personal observation).

10.3.4 Diagnosis and Control

With regard to the localization of adult flukes, complete helminthological dissection of fish is necessary for reliable confirmation/exclusion of infection by aporocotylids. Adult flukes or tissue-migrating juvenile stages can be found almost anywhere in the body of the host. Special attention must be paid to the vascular system, namely, heart and bulbus arteriosus, branchial arteries, aortas and other large arteries, lymphatic system; nevertheless, aporocotylids have been found also in mesenteric veins [99], veins surrounding brain and optic lobes [109] and elsewhere. Due to small dimension, the worms are often hard to find and the use of dissecting microscope is necessary. Methods of collection and preservation of trematodes from fishes for taxonomy and systematics have been published by Cribb and Bray [110].

As for the presence of aporocotylid eggs, various organs/tissues should be checked microscopically. Preparation of compression mounts of soft tissues is the method of choice; alternatively, histological processing can be performed. Determination of fluke species based solely on egg morphology may not be reliable due to relative uniformity of eggs among some species, or lack of data on egg morphology in particular species.

Intravital approaches to diagnosis of infections may include biopsy of gill filaments or cardiac puncture, which require certain skills and equipment. There is

always some risk of unattended bacterial or fungal infection of manipulated fish connected with these invasive methods. Theoretically, diagnosis based on capture of miracidia escaping from live fish isolated in appropriate tanks would be possible.

Immunodiagnosis of aporocotylidiasis based on specific antibody response of the hosts is a new approach and is still in its infancy. The methods are known for a long time, but limitations are linked with poor knowledge of fish immune system, e.g., variations in immune responses of different species of fishes, specific responses against diverse blood flukes, unknown nature of fluke antigens and their cross-reactivity with those of other parasites. Thus, the application of immunodiagnostics may be useful in particular cases and conditions, and will rather be targeted to aquaculture, where the risk of infection by a specific parasite can be assessed and immune response of particular host species has been studied. First promising results in immunodiagnosis of *Cardicola forsteri* infections have been reported by Aiken et al. [111] and Kirchhoff et al. [112].

Molecular diagnostic tools are also available and constantly develop. The most used genetic markers include ribosomal 28S DNA, 18S DNA, and ITS2 region, and mitochondrial gene coding for CoxI [82, 89, 100, 113, 114, 115]. The practical applicability and outputs of molecular techniques largely depend on the set of sequence data available.

Various measures can be taken to control the abundance of fish blood flukes. Indeed, regular monitoring of stocks is desirable on the site of production as well as veterinary inspection before transfer, and examination and quarantine of imported fish. Wherever possible, the chance of contact between farm and wild populations of fish should be avoided or kept to a minimum, because the intermediate hosts serving for transmission may co-occur at the same time.

Several approaches can lead to interruption of the life cycle of aporocotylids. Intervention to and management of the environment in order to decrease populations of intermediate hosts is one of the possibilities. Thus, "summering" or "wintering" (i.e., keeping ponds without water over the summer or winter) has traditionally been practiced in the Czech Republic and surrounding countries with carp farming in order to interrupt transmission of *Sanguinicola inermis* and various other digeneans and cestodes. It can be accompanied by application of compounds relatively harmless to the environment, like burnt lime, on the surface of mud. Application of other chemicals used in the past (e.g., copper sulfate) is arguable due to their ecotoxicity. Macrophytes serving as diet for some freshwater gastropods can be removed from litoral zones, which increases expenses and therefore it is practiced just in restricted areas, e.g., in little startup ponds used for small fry and fingerlings. Introduction of a proportion of snail-eating fish into the stocks of fish of main economical importance in extensively managed ponds may cut down the population of snails.

In marine fish farming, careful selection of sites for location of pens or net cages is important as the excessive occurrence of intermediate hosts will promote parasite transmission; moving aquaculture further offshore may be a solution. Comparison of prevalences of *Cardicola forsteri* in caged southern bluefin tuna showed 85 % of infected fish kept near shore in contrast to 0 % in the offshore cohort [116].

Possibilities of antihelminthic therapy have recently been checked in bluefin tunas (*Thunnus orientalis*). Among four compounds tested, praziquantel was the most effective against adult flukes and therefore being considered as the drug of choice for treatment of *Cardicola* infections [117, 118]. However, this treatment had no significant effect on the viability of miracidia inside the eggs, thus not guaranteeing interruption of transmission to intermediate hosts.

There are few examples of aporocotylids, both marine and freshwater, causing damage to fish industry on a larger scale. Some of them arose from recent increase of the number of pen-reared fish species in marine aquaculture [106].

10.3.5 Cardicola

Cardicola is the largest marine aporocotylid genus [86] found in teleost fishes representing nine families, reported from the Atlantic and Pacific Oceans and South China Sea [79]. *Cardicola forsteri* have recently become an emerging pathogen causing massive mortalities in cage-reared southern bluefin tuna in coastal areas of Australia [119, 120]. It has a big potential of further emergence in aquacultures in other parts of the world, being able to infect other tunas, like northern bluefin suffering from cardicoliasis in Spanish coastal farms [100]. In Japan, mortality of pacific blue tunas is caused by *C. orientalis* and *C. opisthorchis*; in a heavily infected individual, a total number of >4.5 million eggs were estimated in the gills at one side of the fish [121]. Another cardicoline species causes trickling mortalities in cultured sea bram *Sparus aurata* in Spain [102].

10.3.6 Sanguinicola

Sanguinicola spp. are mostly parasites of cyprinid and other freshwater teleosts of Europe, Asia, northeast Africa, and North America [79], and probably also South America. There is only one species reported so far from marine teleosts (*S. maritimus*) in Australian waters [122]. *Sanguinicola inermis* was reported from lymnaeid snails of the genus *Radix* in Europe (*R. auricularia* and *R. peregra*, although the identity of the latter host is uncertain due to confusion in taxonomy of the genus [123]). Its main definitive host is common carp (*Cyprinus carpio*), and some other cyprinids serve as less efficient hosts [124]. The seasonal cycle of development in carp fisheries with peaks of fish infection in spring/early summer and late summer in temperate regions was summarized by Kirk [107]. In extensive carp breeding, massive mortality caused by *S. inermis* may occur, especially in young fish (year 0–1+). Serious outbreaks in carps have repeatedly been reported from various parts of Europe; salmonid hatcheries in North America have encountered problems with huge losses due to infections by *S. davisi*, *S. klamathensis*, and *S. fontinalis* [124]. Currently, as a result of integrated approaches to the control of sanguinicoliasis, large outbreaks are not reported from Europe and North America.

10.4 Spirorchiidae

Spirorchiids are one of the three families of blood flukes. They have been found in both marine and freshwater turtles, and in crocodiles. Although their economic importance is rather inconsiderable in comparison to blood flukes of mammals and fish, they may seriously threaten populations of endangered species of turtles in some parts of the world [125, 126]. Besides, the group has drawn much attention in phylogenetic and evolutionary studies within Schistosomatoidea [127–129]. The diversity of the group is yet not fully understood, as the majority of turtle genera have probably never been examined for parasites.

Biology, diversity and taxonomy of spirorchiids have been reviewed by, e.g., Yamaguti [76], Smith [83–85], and Platt [130]. Besides localization within the host, they share more common features typical for the other families of blood flukes, having dixenous life cycle, furcocercariae that actively penetrate the definitive host, double surface membrane in adults, and eggs sequestered in the host tissues that are the main source of pathologies. As in the aporocotylids, they are hermaphrodites (except for *Griphobilharzia amoena*). Eggs are released from the digestive tract of the host with feces, thus resembling most schistosomatids. Postmortem liberation of eggs from host tissues to the aquatic environment seems to be likely due to natural disintegration of carcasses or digestion in the predator alimentary tract. Larval development, where known, takes place in gastropods.

Adults can be found in various parts of the host body, including heart, pericardial cavity, arteries, veins, esophagus, and even abdominal coelom or connective tissues [131], but some species can be more organ-specific. Eggs are typically disseminated in virtually all tissues. Chronic infections can result in generalized disease and may be accompanied by high morbidity and mortality rates. Spirorchidiasis can produce neurological signs in infected animals, like hemiplegia and loss of reflex activity caused by focal destruction of brain tissues due to inflammatory reactions surrounding the eggs [132]. Further pathological effects involve generalized granulomatous response, aneurysms, arteritis, endocarditis, vasculitis, and hemorrhagic lesions [133–136]. In green turtles (*Chelonia mydas*) and some other marine species, spirorchidiasis is often associated with a herpes virus-related fibropapillomatosis; this leads to general emaciation and anasarca, and neoplasy in the gastrointestinal tract, lungs, liver, kidney, muscles, etc. [137]. However, the causal relationships require further research [138].

Several reports have been published on high prevalence of infections in both freshwater and marine turtle species, often reaching more than 80 % [132, 134–136]. However, the impact on wild populations of particular host species is largely unknown.

Methods for the collection and preservation of spirorchiid trematodes from turtles are based on necropsy and helminthological dissection. Some improvements using citrated saline and a separatory funnel were described by Snyder and Clopton [139]. Treatment of spirorchidiasis is possible, and praziquantel has been proved an efficient drug in a few studies [140, 141]. The practical use is related to wild turtles caught and introduced to farms or aquaria.

Griphobilharzia amoena is the only species reported so far from a non-testudine host, that is, the crocodile *Crocodylus johnstoni* living in Australia and Irian Jaya. Historically, this fluke has been considered as a basal schistosomatid [127, 142], the missing link in schistosome evolution, and was presented in this way in the *Keys to the Trematoda* [143]. However, the study of Brant and Loker [144] revealed that it is more closely related to spirorchiids from freshwater turtles;therefore, dioecious condition and unique morphology of *G. amoena* [145], together with its phylogenetic position among flukes require further study.

10.5 Diplostomidae and Strigeidae

Both families comprise parasites with adults living in the digestive tract of birds and mammals. Members of these digenean taxa are remarkable in two aspects: (a) Body of the adult worms is composed of two parts—foliate, spatulate, calyciform, or bulbiform forebody, and cylindrical or coniform hindbody. Besides oral and ventral suckers, the forebody has a ventrally located massive holdfast (tribocytic organ) and, in some representatives, pseudosuckers (lappets). The holdfast probably serves also as a digestive–absorptive organ. (b) Some life cycles include the stage of an unencysted mesocercaria, an additional type of larva that follows the stage of furcocercous cercaria, and precedes the stage of metacercaria. Therefore, these flukes can have three- or four-host life cycles that can be extended by paratenic hosts with mesocercariae (see below).

10.5.1 Alaria

10.5.1.1 Life Cycle

The life cycle of *Alaria* spp. has been reviewed by Möhl et al. [146]. Various freshwater pulmonate snails serve as the first intermediate host; asexual reproduction of larval stages within these snails leads to the formation of furcocercous cercariae leaving the snail. Highly motile cercariae search for a frog (second intermediate host) in which they transform to mesocercariae. If a tadpole is infected, mesocercariae survive metamorphosis of the host. The infected frogs can be caught by a predator, and mesocercariae released in the intestine migrate through the predator body, settle in different organs/tissues, but they do not develop further. Therefore, these predators serve as paratenic (transport) hosts. Mesocercariae can survive several host transitions unharmed. Further development of mesocercariae takes place in a broad spectrum of definitive carnivore hosts (Canidae, Felidae, Mustelidae). Mesocercariae transform to diplostomulum-type metacercariae that occur in the lungs, and the adult worms (2–4 mm long) can be found in the small intestine of

these hosts. Migration in the definitive host does not include a true stationary phase and is accompanied by a continuous transformation: mesocercaria–metacercaria–adult worm. The eggs produced by adults leave the host via feces, and miracidia develop in the outer environment.

Some definitive hosts (e.g., felids) may serve, under certain circumstances (e.g., pregnancy), as paratenic hosts (amphiparatenesis). In this case, mesocercariae do not transform to metacercariae, and transmammary transmission of mesocercariae to the offspring may occur [147].

10.5.1.2 Occurrence

The genus *Alaria* consists of about eight species; *A. alata* parasitizing carnivores occurs in Europe and the former Soviet republics, whereas the remaining species (including *A. americana* and *A. marcianae*) can be found in North and South America [148]. Prevalence of infection in the definitive hosts in some areas can reach 70–90 % (European wolves in Estonia [148], red foxes in Poland [149], raccoon dogs in Germany [150]). Prevalence rates of mesocercariae in wild animals can also be quite high, particularly in omnivores like wild boars.

10.5.1.3 Damage to the Intermediate or Paratenic Hosts

From the veterinary and medical viewpoints, mesocercaria seems to be the stage of enormous interest. It is particularly nonspecific towards its hosts, because diverse groups of vertebrates can be infected (snakes, rodents, moles, birds, felids, etc.). Mesocercaria is probably the most pathogenic stage causing tissue lesions. It can cause damage to the abdominal organs, lungs, eye, somatic muscle, and subcutaneous tissue; severe and sometimes fatal illnesses were reported also in humans [151].

10.5.1.4 Diagnosis and Treatment

Whereas the methods to detect *Alaria* spp. in the definitive host (proof of the eggs in stool samples, postmortem examination of the intestine for adult worms) are available, detection of mesocercariae in the meat of paratenic hosts (mainly wild boar) is under discussion. Use of compression method or pooled digestion method according to the protocols for *Trichinella* larvae seems to have limited sensitivity. Recently, a new detection method based on larval migration technique has been introduced [152]. As well as, a new PCR assay for identification and characterization of *A. alata* mesocercariae has successfully been tested; it can be used for diagnostic and epidemiological purposes [153]. Praziquantel can be used for the treatment of intestinal infections with adult worms.

10.5.2 Diplostomum

Members of the genus are widely distributed throughout the world; taxonomy, morphology and biology have been reviewed by, e.g., Niewiadomska [154, 155] and Chappell [156]. Although several species have been recognized according to the morphology of adults, a reliable determination based on morphology of larval stages is often challenging or even impossible, and has led to confusion in the literature. In Europe and North America, diplostomid cercariae from lymnaeid snails or metacercariae from fish eyes have often been referred to as *D. spathaceum*. Recent investigations, commonly supported by molecular data, depicted higher diversity and enabled accuracy in taxonomy of larval stages from snails and fish, and adult flukes from birds [157–159]. Reliable identification is desirable, as pathology, monitoring and control measures may vary among species [156] and *Diplostomum* spp. are often used in veterinary-related, ecological and experimental studies. As Niewiadomska [154] stressed, many early records of different *Diplostomum* spp. in fishes from Europe are based on erroneous identifications; the problem has been similar in cercariae. This fact somewhat disparages the span and accuracy of detected spectra of hosts.

10.5.2.1 Life Cycle

The typical life cycle includes freshwater gastropods and fish as first and second intermediate hosts, respectively. Fish-eating birds (Laridae and several other families) serve as definitive hosts. Furcocercariae (Fig. 10.4) develop within sporocysts in the digestive gland of snails. Occasionally, larval progenesis has been observed to produce metacercariae in sporocysts [160]. Intramolluscan stages of *D. spathaceum* suppress the defense system of *Lymnaea stagnalis* in terms of decreased phagocytic activity of hemocytes and reduced agglutinating activity of the hemolymph [161]. Huge numbers of cercariae may be released from the infected snails, almost 40,000 per individual in 24 h; production of *D. spathaceum* cercariae is lower during night hours [162]. They respond to various cues, either environmental or host-related. In *D. pseudospathaceum sensu* Niewiadomska [163], and Niewiadomska and Kiseliene [164], the stimuli include direction and intensity of light radiation, dark and light stimuli, water currents, touch and gradient of CO_2; enduring contact with the host is stimulated by small hydrophilic compounds of carbohydrate character, whereas penetration is triggered by sialylated *O*-glycoproteins of fish mucus and fatty acids of the skin [165]. Penetration is facilitated by action of muscular head organ and secretion of a cathepsin L-like cysteine peptidase released from four unicellular penetration glands [166, 167]. During invasion of fish, loss of tail and major change in the carbohydrate composition of the surface glycocalyx characterize transformation of cercaria to early diplostomulum (Mikeš and Stiegeler, unpublished). Mortalities may occur especially in fry or small fish due to excessive amount of invading larvae.

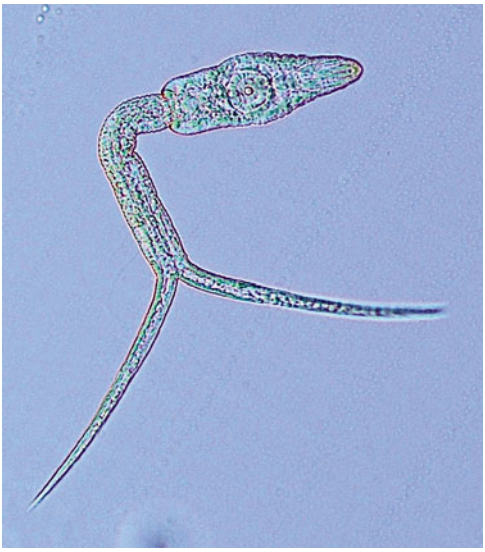

Fig. 10.4 Typical resting position of *Diplostomum parviventosum* furcocercaria (length of about 600 μm) released from the intermediate host, *Radix auricularia*. (Author: Dr. M. Soldánová)

Rapid migration towards the anterior part of the host is accomplished mostly through the subcutaneous connective tissue and muscles of the trunk [168]; however, studies on migration routes revealed inconsistent results and can involve also circulatory system [169]. Within the host, the orientation of *D. pseudospathaceum* is mediated by Cl^- ions, glucose, arginine residues and melatonin [169]. Depending on parasite species, the typical localizations of unencysted metacercariae of diplostomulum type are eye lens, retina, aqueous humor, and/or brain or spinal cord. Temperature-dependent maturation of long-living metacercariae of *D. spathaceum s. s.*, until becoming infective for a final host, takes about eight weeks in the eye [170]. Metacercariae of *Diplostomum* spp. in the eye may be mistaken for some specimens of *Tylodelphys* spp. (e.g., *T. clavata*), which in contrast apparently prefer localization in the vitreous body.

10.5.2.2 Damage to the Intermediate Fish Host

Pathobiology of fish eye flukes of the genus *Diplostomum* has repeatedly been reviewed [171, 172]. The main effect lies in the formation of cataracts, exophthalmia or even destruction of eye lens and complete blindness, especially in chronic and heavy infections. Direct mortality has been only rarely reported, but restraint of vision may alter feeding and antipredatory behavior that may lead to debilitation of fish and effortless catch by predators, including specific definitive hosts.

Example of a species with affinity to CNS can be *D. phoxini*. Its metacercariae express a specific uneven pattern of distribution in minnows *Phoxinus phoxinus*; the parasites are most abundant in brain regions known to be important in the control of

antipredator responses, like cerebellum, medulla oblongata, and optic lobes, suggesting manipulation of host behavior by the parasite [173]. Yet the effect can be a consequence of pathology as significant granular necrotic reactions frequently occur around metacercariae [174]. Some members of a few other genera of diplostomids develop in the CNS. Metacercariae of *Austrodiplostomum mordax* and *Tylodelphys destructor* reported from Americas were mainly observed in the interlobular infoldings, meninges, ventricles, and cerebellum of the brain, causing hemorrhaging, cell necrosis, inflammation, nerve fiber disruption and fibrosis, namely, in case of higher parasite load [175, 176]. In *Ornithodiplostomum ptychocheilus*, the post-penetration larval stages readily associate with cranial nerves to access directly the brain or migrate along/via peripheral nerves to the spinal cord/neural canal first, and then through to the brain [177, 178].

Human infections by fish eye flukes have not been recognized. Cercariae of *D. spathaceum s. l.* are able to enter the cornea of some mammals and humans under experimental conditions. Although being mostly immobilized within 24 h in experimental animals, they represent a potential risk to bathers due to superficial pathological changes in eyes and temporary conjunctival inflammation [179].

Unlike fish blood flukes, the eye flukes do not represent an extreme risk to the fish industry in terms of direct mortality (perhaps except for hatcheries). Nevertheless, the impaired vision/blindness and associated decrease of food uptake mean lower weight gain which, together with the risk of higher predation in open freshwater fisheries, may result in significant economic loss that has still not been well elucidated. Actually, the prevalence and abundance of some species may be quite high both in snail and fish hosts, like in *D. spathaceum* and *D. pseudospathaceum*, the latter being recognized as a dominating species in the hierarchy of larval trematode communities in *L. stagnalis* snails [180–181]. Rapid transmission occurs in places where all three hosts meet extensively due to high population densities and specific conditions (as can be seen in eutrophicated, crowded carp ponds rich in vegetation and nesting colonies of gulls in South Bohemia, Czech Republic).

10.5.2.3 Diagnosis and Control

Diagnosis of diplostomiasis in fish is primarily based on inspection of predilection sites in dissected fish (eye, brain). Alternatively, intravital diagnosis of the disease can be done by ophthalmological microscope [183], indeed, with some limitations in the field. Species diagnosis should be supported by common genetic markers (ITS regions and cytochrome c oxidase I gene), especially in case of larval stages showing high degree of morphological similarity. Sophisticated analyses of trematode parasite communities in fish eye lenses can be currently performed by methods of next-generation sequencing [184].

Prevention of diplostomiasis can rely on removal of snail hosts and management of the environment or water supply to aquaculture ([185]; also see the Chapter 10.2). Immunoprophylaxis is not available, though preliminary attempts have been made to immunize trout by cercarial homogenates or low doses of cercariae, all of them only partly successful [186–187]. Effectiveness of potential antihelminthic

chemotherapy, although not being used, is doubtful due to peculiar localization of parasites within the body of fish.

10.5.3 Black Spot Disease

Members of another group of diplostomid flukes form neascus-type metacercariae in the superficial layers of their second intermediate fish hosts [189]. Again, fish-eating birds serve as definitive hosts.

Fish can be infected by furcocercous cercariae that emerge from freshwater snails and penetrate the skin of fish. Once in the skin or the underlying tissues, the larvae release secretions to form a hyaline metacercarial cyst wall. Usually, the fish host reacts by formation of the outer fibrous capsule, and the space between the cyst and the capsule is filled with a viscous material [190]. These processes attract fish melanocytes and, as a consequence, melanin is deposited around the parasite (Fig. 10.5a); black spots in the surface layers of fish are then visible by naked eye (Fig. 10.5b). Parasite-induced black spots can be differentiated from other patterns formed by melanin-containing cells by their size, intensity and random distribution [191].

As for pathogenic effect, the penetrating cercariae may cause mechanical damage, hemorrhage, and secondary infections. Loss of fish weight and body lipids, together with increased oxygen requirements, may occur. As a consequence, fish ability to overwinter (survive) may be diminished [189]. In addition, poor condition and parasite-induced changes in fish behavior may increase susceptibility to predation. For example, the Prussian carp *Carassius auratus* infected by *Posthodiplostomum cuticola* can be more often consumed by its predator, the perch *Perca fluviatilis* [192].

10.5.3.1 *Uvulifer*

Uvulifer spp. is one of the causative agents of black spot disease. For example, *U. ambloplitis* parasitizes the intestine of the belted kingfisher (*Megaceryle alcyon*) in the USA [189]. The unembryonated eggs are voided to the outer environment with feces. After several weeks, miracidia leave the eggs and penetrate the body surface of intermediate snail host (*Planorbella* spp.). Cercariae produced by sporocysts after several weeks of the asexual reproduction leave the snail host and enter the skin of fish (e.g., sunfish *Lepomis* spp. and yellow perch *Perca flavescens*). Black spots of 1–3 mm can be found in the skin, tail base, fins and musculature. When kingfisher catches the infected fish, the life cycle is completed.

10.5.3.2 *Posthodiplostomum*

Posthodiplostomum cuticola is another fluke causing black spot disease (black spots of about 1 mm). It is a common parasite of freshwater fishes; it has been found in over 70 fish species in Palearctic region. Within the life cycle, planorbid snails

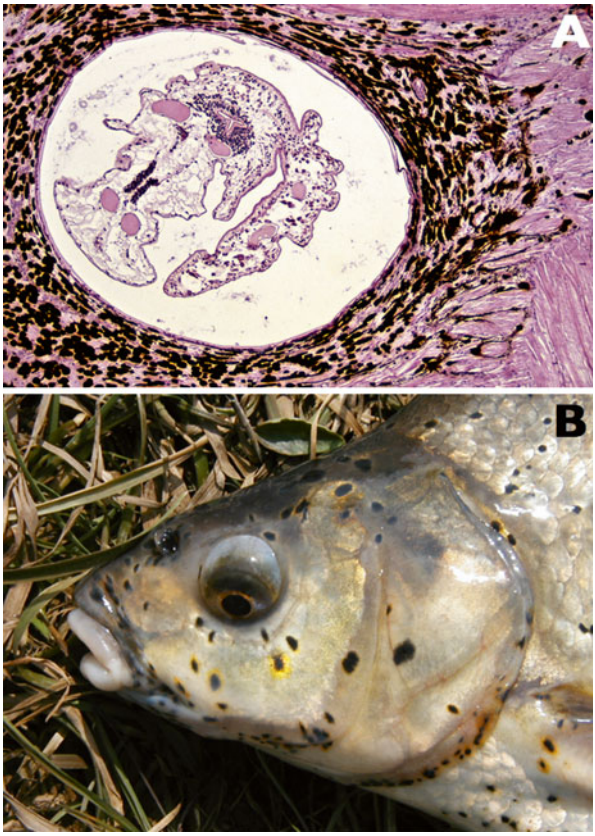

Fig. 10.5 (**a**) Section showing neascus-type metacercaria of *Posthodiplostomum cuticola* from the European bitterling, *Rhodeus sericeus. Dark-brown* deposits of melanin around the parasite cyst are visible. (Author: Prof. I. Dyková); (**b**) Black spot disease caused by metacercariae of *Posthodiplostomum* sp. in the common bream, *Abramis brama* (Courtesy of Dr. M. Ondračková; Archive of the Department of Fish Ecology, Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic)

(*Planorbis planorbis*, *Planorbarius corneus*) serve as the first intermediate host [193]. There is a broad range of second intermediate hosts, but the members of *Cyprinidae* are the most frequently attacked fishes; in some cases (e.g., in Croatia) 100 % prevalence of infection has been recorded [194]. Fish-eating birds, usually a heron or other members of Ardeidae, complete the life cycle as definitive hosts.

Besides poor conditions and potential mortality of fish, black spots often render the infected fish undesirable to consumers. Concerning preventive measures in fish farming, contact of fish with cercariae should be avoided, because the infected fish cannot be treated. Therefore, a control of fish-eating birds and snails at the locality should be used.

It should be noted that species of the same genus have also a different location in the body of their second intermediate hosts. For example, *Posthodiplostomum minimum* encysts within the body cavity [195]; the parasite is frequently found in North America—in lakes in north-central Alberta, Canada, the prevalence of infection in adult minnows is typically 100 % [196].

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Chapter 11
Amphistomes

Veena Tandon, Bishnupada Roy, Jollin Andrea Shylla, and Sudeep Ghatani

11.1 General Morphology

The body of the amphistome fluke is elongate, conical, cylindrical and flattened dorsoventrally. In some species, the body may be differentiated into two parts: cylindrical anteriorly and somewhat expanded posteriorly, with a well developed posterior sucker (acetabulum). Amphistomes are variable in size, those of the domesticated ruminants measure from 3 to 20 mm in length and 1.5–7 mm in width. A live amphistome appears light pink to red, whereas some species may be slightly deeper in colour.

The acetabulum is a strong muscular organ placed at the posterior end of the body. It may be terminal, subterminal or almost ventral. It varies in size in different amphistomes and its dorsoventral diameter in relation to body length is of some importance in the taxonomy of the group. The size of the acetabulum may be characteristic in some genera (e.g. *Explanatum*) and due to its strongly muscular nature it can be regarded as a relatively stable morphological character. The much more valuable taxonomic characteristics are, however, exhibited by the structure of the muscular series of the acetabulum. The muscles are oblique, circular, longitudinal and radial; on the dorsal and ventral sides, particularly along the exterior and interior borders, the circular muscle fibres are arranged in a definite pattern.

The digestive system of amphistomes comprises the mouth, pharynx, oesophagus and two intestinal caeca. The mouth is a small circular aperture at the anterior end of the body. The pharynx is a globular muscular organ situated anteriorly. It may be terminal or subterminal, with a spindle-shaped pharyngeal cavity. The pharynx may be simple, with or without a lip sphincter or with two primary sacs, one on

V. Tandon (✉) • B. Roy • J.A. Shylla • S. Ghatani
Department of Zoology, North-Eastern Hill University, Shillong 793 022, Meghalaya, India
e-mail: tandonveena@gmail.com; bishnuroy12@rediffmail.com; jolleneandrea@gmail.com; sudeep.ghatani@gmail.com

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either side, or with a pharygeal bulb with paired secondary sacs. The pharyngeal bulb is a transversely elongate, muscular structure arising from the posterior region of the pharynx and separated from it by a constriction. It occurs only in the family Gastrodiscidae and the Brumptidae. The relation of the length of the pharynx to that of the body and the structure of musculature of the pharynx are of considerable importance in the taxonomy of amphistomes. The oesophagus is a thin tube arising from the basal portion of the pharynx, which distally bifurcates into two intestinal caeca. It may be a straight tube or J-shaped and in some species, provided with an oesophageal bulb at the distal end. The intestinal caeca run laterally towards the posterior end of the body and end blindly, in the pre- or mid acetabular zone. They may be wavy, straight, coiled or forming loops.

The excretory system consists of two main excretory canals, each passing laterally outer to the pharynx and following the course of the intestinal caecum of its side. Posteriorly dorsal or anterodorsal to acetabulum, the two canals bend inwards and meet to form the excretory vesicle. The excretory canal arises from the vesicle that opens on the dorsal side, somewhat medially close to the posterior extremity of the body. The opening may be pre-vesicular, invesicular or post-vesicular. The relationship of the opening (excretory pore) to the Laurer's canal opening is of great taxonomic importance.

The reproductive system of this hermaphroditic parasite group consists of the male and female genital systems. The male genital system comprises the testes, vasa efferentia, a vas deferens and an ejaculatory duct. Testes are usually two in number, occasionally single, usually situated between the intestinal caeca but may be caecal or extracaecal. Testes may be in tandem, diagonally arranged or juxtaposed; they may be entire or lobed or deeply lobed. A vas efferens arises from each testis and the two vasa efferentia unite in front of the anterior testis to form the vas deferens. It is divided into three parts: the first part is known as the vesicula seminalis, the second as the pars musculosa and the last as the pars prostatica, which continues into the ejaculatory duct. The degree of development of the pars prostica and pars musculosa is a character of generic significance. Most paramphistomoids parasitic in mammals do not have a cirrus sac, but it is usually present in taxa from lower vertebrates. There is no cirrus and it is functionally replaced by a genital papilla composed of erectile tissue and containing lymph vessels. In some species, the ejaculatory duct unites with the terminal portion of the uterus and leads into a delicate chamber—the hermaphroditic sac, which opens out through the genital papilla. The genital papilla is surrounded by a cavity or depression called the genital atrium and the walls of the genital papilla may be studded with sphincter papillae. The wall of the genital atrium is strengthened by genital atrial-radial muscles and, in some cases, may be additionally presented with a cluster of muscle fibres forming the genital sphincter. In some amphistomes, another cavity or depression, the ventral atrium, is seen around the genital atrium. In case of the family Gastrothylacidae, the vental atrium is enormously developed to form the ventral pouch, an internal blindly ending sac that opens to the exterior by a single aperture on the ventral surface of the body close to the anterior extremity. The genital pore may be surrounded by a distinct genital sucker in some species.

The genital papilla and various structures, which may or may not be present, such as the sphincter papillae, genital and ventral folds, genital and vental atria, genital sphincter, genital sucker, genital pillar, muscle fibres and tegumental papillae are collectively known as the terminal genitalium, a specialized term used only in case of paramphistomoids but not in other digeneans. The term was introduced by Eduardo [1] and has been well adopted and established in the literature. The majority of the components hold taxonomic importance at species-level identification but possession of a true genital sucker has generic-level significance. The degree of development and the size of the terminal genitalium are also characters in themselves and can be significant at the generic level.

The female genital system consists of the ovary, oviduct, Mehlis' gland, Laurer's canal, vitellaria and their ducts, uterus and metraterm. The ovary is more or less spherical in shape and is usually situated in the post-testicular region in front of the acetabulum. The oviduct emerges from the ovary, and after a short free course enters the Mehlis' gland complex, emerging from it as the uterus. The Laurer's canal arises from the oviduct at a place where the latter enters the Mehlis' gland complex, which then runs towards the dorsal side of the body to open either anterior or posterior to the excretory pore. The vitellaria are usually follicular and confined to the lateral regions of the body. Their extent is variable at the anterior and the posterior region. The Mehlis' gland complex is a compact structure enclosing the dilated part of the oviduct, the ootype. The ootype receives a duct of the yolk reservoir whichs opens into two transverse vitelline ducts, one from each side. The uterus traverses its way towards the anterior side and opens into the hermaphroditic sac through a metraterm—the muscular distal end of the uterus. The hermaphroditic sac is formed from the union of the ejaculatory duct and the metraterm and opens at the tip of the genital papilla. The genital pore lies in the ventro-median line just below the bifurcation of the intestinal caeca [2–4] (Fig. 11.1).

11.2 Classification

Several schemes have been proposed in the history of amphistome systematics, according to which the amphistomes are represented by various ranks—familial [5–8], super-familial [9–14], subordinal [15–17] or ordinal rank [18–20].

Fischoeder [21] coined the word '**Paramphistomidae**' and proposed it as a family name for all amphistomes. The family Paramphistomidae was raised to the rank of a superfamily, **Paramphistomoidea** by Stiles and Goldberger [9] who included in it three families: **Paramphistomidae** Fischoeder, 1901 for those amphistomes in which the ventral pouch is absent; **Gastrothylacidae** Stiles & Goldberger, 1910 for those with a ventral pouch; and **Gastrodiscidae** Monticelli, 1892 in which the ventral pouch is absent and the body is divided into cephalic and caudal portions. Maplestone [10] treated the group as a suborder under the name '**Amphistomata**' and placed under it the families Paramphistomidae, Gastrothylacidae and Gastrodiscidae. Stunkard [6], on the other hand, recognized the family Paramphistomidae and

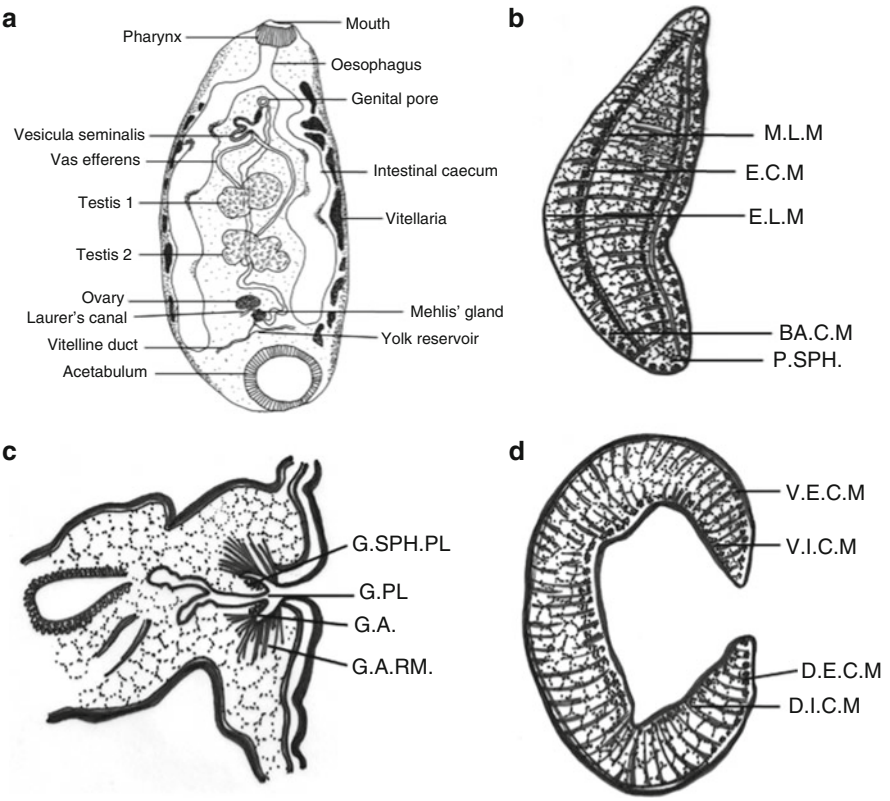

Fig. 11.1 (**a**) A generalized diagram of a typical amphistome fluke. (**b**) A median sagittal section of acetabulum. (**c**) A median sagittal section of one half of pharynx. (**d**) A median sagittal section of one half of genital atrium. *BACM* basal circular muscles, *DECM* dorsal exterior circular muscles, *DICM* dorsal interior circular muscles, *ELM* external longitudinal muscles, *GA* genital atrium, *GARM* genital atrial radial muscles, *GPL* genital papilla, *GSPHPL* genital sphincter papilla, *MLM* middle longitudinal muscles, *VECM* ventral exterior circular muscles, *VICM* ventral interior circular muscles. (Adopted from [3])

included in it the subfamilies Diplodiscinae Cohn, 1904; Schizamphistominae Looss, 1912; Paramphistominae Fischoeder, 1901; **Cladorchinae** Fischoeder, 1901; **Gastrodiscinae** Monticelli, 1892; **Gastrothylacinae** Stiles and Goldberger, 1910; **Zygocotylinae** Stunkard, 1916; **Balanorchinae** Stunkard, 1917 and **Brumptinae** Stunkard, 1925. Fukui [22] treated the amphistomes as a single family, although he grouped the families Angiodictyidae Looss, 1902, and two new families created by him—Opistholebetidae and Opisthoporidae alongside them in the **Amphistomata**. Travassos [11] recognized the superfamily **Paramphistomoidea** and included in it six familes, namely Paramphistomidae, Gastrodiscidae, Opistholebetidae, Gyliauchnidae Fukui, 1929, Cephaloporidae Yamaguti, 1934 and Microscaphidiidae Looss, 1900. Näsmark [8] revised the family Paramphistomidae on the basis of

histological structures of the muscular organs such as pharynx, genital atrium and acetabulum. Principles elaborated by Näsmark [8] are important contributions to the classification of amphistomes.

Skrjabin [16] recognized two superfamilies, the Paramphistomoidea Fischoeder, 1901 and the Cladorchoidea Fischoeder, 1901, which he was the first to elevate to superfamily rank, within the order Paramphistomata. La Rue [17] adopted the suborder Paramphistomata and the superfamily Paramphistomoidea but not the Cladorchoidea. Yamaguti [14] recognized the Paramphistomoidea, but reduced the Cladorchoidea to family status. Mehra [23] accepted the suborder Paramphistomata with two superfamilies, Paramphistomoidea and Notocotyloidea La Rue, 1957.

Sey [2, 24], following Odening's [19] review of higher-level classification, categorized the amphistomes into three suborders, namely **Heronimata** Skrjabin & Schulz, 1937, **Zonocotylata** Sey, 1988 and **Paramphistomata** Szidat, 1936, all under the Order **Amphistomida** (Lühe, 1909) Odening, 1974. Heronimata is a monotypic taxon, its taxonomic rank is determined by monostome type, and by its special morphological structure of the adults, and by the ancient type of reproduction. The taxon Zonocotylata is a reduced one, including one genus and its species. It has morphological characters of its own (reproductive system, fixative apparatus, etc.); which justified its taxonomic position in the systematics of the Amphistomida. Among the three suborders, Paramphistomata has proven to be the most successful developmental line whose representatives can be found in all higher taxa of the vertebrate definitive hosts. This group includes the typical forms of amphistome species comprising the amphistomes sensu stricto. As per the classificatory scheme suggested by Sey [2, 24], Paramphistomata is divided into two superfamilies: **Cladorchoidea** Skrjabin, 1949 and **Paramphistomoidea** Stiles & Goldberger, 1910, based on the presence or absence of a cirrus pouch. Recently, based on molecular data pertaining to complete small subunit rDNA and partial (D1–D3) large subunit rDNA sequences, Olson and colleagues [25] indicated a close relationship of the superfamily Paramphistomoidea with members of the superfamilies Microscaphidioidea Looss, 1900 and Pronocephaloidea Looss, 1899. Based on these data and also considering the fact that these digenean groups lack an oral sucker, the superfamilies Paramphistomoidea and Microscaphidioidea were placed together in the new suborder Pronocephalata along with the pronocephaloid families. However, treating the superfamily as the basic unit of classification, Jones and colleagues [26] proposed a different taxonomic hierarchy for the group of amphistomid digenea. As per this system of classification, amphistomes are placed under the superfamily Paramphistomoidea, the latter being one of the seven superfamilies under the Order Echinostomida [27], with the superfamily Cladorchoidea treated as a synonym of Paramphistomoidea. Jones [4] reduced the Heronimata to superfamily status treating it as a distinct superfamily, the Heronimoidea Ward, 1919, which is represented by a single genus. Similarly, Zonocotylata was reduced to family level and was placed together with other amphistome families.

11.2.1 Superfamily Paramphistomoidea Fischoeder, 1901 (Syn. Cladorchoidea Fischoeder, 1901)

Diagnosis as per Jones [4]: Body tiny to large, may be conical or dorsoventrally flattened. Acetabulum (ventral sucker) at or close to posterior extremity, usually simple but may show various modifications; rarely, non-acetabulate, modified attachment organ present. Tegumentary papillae often present but tegument always unarmed. Ventral pouch present or absent. Oral opening terminal or subterminal. Oral sucker absent. Pharynx present, with or without pharyngeal sacs; if present, sacs may be paired intra- or extramural or, rarely, single; paired extramural sacs may be primary, arising directly from pharynx, or secondary, arising from pharyngeal bulb. Oesophagus with or without the pharyngeal bulb or sphincter. Intestinal bifurcation in anterior half of body. Caeca two, end blindly; posterior extent variable. Testes usually two, occasionally single, usually intercaecal but may be caecal or extracaecal; relative positions variable. Male duct may be differentiated into tubular, coiled, seminal vesicle (pars seminalis), muscular, tubular pars musculosa, prostatic region (pars prostatica) and ejaculatory duct. Cirrus sac present or absent. Hermaphroditic sac present or absent. Genital sucker present or absent. Cirrus absent, functionally replaced by genital papilla. Genital pore opens on midventral surface, usually near level of intestinal bifurcation, or into ventral pouch if present. Ovary usually post-testicular. Mehlis' gland close to ovary. Laurer's canal present, crosses excretory vesicle or duct or not. Canalicular seminal receptacle absent. Uterine seminal receptacle often present. Uterus mainly intercaecal, usually dorsal to testes and ventral to male ducts, may pass between testes. Vitellarium usually follicular; follicles usually in two lateral fields, variable in anterior and posterior extent, confluent or not in midline. Excretory vesicle usually dorsal or anterodorsal to acetabulum, rarely intertesticular, often saccate; pore usually opens close to posterior extremity, rarely at intertesticular level. Lymphatic system present. In gastrointestinal tract of all groups of vertebrates, larval stages in molluscs, cercariae encyst on vegetation; cosmopolitan. Type genus: *Paramphistomum* Fischoeder, 1901.

In a combination of the classificatory system proposed by Jones and colleagues [26] and Sey [2, 24], the superfamily **Paramphistomoidea** Fischoeder, 1901 is treated as the basic unit of classification and further divided into 12 families [27]. Of these, only six viz., **Paramphistomidae** Fischoeder, 1901, **Gastrodiscidae** Monticelli, 1892, **Gastrothylacidae** Stiles & Goldberger, 1910, **Olveriidae** Yamaguti, 1958, **Balanorchiidae** Stunkard, 1925 and **Stephanopharyngidae** Stile & Goldberger, 1910 are represented in mammalian hosts of veterinary significance (Fig. 11.2).

Key Characters of Amphistomid Genera of Veterinary Significance

1. ***Gigantocotyle* Näsmark, 1937**
   Acetabulum enormous; genital sucker absent; pars musculosa well developed. Type species: *G. gigantocotyle* (Brandes in Otto, 1896) Näsmark, 1937.

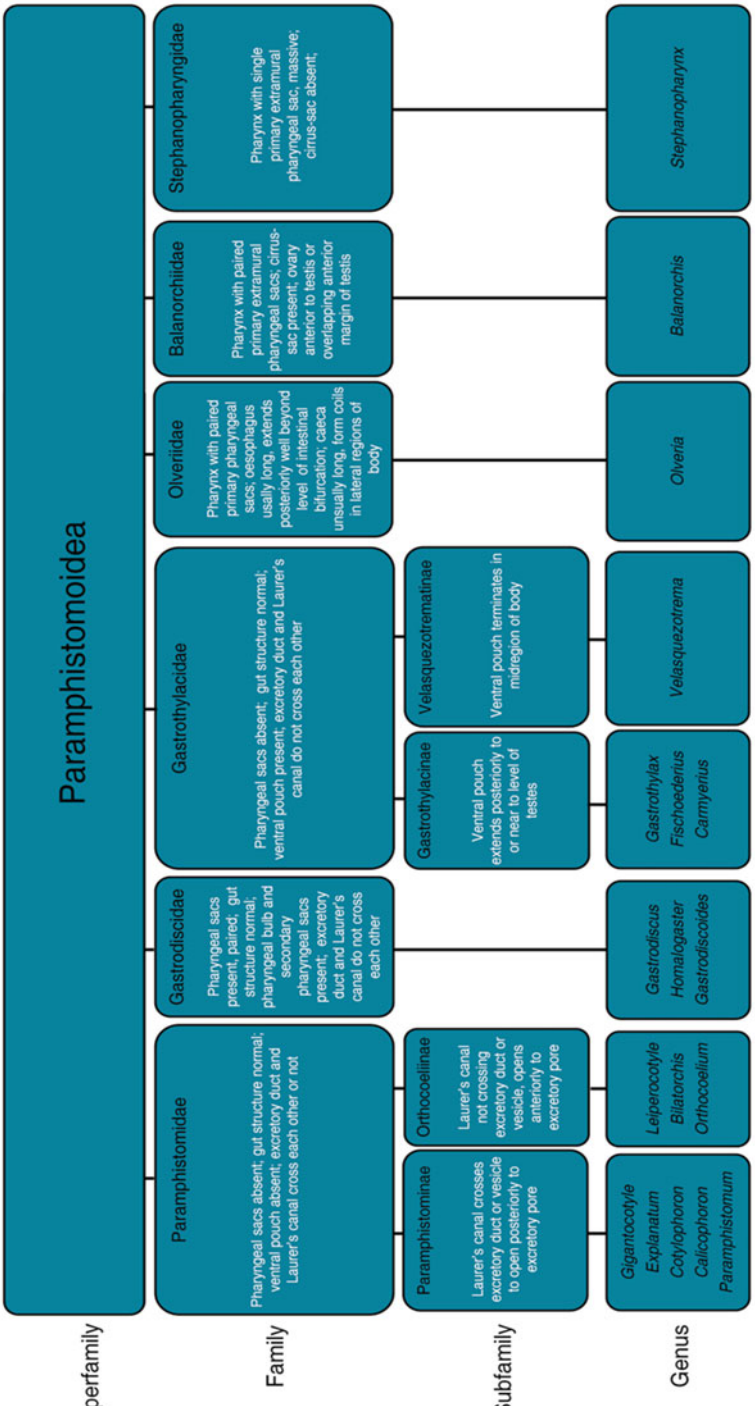

Fig. 11.2 Schematic representation of the classification of amphistomes (following Sey, 1988, 1991 [2, 24]; Jones et al., 2005 [4]) of veterinary significance up to genus level along with key characters up to subfamily level

2. ***Explanatum*** **Fukui, 1929**
   Acetabulum enormous; genital sucker absent; pars musculosa weakly developed. Type species: *E. explanatum* (Creplin, 1847) Fukui, 1929.
3. ***Cotylophoron*** **Stiles & Goldberger, 1910**
   Acetabulum small to medium; genital sucker present; pars musculosa strongly developed. Type species: *C. cotylophorum* Stiles & Goldberger, 1910.
4. ***Calicophoron*** **Näsmark, 1937**
   Acetabulum moderate in size; genital sucker absent; pars musculosa well developed. Type species: *C. calicophorum* (Fischoeder, 1901) Näsmark, 1937.
5. ***Paramphistomum*** **Fischoeder, 1901**
   Acetabulum medium-sized; genital sucker absent; pars musculosa very weakly developed. Type species: *P. cervi* Fischoeder, 1901.
6. ***Leiperocotyle*** **Eduardo, 1980**
   Genital sucker present; pars musculosa strongly developed; in rumen of Giraffidae and Bovidae. Type species: *L. okapi* Eduardo, 1980.
7. ***Orthocoelium*** **Stiles & Goldberger, 1910**
   Genital sucker absent; ventral atrium of normal size; testes tandem or diagonal; body not ringed with ridges; body conical, not flattened with inrolled margins; pars musculosa strongly developed; in rumen of Bovidae and Cervidae. Type species: *O. orthocoelium* Stiles & Goldberger, 1910.
8. ***Gastrodiscus*** **Leuckart in Cobbold, 1877**
   Ventral surface bears large conspicuous invaginable papillae; anterior region conical; posterior region discoid with inrolled margins, covered ventrally with large invaginable papillae; testes diagonal. Type species: *G. aegyptiacus* (Cobbold, 1876) Leuckart in Cobbold, 1877.
9. ***Homalogaster*** **Poirier, 1883**
   Ventral surface bears large non-invaginable papillae; anterior extremity roughly triangular in ventral view; posterior region without inrolled margins, covered ventrally between triangular anterior region and acetabulum with large non-invaginable papillae; testes tandem. Type species: *H. paloniae* Poirier, 1883.
10. ***Gastrodiscoides*** **Leiper, 1913**
    Ventral surface without conspicuous papillae, bears papillar ridges; surface of acetabulum not covered by papillae; testes tandem, lobed; genital pore prebifurcal at level of oesophageal bulb; anterior region of body conical, posterior region discoidal and dorsoventrally flattened. Type species: *G. hominis* (Lewis & McConnell, 1876) Leiper, 1913.
11. ***Gastrothylax*** **Poirier, 1883**
    Ventral pouch extends to or near to level of testes; uterus loops from one side of body to the other; testes symmetrical. Type species: *G. crumenifer* (Creplin, 1847) Poirier, 1883.
12. ***Carmyerius*** **Stiles & Goldberger, 1910**
    Ventral pouch extends to or near to level of testes; uterus in midline throughout its length; testes symmetrical. Type species: *C. gregarius* (Looss, 1896) Stiles & Goldberger, 1910.

13. ***Fischoederius* Stiles & Goldberger, 1910**
    Ventral pouch extends to or near to level of testes; uterus in midline throughout its length; testes tandem in midline, one anterodorsal to other. Type species: *F. elongatus* (Poirier, 1883) Stiles & Goldberger, 1910.
14. ***Olveria* Thapar & Sinha, 1945**
    Key characters same as that of family—oesophagus long and J-shaped. Type species: *O. indica* Thapar & Sinha, 1945.
15. ***Balanorchis* Fischoeder, 1901**
    Key characters same as that of family. Type and only species: *B. anastrophus* Fischoeder, 1901.
16. ***Stephanopharynx* Fischoeder, 1901**
    Key characters same as that of family. Type and only species: *S. compactus* Fischoeder, 1901.

11.3 Life Cycle

Amphistomes require two hosts to complete their life cycle (Fig. 11.3); a vertebrate definitive host and a snail intermediate host. Infected animals excrete eggs in the faeces. The eggs are large in size measuring approximately about 160 × 90 μm, with a thin (freshwater developed) or thick (marine developed) eggshell, operculated with a distinct operculum. As the eggs are laid, the development of the embryo/miracidium continues within the egg shell. The eggs develop and hatch under suitable conditions (of temperature and moisture) when the eggs have been freed from the faecal mass [28]. The newly emerged miracidium finds a suitable intermediate aquatic snail host belonging to the families Lymnaeidiae and Planorbidae. Its body shape is variable and is visible to the unaided eye. The miracidium shows a 6:8:4:2 and 6:6:4:2 epidermal cell formula and is without pigment spots. The miracidium locates the molluscan host, the snail, either by innate behaviour, random chance or chemotaxis and enters its soft tissues. Generally the younger snails are more susceptible to miracidium penetration. The miracidium makes its way to the mantle cavity and then to the heart, where the ciliated epidermal plates (cells) are shed after sometime and differentiates into a sporocyst. A mature sporocyst is formed in the mantle tissue, sometimes in the digestive tissue and head-foot organ of the snail host. As the sporocyst grows the germ balls from the miracidium develop further and diffrentiate into the redia stage [29]. A mature sporocyst contains developing rediae of different sizes and germ balls. Each developing redia possesses a pharynx, gut and three pairs of flame cells and some primordial germ cells. The rediae come out of the sporocyst by rupturing its anterior body wall or through a terminal opening in the sporocyst. After liberation from the sporocyst, the redia is still immature and continues its growth while in the digestive gland, ovotestis, mantle tissue or the alimentary tract. A mature redia is elongate and has neither collar nor procrusculi. The germ cells in the redia are exhausted in the formation of cercariae. Immature cercaria leaves the redia and completes development in tissue of the snail host [30].

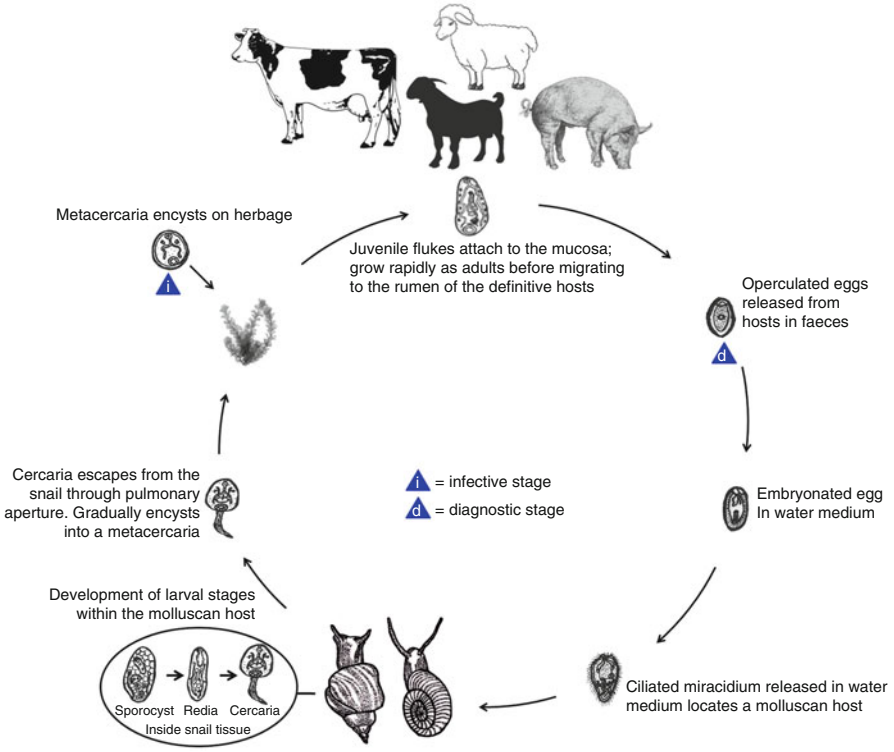

Fig. 11.3 Generalized life cycle of a typical amphistome fluke

A mature cercaria is released from the snail from the pulmonary aperture and swims actively in the water. The cercaria is large and of 'amphistome' type with an unforked tail; the acetabulum lies at the posterior end of its body; the body is either with dense pigmentation (*Cercaria pigmentata*) or with light pigmentation (*Cercaria intermedia*) or without pigmentation (*Cercaria diplocotylea*). A pair of eyespots and a lot of cystogenous cells are present (except in *Heronimus*). The protonephridial system is well-developed, where the excretory bladder is non-epithelial [2, 3]. Infected snails can live and shed cercariae for up to 1 year. The free cercaria after its escape from the snail lives in that condition for a short period, from a few minutes to a few hours. Cercariae encyst to form metaceraciae on the ventral surface of subaquatic plants where they can remain viable for up to 6 months. The metacercariae usually encyst freely. The final (definitive) host ingests the encysted metacercaria on the herbage. Excystment occurs in the small intestine, and the newly hatched juvenile flukes attach to the mucosa where they grow rapidly before migrating to the rumen after about 3–6 weeks. In the rumen the flukes attach to their predilection sites on the dorsal surface of the anterior ruminal pillar, and dorsal and ventral aspects of the posterior ruminal pillar. Here, they continue to grow to reach their maximum size 5–9 months after infection [3, 31].

11.4 Hosts and Distribution

Amphistomes are found as endoparasites in the digestive tracts, liver and bile ducts of many vertebrates, ranging from fishes to mammals. The majority of the species are found to parasitize mammalian livestock species such as cattle (cow, buffalo, mithun and yak), sheep, goats and pigs. However, representatives of this group are also found to infect a variety of other animal groups such as fishes (both freshwater and marine), amphibians (frogs, toads and salamander), reptiles (turtles, tortoises and snakes), aquatic birds and mammals other than livestock species (Table 11.1).

The distribution of amphistomes infecting cattle has been well documented owing to the economic importance of these parasites from a veterinary standpoint. These parasites have been found to infect cattle globally, but the most severe effects occur in the tropical and subtropical region of the world including Australia, Asia, Africa, Eastern Europe and Russia [32–36].

11.5 Epidemiology

11.5.1 Amphistomiasis in Ruminants

Amphistomes are known to be the causative agent of a debilitating disease 'Amphistomiasis' or 'stomach fluke disease' of domestic and wild animals, mainly ruminants. The disease is characterized by loss of appetite, foetid diarrhoea, dehydration, emaciation, extreme weakness, exhaustion, intermandibular odema and subnormal temperature [37, 38]. Though a neglected trematode infectious disease in ruminants, amphistomiasis is amongst the most pathogenic diseases of parasitic origin in domesticated livestock and has recently emerged as an important cause of productivity loss [39]. The significant damage caused by migrating flukes especially among young stock adds to great economic loss due to high morbidity and mortality [40]. Thus, the disease is virulent when these amphistomes are immature and found in the small intestine eventually migrating to the rumen on maturity. In case of the genus *Explanatum*, the flukes may travel up the bile duct and clog it and the flow of the bile from the gall bladder to the duodenum is obstructed and the gall bladder becomes distended [3].

The outbreaks of amphistomiasis are usually restricted to the drier months of the year. This period, however, varies according to the climate of the region and during rainy season ruminants have more access to graze freely over these areas thus the infection is easily acquired by ingesting vegetation that is metacercariae infested. The affected hosts usually inhabit water bodies due to anorexia, restlessness and polydipsia. The rate of infection also flares up due to heavily contaminated herbage. The rate of migration of these flukes from the intestine to the rumen is usually slow thereby inflicting grave intestinal damage in the hosts infected. In India, domestic ruminants are generally permitted to graze around small ponds or banks of

Table 11.1 Amphistome genera from vertebrates other than mammalian livestock [3, 14, 26, 137]

Host	Genera	Family	Distribution
Fish (freshwater and marine)	*Amurotrema*, *Bancroftrema*, *Basidiodiscus*, *Brevicaecum*, *Caballeroia*, *Cleptodiscus*, *Dadayius*, *Dadaytrema*, *Helostomatis*, *Kalitrema*, *Macrorchitrema*, *Microrchis*, *Neocladorchis*, *Nicollodiscus*, *Ophioxenos*, *Orientodiscus*, *Panamphistomum*, *Pisciamphistoma*, *Protocladorchis*, *Pseudocladorchis*, *Sandnoia*, *Travassosinia*	Cladorchiidae	Africa, Asia, Australia, North America, Caribbean, South America
	Pseudodiplodiscus	Displodiscidae	South America (Brazil)
Amphibians (frog, toad, newt and salamander)	*Allassostomoides*, *Megalodiscus*, *Ophioxenos*, *Opisthodiscus*	Cladorchiidae	Africa, Europe, North America
	Catadiscus, *Diplodiscus*, *Progonimodiscus*	Diplodiscidae	Africa (South Africa), Asia (India), South America, Central America, Europe (Germany)
Reptiles (snake, turtle and tortoise)	*Allassostoma*, *Allassostomoides*, *Halltrema*, *Nematophila*, *Ophioxenos*, *Orientodiscus*, *Parachiorchis*, *Pseudoallassostomoides*, *Pseudocleptodiscus*, *Quasichiorchis*, *Schizoamphistomoides*, *Schizoamphistomum*, *Stunkardia*	Cladorchiidae	Asia, North America, South America, Pacific, Atlantic, Mediterranean
	Catadiscus, *Dermatemytrema*	Diplodiscidae	North and South America
Rodents (agouti, muskrat, rat and mice)	*Chiostichorchis*, *Cladorchis*, *Taxorchis*, *Wardius*	Cladorchiidae	North America, South America (Brazil)
Aquatic birds	*Zygocotyle*	Zygotylidae	North America (the USA), South America (Brazil)
Aquatic mammals (manatee and dugong)	*Chiorchis*, *Indosolenorchis*, *Solenorchis*	Cladorchiidae	Africa, Asia (Sri Lanka), North America (the USA), Australia

Other mammals (wild dog, wild pig, tapir, deer, horse, bison, hippopotamus, elephant and rhinocerus)	*Buxifrons*, *Cotylophoron*, *Gigantocotyle*, *Macropharynx*, *Nilocotyle*, *Orthocoelium*, *Paramphistomum*, *Ugandocotyle*	Paramphistomidae	Africa, Asia, North America, Europe
	Choerocotyle, *Gastrodiscoides*, *Gastrodiscus*, *Pseudodiscus*	Gastrodiscidae	Asia, Africa
	Choerocotyloides	Choerocotyloididae	Africa (Zimbabwe)
	Stephanopharynx	Stephanopharyngidae	Africa
	Cladorchis, *Pfenderius*, *Stichorchis*, *Taxorchis*	Cladorchiidae	Asia, Caribbean, South America
	Brumptia	Brumptidae	Africa
	Balanorchis	Balanorchiidae	South America
Primates (monkey)	*Gastrodiscoides*, *Watsonius*	Gastrodiscidae	Africa, Asia (Singapore and Thailand)
Human	*Gastrodiscoides* (*hominis*)	Gastrodiscidae	Africa, Asia
	Watsonius (*watsoni*)		

streamlets during the months from September to January, which were previously swamped with water and are drying up. In the process, these ruminants ingest many metacercariae in a short period of time. Thus, the outbreaks may appear in the months that follow the monsoons. Amphistomiasis is thus a disease of domesticated animals that are forced by natural reasons or by farming practices to graze upon foliage that is infected by amphistome metacercariae [3].

A recent epidemiological survey in Central France demonstrated that the prevalence of natural infections with *Paramphistomum* in cattle significantly increased from 5.2 % (in 1990) to 44.7 % (in 1999). The apparent spread of the disease was attributed to the efficacy of fasciolosis control that leaves free the intermediate host (*Galba truncatula*); the development of extensive livestock farming or the propagation of metacercariae by mechanical vectors has also been incriminated [41]. The disease is most rampant during monsoon and post-monsoon period. Characterized by sporadic epizootics of acute gastroenteritis with high morbidity and mortality in domestic animals, amphistomiasis constitutes a major health hazard to ruminants particularly in low-lying areas where snails are found abundantly during monsoon and post-monsoon season [42].

Amphistomiasis has a wide geographical distribution in subtropical and tropical areas, where the infection leads to economic losses related to mortality and low productivity. The disease is distributed all around the world, but its highest prevalence has been reported in tropical and subtropical regions, particularly in Africa, Asia, Australia, Eastern Europe and Russia and also from various states of India [35, 36, 43–46].

The causative flukes mainly belong to different genera, viz., *Paramphistomum*, *Cotylophoron*, *Calicophoron*, *Explanatum*, *Orthoceolium*, *Gastrothylax* and *Fischoederius* [42, 47]. The etiological agent implicated in the disease may be different species in specific regions [48]. For example, in Europe, *Paramphistomum cervi* has been reported as the main causative agent of amphistomiasis, while it is *Paramphistomum microbothrium* in Sardinia, Yugoslavia and Hungary [34]. In Australia and New Zealand, species of amphistomes affecting cattle and sheep are *Paramphistomum ichikawai* and *Calicophoron calicophorum* [49]; species responsible for infection in Turkey are mainly *P. cervi*, *P. ichikawai* and *Calicophoron daubneyi* [50, 51]. In Asia, *Paramphistomum explanatum*, *P. cervi*, *Gastrothylax crumenifer*, *Cotylophoron cotylophorum*, *Fischoederius elongatus* and *F. cobboldi* have been recorded in Ceylon and China [32, 52, 53]. In the northeastern region of India, as many as 25 amphistomid species representing 13 genera have been reported as occurring in livestock mammals [54–60] (Fig. 11.4).

Across the world, effects of amphistomes are underestimated, however, the infection have to be considered seriously, particularly when a risk of acute infection is present as the immature worms are very difficult to identify and there is no serological test available and during necropsy small lesions of abomasum and of ileum may be confused with other diseases. Adults are very prolific and many eggs are expelled: their number not being related to the parasitic burdens. Amphistomiasis is one of the most pathogenic diseases of domesticated animals, causing heavy losses to the livestock industry, amounting to several thousand crores of rupees annually [42, 61]. It has been estimated that more than 500 million cattle worldwide are at

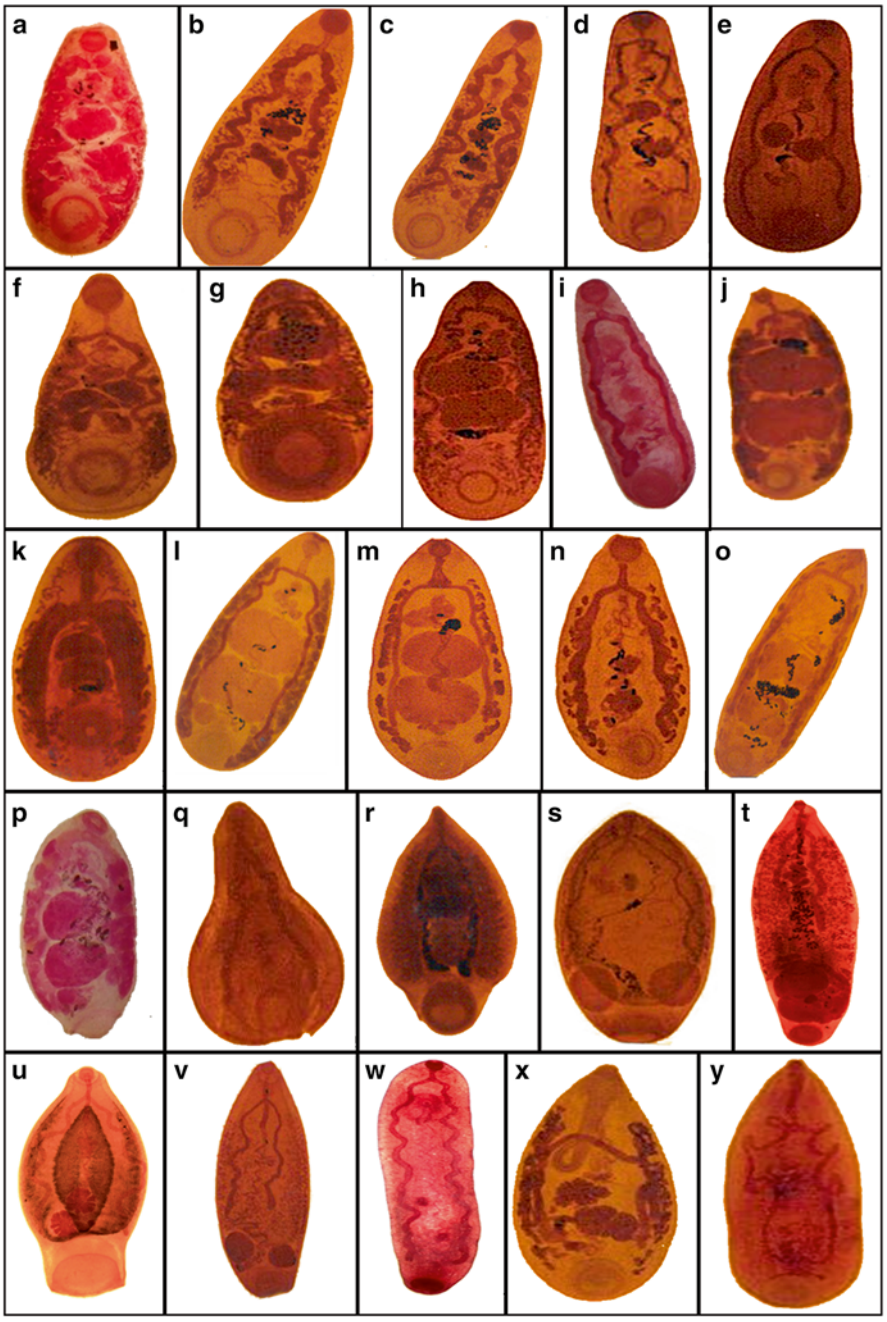

Fig. 11.4 Amphistome species of mammalian livestock reported from Northeast India (Roy and Tandon, 1990, 1992, 1995 [55–58]) (**a**) *Paramphistomum gracile*, (**b**) *P. epiclitum*, (**c**) *P. ichikawai*, (**d**) *Calicophoron papillosum*, (**e**) *C. calicophorum*, (**f**) *C. shillongensis*, (**g**) *Explanatum explanatum*, (**h**) *Cotylophoron cotylophorum*, (**i**) *Leiperocotyle meghalyensis*, (**j**) *Orthocoelium dinniki*, (**k**) *O. orthocoelium*, (**l**) *O. parpvipapillatum*, (**m**) *O. dawesi*, (**n**) *O. streptocoelium*, (**o**) *O. dicranocoelium*, (**p**) *O. scoliocoelium*, (**q**) *Gastrodiscoides hominis*, (**r**) *Homalogaster paloniae*, (**s**) *Gastrothylax crumenifer*, (**t**) *Fischoederius elongatus*, (**u**) *F. cobboldi*, (**v**) *Carmyerius spatiosus*, (**w**) *Velasquezotrema tripurensis*, (**x**) *Olveria indica*, (**y**) *Olveria bosi*. [Source for **b–h**, **j–o**, **q–s**, **v**, **x**, **y**: www. nepiac.bicnehu.ac.in]

risk due to parasitic infection [62]. In some areas of India, the Republic of South Africa and Australia, the mortality of cattle has reached 80–90 % in sheep and cattle [46, 61, 63, 64]. Economic loss caused by amphistome infections has not been estimated, but may be greater than those caused by many other parasites; the cost of the parasite in terms of economic losses to human society is incalculable due to the high mortality and morbidity in young animals [36, 42, 46, 65, 66].

11.5.2 Human Amphistomiasis

Amphistomiasis is not restricted to ruminant hosts given that human infections have also been reported. Three species have been reported to infect man namely, *Gastrodiscoides hominis*, *Watsonius watsoni* and *Fischoederius elongatus. G. hominis* is frequently found in pigs and accidentally in man as reported in Burma, China, India, Kazakhstan, the Philippines, Thailand and Vietnam [67–70]. The exact life cycle is unknown but probably similar as in other species of Gastrodiscidae involving aquatic vegetation as the second intermediate environment that is used for the encystment of the metacercarial infective stage [68, 71, 72]. Human and animal contamination can take place when swallowing encysted metacercariae, by ingestion of vegetation (aquatic plants) or animal products, such as raw or undercooked crustaceans (crayfish), squid, molluscs or amphibians (frogs, tadpoles). Although it is mainly a parasite of pigs, sometimes high prevalence (41 %) of *G. hominis* has also been detected in humans, in mainly children in Kamrup District in Assam, in northeastern India [73]. *G. hominis* has a wide distribution throughout India including the states of Assam, Bengal, Bihar, Uttar Pradesh, Madhya Pradesh and Orissa [74, 75]. Apart from India, it is widely distributed in countries like Pakistan, Burma, Thailand, Vietnam, Philippines, China, Kazakhstan and Russia [67, 69, 70, 73, 76, 77]. In a later study carried out in Meghalaya (India), *G. hominis* was shown to have a pattern of seasonal prevalence [57].

Pathology and symptomatology of *G. hominis* infection are uncertain. In humans, the parasite causes inflammation of the mucosa of the caecum and ascending colon with attendant symptoms of diarrhoea. Ill health in a large number of individuals, and deaths among untreated patients, especially children, has been attributed to this infection [69]. The specimens can be collected from the caecum, especially near the ileocaecal valve [76]. Human infection by *G. hominis* is easily recognizable by finding the characteristic eggs of this amphistome in faeces [72].

The other two species that have been reported from humans are *Watsonius watsoni* (Gastrodiscidae) and *Fischoederius elongatus* (Poirier, 1883) (Gastrothylacidae) and are of lesser significance. *W. watsoni* is a common paramphistome of various species of primates in eastern Asia and Africa. It has been reported only twice in humans in Africa [78, 79]. *F. elongatus* is a parasite of ruminants with the only human infection reported from Guangdong, China [80].

11.5.3 Pathology and Clinical Aspects

There is little evidence regarding the pathogenesis of adult flukes to their hosts, but severe damage to the mucosa of the rumen was provoked in heavy infection in experimentally infected sheep [81]. A high number of immature worms in the duodenum may affect production, since these parasites causing a lower feed conversion, rough hair coat, dullness, weakness, loss of appetite, intestinal haemorrhages, anaemia, reduced milk production, intermandibular swelling, a loss of weight and/or a decrease in milk production are responsible for economic losses, morbidity and mortality [37, 38, 64, 82]. Ruminants suffering from amphistomiasis exhibit a decrease in their appetite, resulting in complete anorexia (loss of appetite). Consumption of water is highly reduced and therefore, sometimes they keep their muzzles dipped in water for long periods. Generally it takes 2–4 weeks to develop diarrhoea in infected animals. The faeces are extremely fluid and foetid (an abnormally frequent discharge of fluid faecal matter from the bowel). In severe cases, diarrhoea may be projectile, tinged with blood and may be present with some immature flukes. The latter present in the duodenum and ileum are plug feeders and cause haemorrhage, which leads to bleeding and diarrhoea; bleeding for a prolonged period may cause anaemia, which further weakens the host [3]. Clinical amphistomiasis is usually diagnosed in cattle 4–18 months of age and is associated with invasion of the duodenum and upper jejunum by large numbers of immature flukes. Counts of up to 30,000 immature amphistomes may be associated with diarrhoea after 8 weeks grazing in tracer (worm-free) calves [83].

The pathological and anatomical effects inflicted on the host can be divided into macropathological and micropathological effects. The macropathological effects include congestion of blood vessels on the peritoneal side of the affected intestine, hyperanaemia, haemorrhages and thickening of the mucosa giving the internal surface a corrugated appearance, cachexia, hydropericardium, hydrothorax, ascites and oedema of the mesentery, abomassum and submandibular space. The micropathological effects include oedema of the epithelial layer and lymphocytic infiltration in the propria and sometimes the submucosa layer; in the vicinity of the worms, the mucosa is necrosed and sloughs with some hypertrophy of the stratum corneum; the tips of the papillae equally degenerate and slough; and the worms also reach the submucosa and muscularis mucosa of the small intestine and this may result in lymphocytic infiltration around the worms [3].

11.5.4 Clinical Signs

The occurrence of the disease depends both on the individual vulnerability and the species susceptibility, as well as on the number of ingested metacercariae. The susceptible categories mainly include young animals, as adults develop immunity easily for longer periods. However, adult cattle and sheep without previous exposure

may produce clinical or subclinical conditions after the ingestion of high doses of metacercariae. It is evident that clinical outbreaks are associated with a high intake of metacercariae from grass, and this fact is related to a high rate of infection of the intermediate host. Doses of 5,000 metacercariae of *C. cotylophorum* produced clinical signs at 116 days after such dosage in a lamb, and death after day 124 post infection. Burdens of 40,000 flukes gave rise to clinical signs and death in sheep, although field infections given by 2,000 flukes were associated with the cause of death [3]. In some cases of acute infection, the death of calves has been observed [84]. The clinical signs include: (1) characteristic and persistent foetid diarrhoea accompanied by weakness, depression, dehydration and anorexia, (2) submaxillary oedema, (3) visible paleness of the mucosa, and (4) death usually occurs 15–20 days after the first signs appear. The necropsy findings include subcutaneous oedema and accumulation of fluid in the body cavities; gelatinous fat depots; the mucosa in the upper part of the duodenum thickened, covered with blood-stained mucus; patches of haemorrhage under the serosa; small, flesh coloured flukes present; and microscopically, immature flukes in the mucosal surface and deeper layers [81, 82, 85, 86].

11.6 Diagnosis

The clinical diagnosis of amphistomiasis remains difficult. Immunological methods and serum antibody detection are not conclusive. As a consequence the diagnosis of amphistomiasis in live animals still depends on faecal detection of eggs [87, 88].

11.6.1 Faecal Egg Counts

Amphistome eggs are clear and measure 160–180 μm in size [89]. The use of either liquids of high density for floatation techniques or sedimentation techniques is required to detect eggs of these flukes since they are particularly heavy eggs, which do not float on water [90]. The filtration technique with sieves and sedimentation is the most accurate to identify eggs in faeces, producing clearer evidence in the sediment of the sample under study. In order to distinguish the differences between eggs more clearly, it is advisable to use contrast stains such as methylene blue or methyl green, instead of Lugol. The proportion of eggs recovered may vary between 18 and 75 % [91, 92].

The McMaster technique is widely used, but sometimes modified according to the parasites being investigated. Modifications are related to the weight of the faecal sample, the specific gravity and the volume of the liquid (more or less dense) and on the surface examined on the McMaster slide: one or two chambers, the whole surface or not. The sedimentation technique appears to be more accurate and sensitive than flotation techniques. In some cases, cup sedimentation using tap water is the simplest and cheapest but more time consuming compared to flotation techniques [90].

11.6.2 Immunodiagnosis

Prolonged prepatency often contributes to the difficulty for detection of mild infection by conventional methods such as coprological examination. Therefore, in addition to chemotherapeutic control, early and accurate immunodiagnosis of the disease is now a focus for study. ELISA has been used by a few workers to detect antiparasitic antibodies [93]. The majority of tests are applied to the detection of blood antibodies and the latest to the detection of antigen in faeces. Nevertheless, many cattle surveys are performed with the ELISA test alone without any confirmatory tests and under those circumstances false positive results may be considered as positive [90]. Whole adult somatic antigens of *Paramphistomum epiclitum*, *Cotylophoron cotylophorum* and *Gastrothylax crumenifer* were prepared; polyclonal antibodies were raised against *P. epiclitum* antigen in rabbits and were tested positive for antibody titre by Dot-ELISA and Plate-ELISA [93, 94]. The use of immune diagnosis is, however, still in its rudimentary stage in case of amphistomiasis.

11.6.3 Molecular Diagnosis

The feasibility of developing PCR-based methods as diagnostic tools for helminth parasites has remarkably been enhanced with the introduction of genomic sequencing and the ample amount of data generated on a daily basis. The growing accumulation of mitochondrial and nuclear genome sequence data in public databases, including NCBI GenBank, facilitates the rapid development of PCR primer design. Comprehensive sequence information is, therefore, required to adapt methods for PCR-based diagnosis, and the remarkable increase of such information for the parasitic helminths over the past decade has facilitated the development, implementation and effectiveness of these types of diagnostic techniques [95]. Additionally variants of PCR like multiplex PCR (mPCR) and real-time PCR (qPCR) assays can aid in detection of multiple distinct helminth species in an individual host [96–99]. These diagnostic techniques are not only useful in human and veterinary medicine but also for environmental monitoring purposes.

Various workers have generated ample amount of data based on the exploration of genetic markers viz., nuclear (18S RNA, 28S RNA, spacers, etc.) and mitochondrial DNA (cytochrome oxidase CO, NADH, etc.) which in the future may be used to yield species specific primers [100–113]. These primers may eventually be used to accurately identify this ambiguous group of parasites. PCR-based techniques providing rDNA ITS2 sequences have proven to be a reliable tool to identify digenean species and to recover their phylogenetic relationships [114, 115]. ITS2 has been found to be a useful marker for species identification of amphistomes as well [59, 60, 115–118]. In particular, the combination of PCR-RFLP analysis, which is a widely used method for the accurate determination of helminth parasites [119, 120], has been successfully used for characterizing some amphistome genera and species;

Itagaki and colleagues [116] characterized three different genera by ribosomal DNA (rDNA) Internal Transcribed Spacer 2 (ITS-2), whereas Rinaldi and colleagues [117] applied a similar technique using the ITS-2 of *Calicophoron daubneyi* from different definitive hosts and Sanabria and colleagues [121] generated restriction profiles for *Paramphistomum* spp. Another variant of PCR, PCR-RAPD has also been used for species identification of three amphistome flukes, viz., *Paramphistomum epiclitum*, *Orthocoelium streptocoelium* and *Fischoederius elongatus*. 50 polymorphic markers were detected and 16 genetic markers were generated by the high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) technique with OPA2, OPA4, OPB18, OPC9, and OPH11 primers [122].

One of the latest techniques for species identification is DNA barcoding, which is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species [123]. The DNA barcoding initiative is one prominent line of research within this field, coordinated by the Consortium for the Barcode of Life (CBoL, http://barcoding.si.edu) [124]. A desirable locus for that DNA barcoding should be standardized (so that large databases of sequences for locus can be developed), present in most of the taxa of interest and sequencable without species-specific PCR primers [125], short enough to be easily sequenced with current technology, and provide a large variation between species yet a relatively small amount of variation within a species [126, 127]. Although several loci have been suggested, a common choice is the mitochondrial COI gene for animals and many other eukaryotes. Concerning the amphistomes found in Northeast, India DNA barcodes based on partial sequences of mitochondrial COI for 18 amphistomes were generated and deposited in the Barcode of Life Database (BOLD) (our unpublished results).

11.7 Control and Treatment

There are several methods, which can be employed for the control of amphistomes in ruminants. These can be categorized as follows:

1. Control in grazing and water sources
   Serious outbreaks may be prevented if pastures with a natural water supply are grazed only in spring and early summer. Late summer, autumn and early winter grazing should be avoided. If wet pastures are grazed continuously, young weaner calves and lambs should not be grazed together with adults. Affected areas must be drained properly and such areas should be fenced. Alternative water supply treated with a suitable molluscicide may be provided in these areas [3].
2. Chemical control
   In case of an outbreak the whole flock or herd must be removed from the source of infection before treatment is instituted. All animals, irrespective of clinical conditions, must be treated, since an apparently healthy animal may die within few days of onset of symptoms [3]. Although treatment for adult fluke has no

direct benefit to the animal, it may reduce the source of infection for the snail intermediate host. This then reduces the size of the next generation of infective fluke larvae on pasture. Treatment with an appropriate drench should be timed for autumn and spring. Effective treatment of immature stomach fluke infection requires removal of stock from the source of infection, usually swampy land, as well as treatment with a drench which is effective against immature flukes. Supportive therapy to treat dehydration and any secondary infection may be needed [47].

There are several anthelminthics that are used for treatment of amphistomiasis in ruminants. These include Terenol, Vermitan, Febantel, Niclosamide, Oxyclozanide, Resorantel, Bithionol, Hexachloroethane and Carbontetrachloride [3, 128]. Terenol is highly effective against both juvenile and adult flukes when applied at a dose of 65 mg/kg in cattle and sheep [129–133]. Vermitan is another anthelminthic which proved to be highly effective in sheep against artificial subclinical intestinal amphistomiasis with an efficacy of 99.2–99.5 % at the dose rate of 20 mg/kg [134]. Febantel was applied against ruminal and intestinal amphistomiasis in cattle; at a dose of 100 mg/kg it had more than 90 % efficacy (93.9 % against juvenile and 94.9 % against adult paramphistomes). Clinical symptoms were shown to disappear in 7–10 days after treatment [135]. Niclosamide is very efficient against immature amphistomes with efficiency of 92–99 % observed against various species in sheep. A single dose of 160 mg/kg or two doses at 3 days apart is effective in cattle and a dosage of 100 mg/kg is effective against immature amphistomes in sheep. Oxyclozanide is highly efficacious against adult as well as immature forms with two doses of 18.7 mg/kg 2 days apart and gave consistent results against immature amphistomes in cattle [3]. A combination of Levamisole and Oxyclosanide is also very effective in treating both adult and immature forms [85]. Resorantel and Bithionol have also been shown to be efficacious against both immature and mature forms [3].

3. Immunological control
   This method of control is by means of immunization and particularly in those areas where amphistomiasis is enzootic. Cattle, sheep and goats have been shown to develop resistance after exposure to the parasite. This immunity protects the animal against the massive infections of immature fluke that cause the most problems. This approach was utilized by Horak [34] by successfully immunizing sheep, goats and cattle against massive artificial infections with *Calicophoron microbothrium*. The results indicated that cattle were the most suitable subjects for immunization. Immunity in adult cattle was attained within 4–6 weeks after immunization and the immunity was effective for at least a year post-immunization.
4. Control of intermediate hosts
   Snails are the intermediate hosts of amphistomes. If snails are controlled, the life cycles of these parasites become interrupted, and the disease is controlled automatically. Therefore, removal of snails from the chain of the life cycle of amphistomes would be the most effective method in the control of amphistomiasis [3]. This would involve fencing off of marshy areas, thus preventing cattle

from grazing in those areas. Moreover, drainage of low lying areas that are the breeding places of snails is useful in controlling the snail population. It is also important to repair any leaks in dams and water troughs as the latter can create an ideal habitat for the survival of water snails. Use of molluscicides like copper sulphate, niclosamide, sodium pentachlorophenate is very effective in the elimination of snails. Reports from several parts of the world indicate that a number of plants have molluscicidal properties. Planting of such trees and shrubs along streams and irrigation channels can reduce the number of snails in a population. The efficacy of this method for control of flukes is yet to be assessed [88].

The control measures discussed above are basically applicable for amphistome infection in ruminants. However, a different control strategy has to be in place for controlling the infection of *Gastrodiscoides hominis* in man. Control methods including the following have been proposed [136]: (1) prevention of the human contamination, (2) actions at human level to cut disease dissemination by humans, (3) control the disease at the animal reservoir level and (4) actions at the level of the intermediate molluscan host.

Trematode infections are some of the most economically important helminth diseases hampering the productivity of domestic ruminants worldwide. Amphistomiasis, at present emerging as an important livestock parasitosis, is still highly underestimated. It is often not diagnosed and the importance of subclinical infection has not been determined. The biodiversity of amphistomes is still highly underestimated both at the morphological and molecular level. Amphistomiasis being a debilitating disease of livestock necessitates the importance of an in-depth study of the causative agents, the amphistomes, which will therefore speed up the estimation and prompt the control of this group of parasites.

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Chapter 12
Dicrocoeliidae Family: Major Species Causing Veterinary Diseases

M. Yolanda Manga-González and M. Carmen Ferreras

12.1 Introduction

Family Dicrocoeliidae Odhner, 1911 includes more than 400 species of Digenea parasites [1] which infect the liver, bile ducts, gall bladder, intestine or pancreas (amongst others) of amphibians, reptiles, birds and mammals [2]. The type genus is *Dicrocoelium* Dujardin, 1845.

12.2 Taxonomy of Dicrocoeliidae Family

There is no unanimous criterion regarding the inclusion of family Dicrocoeliidae in higher taxa. Some authors [2, 3] include it in the order Digenea, suborder Prosostomata, although some of them also consider the superfamily Plagiorchiida [3]. The order Dicrocoeliata was created later [4]. However, other authors [5] include family Dicrocoeliidae in the order Plagiorchiida.

M.Y. Manga-González (✉)
Departamento de Sanidad Animal, Instituto de Ganadería de Montaña, Consejo Superior de Investigaciones Científicas (CSIC)—ULE, Grulleros, 24346 León, Spain
e-mail: y.manga@csic.es

M.C. Ferreras
Facultad de Veterinaria, Departamento de Sanidad Animal, Universidad de León (ULE), Campus de Vegazana s/n, 24071 León, Spain
e-mail: mcfere@unileon.es

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Family Dicrocoeliidae falls within the following taxonomic levels [6]:

Kingdom Animalia

Subkingdom Eumetazoa

Phylum Platyhelminthes

Subphylum Neodermata

Class Trematoda

Subclass Digenea

Order Plagiorchiida

Infraorder Plagiorchioidea

Family Dicrocoeliidae

Nor is there any agreement among authors regarding the name and number of lower taxa of family Dicrocoeliidae, as follows:

Travassos (1944) [7]

Subfamilies:
Dicrocoeliinae Looss, 1899

Infidinae Travassos, 1944

Mesocoeliinae

Yamaguti (1958) [2]

Subfamilies:
Anchitrematinae Mehra, 1935

Leipertrematinae Yamaguti, 1958

Dicrocoeliinae Looss, 1899

Tribes:
Euparadistomini Yamaguti, 1899

Controrchiini Yamaguti, 1958

Brodeniini Yamaguti, 1958

Eurytrematini Yamaguti, 1958

Lypersomini Yamaguti, 1958

Athesmiini Yamaguti, 1958

Brachylecithini Yamaguti, 1958

Dicrocoeliini Yamaguti, 1958

Panin (1981) [8]

Subfamilies:
Dicrocoeliinae Looss, 1899

Eurytrematinae Panin, 1971

Proacetabulorchinae Odening, 1964

Infidinae Travassos, 1944

Agrawal and Sharma (1990) [9]

Subfamily:
Neodicrocoeliinae Agrawal and Sharma, 1990

The Following Genera, Amongst Others, are Included in the Tribe Dicrocoeliini [2] and the Subfamily Dicrocoeliinae [8] of the Family Dicrocoeliidae.

Athesmia Looss, 1899
Brachylecithum Strom, 1940
Brodenia Gedoelst, 1913
Canaania Travassos, 1944
Conspicuum (Bhalerao, 1936) Travassos, 1944
Controrchis Price, 1928
Dicrocoelium Dujardin, 1845
Dictyonograptus Travassos, 1919
Eurytrema Looss, 1907
Leipertrema Sandosham, 1951
Lyperosomum Looss, 1899
Metadelphis Travassos, 1944
Platynosomum Looss, 1907
Pseudathesmia Travassos, 1942

The Following Genera are Mentioned Within Family Dicrocoeliidae [6]:

Athesmia Looss, 1899
Brachydistomum Travassos, 1944
Brachylecithum Shtrom, 1940
Brodenia Gedoelst, 1913
Conspicuum Bhalerao, 1936
Corrigia Shtrom, 1940
Dicrocoelium Dujardin, 1845
Euparadistomum Tubangui, 1931
Eurytrema Looss, 1907
Lutztrema Travassos, 1941
Lyperosomum Looss, 1899
Paradistomum Kossack, 1910

Platynosomum Looss, 1907
Prosolecithus Yamaguti, 1971
Pseudoparadistomum Roca, 2003
Skrjabinus Bhalerao, 1936
Stromitrema Skrjabin, 1944
Unilaterilecithum Oschmarin in Skrjabin & Evranova, 1952
Zonorchis Travassos, 1944

12.2.1 Major Dicrocoeliidae Genera and Species Causing Veterinary Diseases

After analyzing the taxonomic position of family Dicrocoeliidae, whose morphological traits have been indicated [7] and outlined [10], we will now concentrate on the most important genera and species in veterinary parasitology, mainly those affecting ruminants.

Genus *Dicrocoelium* Dujardin, 1845

D. dendriticum (Rudolphi, 1819)
D. chinensis Tang and Tang, 1978
D. hospes Looss, 1907
D. orientale Sudarikov and Ryjikov, 1951
D. petrowi Kassimov, 1952
These species of genus *Dicrocoelium* have been mentioned as valid [6].

Genus *Eurytrema* Looss, 1907

E. coelomaticum (Girad and Billet, 1892)
E. pancreaticum (Janson, 1889)

Genus *Platynosomum* Looss, 1907

P. fastosum Kossack 1910

12.3 Biology of the *Dicrocoelium* Species

Dicrocoelioses are parasite infections caused by different species of the type-genus *Dicrocoelium* Dujardin, 1845. The diagnosis of the adult parasites of this genus is as follow [1]: Body elongate, lanceolate or fusiform. Tegument unspined. Oral sucker usually slightly larger than ventral. Pharynx small. Oesophagus relatively long. Caeca do not reach posterior extremity. Testes large, immediately posterior to ventral sucker, diagonal, may be lobed. Cirrus-sac elongate, may reach anterior border of ventral sucker. Genital pore median, just posterior to intestinal bifurcation. Ovary smaller than testes, close to posterior testis. Uterus occupies entire hindbody, intercaecal.

Vitellarium in two bands composed of small follicles, limited to middle third of body. Excretory vesicle I- or Y-shaped; stem may reach close to ovary; pore terminal. The adult parasites live in the bile ducts and gall bladder of mammals and birds; cosmopolitan.

The most important species of this genus which infects ruminants are: *Dicrocoelium dendriticum* (Rudolphi, 1819) Looss, 1899; *Dicrocoelium hospes* Looss, 1907 and *Dicrocoelium chinensis* Tang and Tang, 1978.

12.3.1 Dicrocoelium dendriticum

This species is one of the most widespread trematodes in mammals, mainly ruminants, in many countries of Europe, Asia, North Africa and North America [11–15]. This trematode could affect humans.

This parasite was confused with an immature form of *Fasciola hepatica* for a long time, because both trematodes are frequently found together in the liver of ruminants at the same time. Its description thus came late. The synonymy of this parasite is complex, due to the different generic and specific denominations received [10, 16]. This could be due primarily to the fact that Rudolphi described the same species twice: once as *Fasciola lanceolata* and again as *Distoma dendriticum* in 1803 and 1819, respectively. On re-examining the specimens studied by Rudolphi on both occasions, it was concluded that *Distoma dendriticum* was identical to *Fasciola lanceolata* [17]. Nevertheless, since the name *lanceolata* had previously been invalidated, the law of priority meant that *dendriticum* was the correct species denomination. *Dicrocoelium dendriticum* (Rudolphi, 1819) Looss, 1899, appears as a valid name on the "Official list of specific names in zoology", whilst *Dicrocoelium lanceatum* and *Dicrocoelium lanceolatum* are mentioned in the "Official index of rejected and invalid specific names in zoology" [16].

12.3.1.1 Life Cycle of *Dicrocoelium dendriticum*

The life cycle of *Dicrocoelium dendriticum* is extremely complex (Fig. 12.1) because land molluscs and ants are required as first and second intermediate hosts, respectively [18]. It was completed for the first time [19, 20] after numerous studies had been carried out over a century [10, 21]. This research showed that the eggs were fully mature when laid, but did not hatch in the bile duct [22] or in water [23] or in aquatic molluscs [24, 25]. Later a long-tailed cercariae was found which was associated with *D. dendriticum*, in land molluscs which live in enzootic zones of the parasite [26–29]. Based on this, and after finding two small tailed forms found in a sheep severely parasitized with *D. dendriticum*, it was suggested that the definitive host was infected via drinking water [30]. After various failures on attempting to infect definitive hosts by feeding them with infected molluscs and cercariae, the possible existence of a second intermediate or auxiliary host was considered.

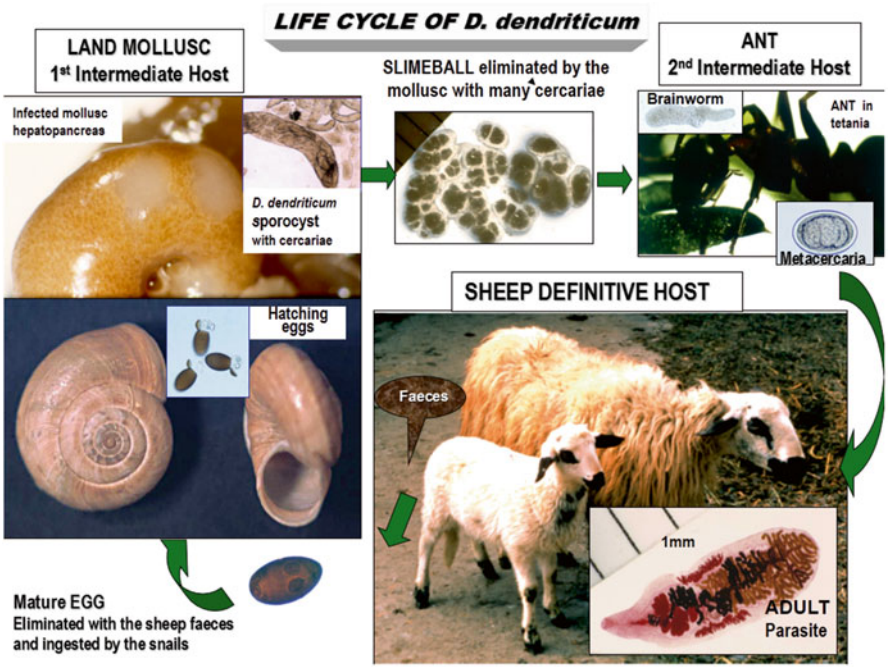

Fig. 12.1 Life cycle of *Dicrocoelium dendriticum*. (1) The sheep definitive host which harboured the adult parasite in the liver. (2) The land molluscs first intermediate host eat the parasite eggs which when they hatch occur the miracidium liberation; finally the parasite evolved to daughter sporocyst harbouring mature cercariae. (3) Many of these mature cercariae goes to the respiratory chamber of the mollusc and are covering in slime forming small spherules that constitute a slime-ball, which is eliminated by pneumostoma of the molluscs. (4) The ants, second intermediate host, became infected when they ingest theses slimeballs; the parasite that go to the brain "brainworm" causes tetania to the ant, the rest "metacercariae" go to the abdomen. The ruminant became infected when ingest the infected ants

Given the failure to detect any infection in fly larvae, parasitic nematodes and beetle larvae [31] or to make the long-tailed cercariae penetrate ants, slugs or earthworms [32], it was questioned whether that parasite belonged to *D. dendriticum*. However, this doubt disappeared when the full development of the parasite was followed in snails and they were seen to shed slimeballs containing cercariae [31, 33–35]. Some authors state that they have been able to infect sheep with slimeballs [31, 33, 34], but must have made a mistake, as all the later attempts to repeat the experiment failed [36]. Due to this a second intermediate host was looked for again and the conclusion that ants were the hosts of the *D. dendriticum* metacercariae and they were used to successfully infect lambs in an experiment [19, 20]. These authors established that three hosts are required for the complete cycle of *D. dendriticum* and that, in New York State, the first intermediate host was the mollusc *Cionella lubrica* (= *Cochlicopa lubrica*), the second one was the ant species *Formica fusca* and that the definitive hosts could be mammals such as sheep, oxen, deer, marmots or rabbits.

Fig. 12.2 Several *D. dendriticum* adults inside of one lamb hepatic bile duct. *Bar* = 0.5 cm

The first ants found infected with metacercariae of *D. dendriticum* in Europe (Florshein region, Germany) were *Formica fusca* too [37]. These authors also followed the experimental metacercaria development in *Formica fusca*, *Formica rufibarbis* var. *fuscorufibarbis* and *Formica gagates* ants feeding them with slime-balls containing *D. dendriticum* cercariae. Likewise, for the first time they completed the life cycle by experimentally infecting one rabbit, two sheep and one mouse by administering mature metacercariae from natural or experimentally infected ants.

Considering the mentioned research and that carried out later by several authors, the development of the complex life cycle of *D. dendriticum* can be summarized as follows.

12.3.1.2 Adult *D. dendriticum* in Definitive Hosts

The adult parasite, distome type, measuring 5–12 mm in length by 1.5–2.5 mm in width live in the mammal's definitive hosts, have a pale body, flattened in a dorsal-ventral direction with the maximum width about half-way along and more pointed at the front than at the back [38–40]. The smooth tegument allows the uterus and vitellaria, which are reddish-brown, to become transparent. These adults, which live in the bile ducts (Fig. 12.2) and gall bladder, deposit the embryonated eggs in the bile ducts after fertilization. The eggs then pass through the common bile duct to the intestine to be shed with the host's faeces. The 36–50 × 22–30 μm eggs, have an operculum, a thick wall and are dark brown in colour, with two marks which correspond to the germinal masses [38, 39, 41].

12.3.1.3 Larval Stages in the Mollusc First Intermediate Hosts

Egg hatching and live miracidium liberation only occur in the digestive tract of mollusc intermediate hosts due to various stimuli, presumably physical–chemical [42–44]. Numerous species belonging to different families, genera and species of land molluscs act as first intermediate hosts [13, 18, 21, 45–47]. The miracidium (20 by 25 μm) has cilia at the front and a papilla endowed with a stylet, but no ocular markings. The free miracidium crosses the intestinal wall of the mollusc, loses the cilia and settles in the interlobular spaces of the hepatopancreas near the heart and kidney (observations not yet published). There it becomes a mother or first generation sporocyst which, as it has no wall of its own, takes on the form of the space it occupies. The daughter or second generation sporocysts are differentiated from the mother sporocyst germinal masses as their size varies between 140 and 4,160 μm, mainly according to their maturity and the mollusc species hosting them [18, 38, 39, 48]. The daughter sporocysts are sacciform and have their own wall. They migrate until they settle in different parts of the mollusc hepatopancreas and, in the case of intense infections, they can also be found in the reproductive apparatus. The germinal masses, which the immature daughter sporocysts fill, are transformed into Distoma and Xiphidiocercaria type cercariae. The body of these cercariae measures 360–760 μm in length by 50–165 μm in width has the oral sucker on its front which includes the mouth and perforator stylet, and has the ventral sucker on the central section. In addition the cercaria has a 200–1,000 μm long tail. The time between egg ingestion by the snail until the cercaria is mature, a minimum of 3–6 months, varies mainly with the mollusc species and temperature [36, 49–51]. The mature cercariae leave the sporocysts and go to the mollusc's respiratory chamber through the circulatory system. There they are covered in slime forming small spherules (Fig. 12.3) measuring 1–2 mm, which contain a generally high number of cercariae (108–600, mean 364) [21].

Various of these spherules (from 3 to 13) form a bunch and constitute a slimeball [21], which is brilliant white when newly emitted and can be as large as 1 cm in diameter and host from 300 to 7,800 cercariae [21, 31, 52]. These slimeballs are expelled by the breathing movements of the snail through the pneumostome (respiratory orifice) and remain attached to the mantle edge of the mollusc until they are deposited on plants or other supports as the snail moves. While some authors state that temperature and humidity are the factors with the greatest influence on slimeball emission, others consider that light has the greatest influence. In a few hours the sun affects appearance, size and consistency of the slimeballs and thus the viability of the cercariae. The infective capacity of these for ants is prolonged by at least 18 h if the slimeballs are stored at 4 °C. At this temperature a very small number of cercariae maintain all their mobility after 72 h [21].

12.3.1.4 Larval Stages in Ant Second Intermediate Hosts

When the slimeballs are ingested under suitable conditions by different species of ants of the family Formicidae (*Formica fusca*, *Formica rufibarbis*, *Formica pratensis*, *Formica sanguinea*, amongst others) which act as second intermediate hosts

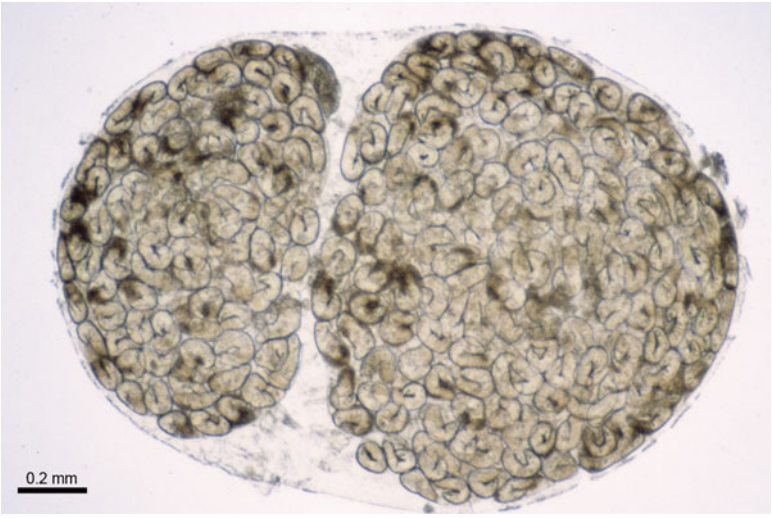

Fig. 12.3 Photomicrography of a spherule from a slimeball emitted by a *D. dendriticum* infected mollusc, which contain a high number of *D. dendriticum* cercariae. *Bar* = 0.2 mm

[18, 53, 54], the cercariae cross the craw of the ants where they leave scars [55] with the help of the stylet, lose their tail in the first few hours after ingestion and migrate to their final position. One of these (sometimes 2 or 3), called a "brainworm", settles in the suboesophageal ganglia of the ant [18, 55–57] and the rest become metacercariae, with a more consistent cystic wall, which settle in the abdomen in numbers varying (from 1 to 580) according to the ant species and different authors [18]. However, metacercariae have also been found in the trachea and muscles of these Formicidae [58] and in the thorax [59]. According to the studies on the movement of the cercariae, once they had been ingested by the ants until they settled in the brain and became encysted in the abdomen, it seems that 30 % of the ingested cercariae become active and begin their migration from the abdomen to the head [60, 61]. When one penetrates and settles in the ant's brain—the brainworm, the rest of the cercariae initiate their return to the abdomen where they become encysted. The time needed for this process to be completed varies according to the temperature. So the final distribution of the metacercariae occurs after 3 days at 26 °C, after 3½ days at 24 °C and after 8 days at 16 °C. A correlation between the number of scars on the craw and that of metacercariae in the abdominal cavity has been observed [62]. Nevertheless, it seems that the presence of a high number of metacercariae in the abdomen produces very few harmful effects [63].

The size of the *D. dendriticum* encysted metacercariae obtained from the abdomen of different species of ants by several authors [18] varies according to their species and the number of metacercariae per ant. The largest size of the metacercariae (481–596 μm length, 260–311 μm width) was obtained in *Formica pratensis* [64]. Moreover, the cyst wall thickness was also very variable (5–96 μm) according to the species and authors [18].

When the temperature falls, the brain worm (or worms) in the suboesophageal ganglion changes the ant's behaviour by causing tetany of its mandibular muscles. This makes it attach to the upper part of the plants [55, 56], which facilitates ingestion of the ant with metacercariae by the definitive host when grazing. The time needed for the metacercariae, once ingested by the ants, to become infective for the definitive host varies according to the ant species and the temperature: 38–63 days in *Formica fusca* [19, 20]; 38–56 days at 26 °C in *Formica fusca*, *Formica rufibarbis* var. *fuscorufibarbis* and *Formica gagates* [37]; 43 days in *Formica rufibarbis* [65]; 35–38 days at 28–32 °C, 40–62 days at 19–20.5 °C in *Formica rufibarbis* and *Proformica nasuta* [66]; 45 days in *Formica sanguinea* [67]; 40 days or less at 25 °C [68]; 40 days at 25 °C in *Formica rufibarbis* [59].

12.3.1.5 Infection of the Definitive Hosts

When the definitive hosts ingest these infected ants, the mature metacercariae excyst in the digestive tube and the young flukes go through the opening of the common bile duct [69–71], and sometimes the portal circulation [72], to the liver, settling in the bile ducts where they become adult worms. When these are mature they lay eggs which are shed in the faeces of the host and allow the life cycle of *D. dendriticum* to begin again. The prepatent period in sheep varies according to different authors: 96 days p.i. [59], 56 p.i. [50], 48–49 p.i. [73] and 49–79 p.i. [40].

12.3.2 Dicrocoelium hospes

Some authors [74], based on the morphological variability of Dicrocoeliidae adults from the former Soviet Union and Czechoslovakia, concluded that *Dicrocoelium hospes* Looss, 1907 was synonymous to *D. dendriticum*. However, other authors [12, 75–80] considered that *D. hospes* was a valid species. Molecular characterization of the 28S region and the ITS-2 of this species has been carried out [81]. This liver fluke species is widely distributed in ruminants (cattle, sheep, goats and buffalos) from the savanna areas of Africa south of the Sahara [79]. It uses Stylommatophora molluscs, from the genus *Limicolaria* (family Achatinidae), in its biological cycle [75, 79], which act as first intermediate hosts. It also uses family Formicidae ants, belonging to species of genus *Camponotus* (Formicinae), *Crematogaster* (Myrmicinae) and *Dorylus* (Dorylinae) [57, 75, 79].

12.3.3 Dicrocoelium chinensis

Dicrocoelium chinensis (Sudarikov and Ryjikov, 1951) has been mainly found in ruminants from East Asia [82–85] and in sika deer (*Cervus nippon*) from different countries of Europe, probably imported from Asian countries [86–88]. The adults of this species are morphologically larger than those of *D. dendriticum*

and both species are molecularly different [88]. *Dicrocoelium chinensis* uses Stylommatophora molluscs, genus *Bradybaena* (Bradybaenidae), *Xeropicta* (Helicidae) and *Ganesella* (Pleurodontidae) as first intermediate hosts [83, 89]. The ants mentioned as second intermediate hosts of *Dicrocoelium chinensis* belong to genera *Formica* and *Camponotus* [83, 89].

12.4 Epidemiology of Dicrocoeliosis

We refer mainly to the dicrocoeliosis produced by *D. dendriticum*. Although there are various papers on the different aspects of the complex life cycle of *D. dendriticum*, some of which were mentioned above, integrated studies of the epidemiology of dicrocoeliosis—mutual relationships between the parasite, the definitive and intermediate hosts and the environment—are scarce. These studies will allow discovery of the source of contamination and the periods with a risk of infecting ruminants, essential information for applying effective strategic treatments against the parasite in a specific zone [90].

Regarding the complex epidemiology of dicrocoeliosis the following aspects, among others, should be considered: (1) Diagnosis of infection by the *D. dendriticum* parasite of the definitive host mammals, mainly domestic and wild ruminants; (2) Kinetics of trematode egg elimination in the faeces of the definitive host and contamination of pasture by these eggs; (3) Times of year and environmental factors which can affect the survival and viability of the parasite eggs; (4) Presence in the zone of appropriate species of land molluscs, the first intermediate hosts which ingest the eggs and permit the miracidium to hatch, followed by larval development of the parasite inside them; (5) Life cycle of those molluscs, periods when adult or young molluscs are predominant and their activity; (6) Influence of the environmental conditions on the development of the parasite in the different mollusc species; (7) Appropriate meteorological characteristics and time of year for the slimeballs to be shed with cercariae inside; (8) Ingestion of these balls by specific species of ants, the second intermediate hosts, which live in the same biotopes; (9) Times of ant activity and inactivity throughout the year; (10) Behaviour of the ants infected by *D. dendriticum*, with reference to the time, temperature and light intensity; (11) Times when the definitive hosts are infected by ingesting infected ants with mature metacercariae; (12) Transformation of the ingested metacercariae into adult worms which, after the prepatent period, shed the parasite egg, thus contaminating the pasture.

12.4.1 Prevalence of Infection in Livestock

Various coprological studies have been carried out to discover the prevalence (%) of *D. dendriticum* infection in livestock in several, mainly European and Asian, countries. Nevertheless, the data provided vary according to the host species, geographical area and ecology, time of year, time when the samples are taken and the methodology used.

The data on infection prevalence (%) by *D. dendriticum* in ovines, obtained via conventional coprological studies, vary among countries and within the same country [15, 91, 92]. Prevalences of between 60 and 100 % have been recorded in animals from: Azerbaijan [93], Bulgaria [94], China [95], France [96], Greece [97], Italy [98, 99], Macedonia [100], Russia [101], Spain [21, 91, 102]) and Turkey [103], amongst others. Other lower prevalence % rates have also been recorded. Animal age clearly affects egg elimination, as the highest infection rates are in the oldest animals [92, 104].

The prevalence rates obtained by coprology in cattle were generally lower than those in sheep: 38 % in Spain [105], 0.5–21 % in Bulgaria [106], 5 % in Italy [107], 1.51 % in Pakistan [108], among others. Slaughterhouse studies have revealed 27–35 % infection in cattle in Libya [109]. The percentage among infected caprines (via bile ovoscopy) was 45 % in Spain [110].

In addition, various species of wild Leporidae, mainly rabbits, have been found infected with *D. dendriticum* in the lower reaches of the French Pyrenees and in Northwest Spain [111, 112], as well as parasite egg shedding by chamois, mountain goats and mouflons [113]. Prevalence of 20 % has been obtained by necropsy in roe deer (*Capreolus capreolus*) from Northern Turkey [114].

12.4.2 Periods of Pasture Contamination and Egg Survival

The kinetic of *D. dendriticum* egg shedding by ruminants varies from one country to another. Therefore in countries with a continental climate within the Mediterranean Atlantic transition, like Spain (León Province), with cold winters and hot summers, the shedding of *D. dendriticum* eggs in the faeces of ruminants occurs uninterruptedly throughout the year in both ovines and bovines [15, 90–92, 105], although the highest values are recorded at the end of the autumn and mainly in the winter. In Germany the highest elimination of eggs by sheep was observed in the spring and by cows in the autumn [115]. In Italy, the highest rate for egg deposition in sheep was observed between February and May [98].

The viability of *D. dendriticum* eggs in the field depends to a great extent on the consistency of the faeces shed by the definitive host and the environmental conditions they are exposed to. The resistance of *D. dendriticum* eggs to environmental conditions means that pasture contamination continues for long periods of time. Viable parasite eggs have been obtained after 5 years from dried sheep faeces and after 16 months on grass in the former Soviet Union [116]. *D. dendriticum* eggs are more resistant to low temperatures than high ones [52]. They have been experimentally proven to resist temperatures between −20 and −50 °C [117]. Studies carried out under controlled field conditions in León (Spain) [118] have shown that egg mortality in faeces exposed to the environment is a phenomenon regardless of the time they have been outside and, in contrast, show marked seasonality. Thus, while the eggs deposited in the environment between January and July have high resistance (75–85 %), those shed between July and August suffer extremely high mortality (almost 100 %). A significant survival rate is observed from September to

December, as in the first half of the year, so the eggs from that period are not destroyed until the hot months of the following year [118]. In conclusion, the cause of *D. dendriticum* egg mortality under natural conditions seems to be exclusively due to the lethal effect of the high temperatures reached inside the faeces directly exposed to the sun, which causes miracidium death. Thus in the colder period of autumn-winter, when the greatest egg elimination takes place in the province of León (Spain), the survival of *D. dendriticum* eggs is very high. Due to this, pasture contamination by viable eggs is considerable in the spring, when molluscs (mainly the young ones) become active and are very abundant [15, 18].

12.4.3 Mollusc First Intermediate Hosts

The role played by land molluscs in the epidemiology of dicrocoeliosis is very important as they are generally coprophagous and easily ingest the faeces of the definitive hosts containing parasite eggs. Egg hatching and miracidium release appear to occur due to several physical-chemical stimuli of the mollusc intestine, low pH and reducing conditions [43, 44]. Recently *D. dendriticum* eggs have been hatched by freezing (at −80 ºC or in liquid nitrogen) and then thawing, extracting the miracidium DNA and its molecular identification [41].

Dicrocoelium dendriticum egg hatching and miracidium liberation only occur in the intestine of the appropriate molluscs that act as intermediate hosts. Inside the mollusc the parasite multiplies enormously by asexual reproduction (numerous cercariae can be formed from one ingested egg). This increases the possibilities of parasite transmission. Since the long-tailed cercaria in *Helix carthusiana* (= *Monacha* (*M.*) *cartusiana*) was encountered for the first time [26] and later described as *Cercaria vitrina* (from *Zebrina detrita*) [27] and associated with *D. dendriticum*, many studies have been carried out to discover the mollusc species which act as first intermediate hosts for this parasite. More than 100 mollusc species (Gastropoda, Pulmonata, Stylommatophora) have been found receptive to *D. dendriticum* under natural and laboratory conditions. They mainly belong to the following families: Bradybaenidae, Buliminidae, Clausiliidae, Cochlicopidae, Helicidae, Hygromiidae (previously included in Helicidae) and Zonitidae [14, 15, 18, 21, 45, 46, 48, 103, 117, 119–125] among others.

This parasite shows markedly little specificity as regards its first intermediate host. In addition the parasite can develop in various species of mollusc in the same area. In Spain the most important species of mollusc in the epidemiology of dicrocoeliosis are *Cernuella* (*Xeromagna*) *cespitum arigonis* on plateaux and plains and *Helicella* (*Helicella*) *itala* mainly in the northern mountains [18, 21, 126]. The latter species mentioned has also been found naturally infected with *D. dendriticum* in the UK [50] and Bosnia-Herzegovina [119]. Other significant species recorded in the transmission of *D. dendriticum* are: *Helicella* (*Helicella*) *obvia* in Germany, Bosnia-Herzegovina, Bulgaria and Turkey; *Zebrina* (*Zebrina*) *detrita* in Germany, Bosnia-Herzegovina, Bulgaria, France, Italy, Russia and the USA; *Cochlicopa lubrica*

(=*Cionella lubrica*) in the USA, Spain, France, Russia and the Ukraine. Many other species of molluscs have also been recorded as infected with *D. dendriticum* in different countries and with infection percentages varying between 0.5 and 62 % [18]. Nevertheless, the prevalence data indicated by the different authors must be higher, as the first larval stages of the parasite are not detected under the stereomicroscope until at least 50 days p.i. [51] in molluscs experimentally infected and kept in the laboratory and until a maximum of 9 months in molluscs experimentally infected and kept in a natural environment [127]. Therefore, histological [128], isoenzymatic or molecular biology [54] techniques are necessary to detect the first larval stages. It must also be borne in mind that the infection prevalence increases with the age of the molluscs [18, 48, 125].

The life history of the mollusc intermediate hosts is of great epidemiological interest, as regards the abundance, age, activity for both the ingestion periods of *D. dendriticum* eggs and the survival of the parasite in them. The abundance and activity of *H. itala* in Spain were greater in spring and autumn, with young specimens being more abundant in spring, whereas adults were more abundant in autumn [18, 48]. The same is true for *H. obvia* in Germany [125]. Age and nutritional state of the molluscs, infective dose, ambient temperature and relative humidity, amongst other aspects, influence the long development of larval stages of this Digenea in the first intermediate hosts [18, 51, 125, 129]. Both *H. itala* and *H. obvia* adults were more abundant in autumn while the young ones were in spring [18, 125].

The egg ingestion periods of *D. dendriticum* are dependent on mollusc activity, and the survival and development of the larval parasites are important in the transmission of dicrocoeliosis. The sporocysts require a temperature of over 4 ºC to mature, with development increasing with a rise in temperature [125]. Under controlled field conditions, in hot months, larval development of *D. dendriticum* is accelerated within the mollusc host [15, 127]. The mollusc species can also influence *D. dendriticum* development [129].

The infection prevalence increased with mollusc age [18, 125, 130]. In general, the highest rate of infection in molluscs occurred in the autumn and spring. Moreover, immature daughter sporocysts (with germinal masses) predominated in adult and young molluscs collected in the autumn in León (Spain), whilst sporocysts with mature or nearly mature cercariae predominated in adults collected in the winter and spring [18]. In the Marmara (Turkey) region the lowest parasite percentages were generally detected at the end of the summer and in the autumn [103]. In France the infection of the molluscs occurs between the end of one hibernation period and the beginning of the next [131]. The results obtained in León (Spain) seem to indicate that young molluscs, which are more abundant in spring, are infected between the beginning of spring and the beginning of summer [15, 18]. So the first infected ones can shed slimeballs with cercariae in the summer or at the beginning of the autumn, whilst those infected later can shed slimeballs (as adults) in the first half of the following year, provided they survive the harsh winter. In Germany most specimens of *H. obvia* are infected in the autumn of their second year of life, when the shell sizes are intermediate [125]. In addition, the percentage of molluscs with daughter sporocysts is higher in the spring and the shedding of

slimeballs occurs in May and June. Likewise the first slimeballs shed by *Z. detrita* were also observed in Germany at the beginning of May and the last ones in mid-October [59]. The sun affects the appearance, size and consistency of slimeballs in only a few hours and thus influences the viability of the cercariae. Their infective capacity for ants is prolonged at least 18 h, if slimeballs are stored at 4 ºC, although a very low number of cercariae maintain mobility after 72 h at this temperature [21].

12.4.4 Ant Second Intermediate Hosts

When slimeballs are ingested by appropriate species of ants, they become infected. The "brainworms" settle in the suboesophageal ganglion of the ant whilst the rest of the metacercariae are located in the abdomen. The infection prevalence obtained by the authors varied among the different species (*Formica pratensis*, *Formica rufibarbis*, *Formica fusca*, amongst others), but mainly due to the type of sampling [18]. The higher infection percentage is obtained when the ants are collected in tetany. So the infection of *F. rufibarbis* and *F. pratensis* collected directly from the anthills in León (Spain) were 6.59 % and 4.05 %, respectively, while the infection percentage recorded in the specimens of the same ant species collected in tetany was 95.39 % and 100 %, respectively [18]. Similar results were obtained in *F. pratensis* (1.1 % and 97 %, respectively) from Bosnia-Herzegovina [132] and in *Formica lugubris* (0.14 % and 70 %, respectively) collected in the same country [119]. The number of *D. dendriticum* metacercariae given by different authors (from 1 to 580) varied among the different species and even within the same one [18]. The variability could be due to: the time of year, as it is higher in summer [133]; the different affinity of the ant species for slimeballs [134]; the type of vegetation and the ant species [135]; the size of the abdomen [62]; the different species, the size and possible ecological and behavioural causes [53]. It seems that *F. rufibarbis* and *F. pratensis* are the most important species in the epidemiology of dicrocoeliosis in Spain and in many other countries [18].

The importance of ants as second intermediate hosts is mainly due to their abundance, wide distribution and the alteration (tetany) in their behaviour produced by the "brainworm", especially when temperatures and solar intensity decrease, which makes infection of the definitive hosts by ingestion of the parasitized ants easier. The tetany stage of infected ants occurs mainly in the early hours of the morning and late in the afternoon, although on cloudy or warmer days it is also possible to detect ants in tetany at the end of the morning and the beginning of the afternoon [18, 136]. The highest temperatures at which *F. pratensis* and *F. rufibarbis* were observed in tetany in León (Spain) were 26.9 ºC and 28 ºC, respectively [18]. This temperature was higher than the 18–21 ºC reported by other authors [53, 136–138]. On the other hand, active ants were observed between March and November and infected ones from April to November in those collected in León from the nest, and in tetany between May and October [18]. Authors from other countries have also observed infected ants between the spring and autumn in: Kazakhstan [139];

Bosnia-Herzegovina [137]; France [131]; Germany [53]. Transmission to definitive hosts only occurs at times when the ants are not hibernating. Nevertheless, some infected ants survive in their nests during the winter, and they are responsible for infection of the definitive host on becoming active at the beginning of spring [50, 131]. In the following months until November, when ant hibernation starts again, the ingestion of ants containing infective metacercariae by the definitive hosts and the number of adult worms of *D. dendriticum* in their liver increase. As a consequence, parasite egg elimination also increases during this period, reaching its highest values at the end of the autumn but mainly in winter, the cold period when egg survival is at its highest. Recently a multiple regression model has been used to predict risk habitats for the transmission of *D. dendriticum* to grazing ruminants [140].

12.5 Pathology and Clinical Aspects of Dicrocoeliosis

12.5.1 Clinical Signs

Dicrocoeliosis is considered asymptomatic [14, 141]. The fluke usually produces mild non-specific clinical symptoms [142, 143] that are not usually manifested, even in heavy infections with doses of 3,000 metacercariae [40, 144]. Nevertheless, diarrhoea and a decrease in growth in lambs have been observed in sheep tested with 3,905 metacercariae [59]. According to previous reports parasitic burdens of 1,000 or less flukes do not result in clinical manifestations [40, 145]. Animals with dicrocoeliosis may show eosinophilia, anaemia, icterus, oedema, weight loss, reduced meat, milk and wool production as well as impairment of the liver in severely infected cases [13, 14, 143, 146]. In experimentally infected lambs the lowest weight increase was observed 60 days p.i., both in the lambs infected with 3,000 metacercariae (−15 %) and those tested with 1,000 (−12 %) [147]. In this study the highest reduction (−18 %) was detected in the group of lambs with 401–600 worms and no other clinical signs were observed. Previous reports [148] considered that the reduced weight gain was more marked until the parasites reached sexual maturity. Poor growth and body condition, and severe photosensitisation characterized by oedema of the unpigmented areas of the face and ears, yellow crusts and skin necrosis were identified in 14 month-old Texel cross Cheviot ewe lambs, probably associated with dicrocoeliosis [149]. The levels of infection in this case were unusually high, as indicated by their faecal *D. dendriticum* egg counts (909 epg).

12.5.2 Biochemical Parameters

In lambs experimentally infected with 1,000 and 3,000 metacercariae, respectively, which harboured between 30 and 2,063 adults worms in their necropsy carried out at 2 and 6 months p.i., a slight increase in the serum levels of total

bilirubin (7 %) and albumin (3 %) was observed [147]. The hepatic enzyme values: aspartate aminotransferase (AST, 19 %) and alanine aminotransferase (ALT, 22 %) increased, mainly in lambs tested with 3,000 metacercariae. The highest increase in these enzyme values observed at the early stages of the infection (60 days) could be related to the hepatic damage produced by the migration of the worms before reaching sexual maturity, since the first *D. dendriticum* egg elimination was observed in the faeces of the lambs between 49 and 79 days p.i. [40]. No clear relationship was observed between the increased hepatic enzyme values and the parasite burden, although the highest AST, ALT and gamma-glutamyl transpeptidase (GGT) values were detected in the animal with the greatest burden. The GGT values were higher (11 %) in the lambs slaughtered on day 180 p.i. and the increased activity of this enzyme seems to be directly related to the parasite burden [147]. It may also be related to the more severe bile duct lesions observed in this animal group. In cattle infected with *F. hepatica* the increase in GGT serum values coincided with the penetration of the bile ducts by migrating flukes [150].

Serum liver glutamate dehydrogenase (GDH), GGT and bile acid concentrations were markedly elevated, while serum albumin concentration was low, indicative of severe liver parenchymal and biliary disease, in lambs with dicrocoeliosis and hepatogenous photosensitisation [149]. Oxidative liver damage with increased ALT and AST activity as well as bile duct proliferation and chronic cholangitis have been described in experimentally infected hamsters [151–153]. A significant elevation in bile flow (+20 %) and in the biliary output of glutathione (+34 %), bile acid (+59 %), cholesterol (+108 %), phospholipids (+99 %) and alkaline phosphatase (+36 %) was observed in these infected animals [154].

12.5.3 Gross Lesions

In the final hosts, as it was already discussed in the Sect. 12.3, the ingested metacercariae excyst within the proximal intestinal tract and migrate through the small intestinal lumen and common bile duct to the liver, maturing to monoecious adults within the bile ducts [149].

In natural and experimental dicrocoeliosis a different degree of hepatic induration and the presence of whitish, firm, thickened and distended (cholangiectasia) bile ducts were a constant finding. These bile ducts were visible as branching cords on the visceral surface of the liver, mainly on the left and right hepatic lobes. Frequently, the damaged bile ducts were observed dorso-laterally at the renal depression area of the right hepatic lobe. The incised liver surface revealed numerous enlarged bile ducts containing the parasites and bile. In heavy infections a variable number of parasites are also seen inside the extrahepatic bile ducts and gall bladder. The hepatic lymph nodes increased in size [144, 147]. These macroscopic lesions seem to be directly related to the parasite burden [147, 155].

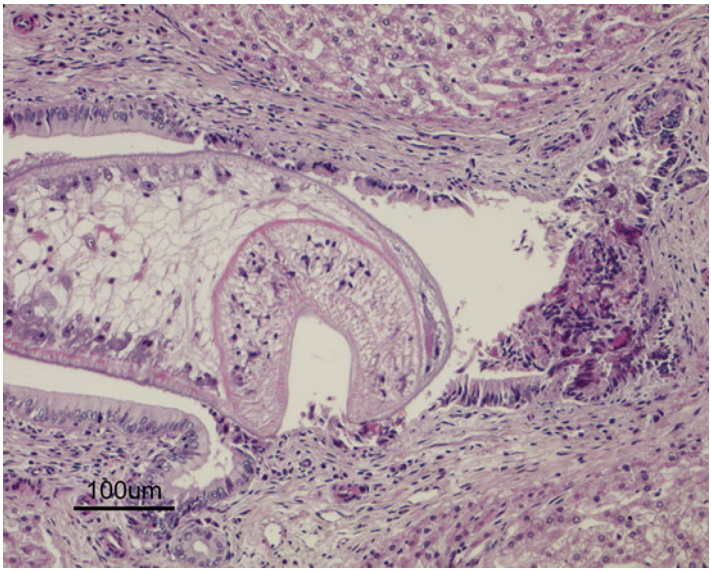

Fig. 12.4 Erosive effect of a *D. dendriticum* adult sucker in the lining epithelial cells of a septal bile duct from a experimentally infected lamb slaughtered a 60 days p.i. *Bar* = 100 μm

12.5.4 Microscopic Lesions

Pathological changes in the liver (cholangiohepatitis) and gall bladder (cholecystitis) are probably caused not only by the action of toxic products formed by the parasite but also by the mechanical irritation of the walls of the bile ducts by the fluke. Due to its buccal stylets, *D. dendriticum* irritates the bile duct surface, causing changes in the septal bile ducts [156, 157]. This parasite is devoid of spines on the tegument, so the hepatic lesions it causes are less significant than those caused by *F. hepatica*, except in the case of massive chronic infection [157]. The adult parasites cause damage to the lining of the bile ducts (Fig. 12.4); however, in comparison with *F. hepatica*, *D. dendriticum* does not cause extensive hepatocellular damage. Hence its potential pathogenicity is frequently underestimated [13]. In experimental dicrocoeliosis in sheep, lesions mainly affecting the biliary system but also hepatocytes were associated with the parasite burden [147]. The septal bile ducts showed extensive hyperplasia, desquamation and necrosis of mucosal epithelium and goblet cell differentiation [147, 156]. The hyperplastic biliary epithelial cells stain positively for alcian blue–periodic acid–Schiff and silver methenamine stains. Thus both neutral mucins and acidic mucins, concentrated close to the apical surface in the epithelial cells of septal bile ducts, could be demonstrated in the same type of cell in natural and experimental dicrocoeliosis [147, 158]. However, the proportion of sialomucins and sulphomucins seen increases in this parasitic cholangitis [158]. A variable number of intraepithelial globule leukocytes and lymphocytes were also observed [147, 156]. The origin and function of globule leukocytes are

not fully understood. These cell populations are considered to be derived from mucosal mast cells that migrate into the epithelial compartment. Dicrocoeliosis and fasciolosis in goats showed an increase in the total number of hepatic mast cells with sulphomucins in their granules, while neutral mucins and carboxy-mucins were revealed in the globules of globule leukocytes [159]. These globule leukocytes seem to be significantly correlated to tissue eosinophils and may play an important role in the response to some parasitic infections [160]. The lumen of these bile ducts frequently contained some worms and a superficial erosive effect of the parasite sucker was seen on the lining of epithelial cells [147, 156]. Leukocytic infiltration (macrophages, eosinophils, lymphocytes and plasma cells) and periductal fibrosis were also observed. Similar histopathological findings were detected in the hepatic and cystic ducts. Their simple columnar epithelium showed numerous goblet cells and the mucous glands present in the lamina propria are very hyperplastic [147, 161]. The presence of abundant mucinous secretion (principally acidic mucins) in the epithelial cells of major bile ducts, confirmed by histochemical staining and electron microscopy, may be a defensive mechanism in the host response against the parasite [147] and has been associated with proliferating cholangitis [162]. A periductal lymphocytic infiltration (diffuse or forming lymphoid aggregates and follicles) is frequently present in both extrahepatic and septal bile ducts. Abundant fibrous tissue, often with smooth muscle cell differentiation, surrounds the extrahepatic bile ducts. The presence of fibrosis of the bile duct walls throughout the liver is a pathological characteristic in severely affected animals with several thousand *D. dendriticum* in the bile ducts [149]. Portal and septal fibrosis were always seen in lambs experimentally infected with *D. dendriticum* but perisinusoidal and centrilobular fibrosis were also found in lambs with the most parasites [147]. Severe extensive biliary fibrosis was a constant finding in heavy infections with both *F. hepatica* and *D. dendriticum* in naturally infected sheep [161]. Liver fibrosis develops as a long-term consequence of chronic liver injury. The hepatic stellate cells, which transdifferentiate into myofibroblasts upon liver injury, are the main fibrogenic cells in the liver [163]. In experimental dicrocoeliosis in sheep [147] cirrhosis was never encountered, in contrast to the studies on natural infections in sheep [155] and on experimentally induced dicrocoeliosis in hamsters [164].

The interlobular bile ducts in the portal tracts were surrounded by an inflammatory infiltrate and showed nuclear pyknosis, necrosis and desquamation of epithelial cells. Parasite eggs or flukes were never seen in these ducts in experimental conditions [147]. Occasional ectopic worms were detected inside the septal hepatic veins in natural and experimental dicrocoeliosis [147] probably due to migration of *D. dendriticum* through the portal circulation.

The gall bladder in experimental dicrocoeliosis in sheep showed focal loss of epithelial cells, subepithelial oedema with lymphatic distension, occasional globule leukocytes in the mucosal epithelium as well as diffuse or nodular lymphoid infiltrates. Parasitosis with *D. dendriticum* is a significant risk factor for cholelithiasis. Cholesterol and pigment stones were found in the major bile ducts and the gall bladder in 18 out of 254 livers in sheep naturally infected with *D. dendriticum*. The assumed mechanism of stone formation was unknown but it seems that the presence of parasites in the bile ducts and the gall bladder impairs bile flow [165].

In the liver parenchyma vacuolar (or hydropic) degeneration, glycogen infiltration (mainly in periportal areas), compression atrophy of liver cell cords, sinusoidal leukocytosis and wall thickening of the portal veins were also observed in experimental dicrocoeliosis [147]. The degenerate hepatocytes from cattle [166] and goats [156] naturally infected with *D. dendriticum* suffer glycogen depletion, although this varied from zone to zone of the liver lobuli. These facts may indicate that the toxic metabolites released by the adult flukes induce hepatic injury both in portal tracts and hepatocytes as has been documented in experimental dicrocoeliosis [147, 153]. The hepatic lymph nodes showed an increase in cellularity of B and T-cell areas, plasmacytosis and histiocytosis.

12.5.5 Immunohistochemical Studies

Immunohistochemical studies focused on characterizing the inflammatory infiltrates associated with hepatic lesions in experimental dicrocoeliosis were carried out on two groups of lambs infected with doses of 1,000 and 3,000 metacercariae [167]. In all infected animals CD3+ T lymphocytes were scattered diffusely or forming lymphoid aggregates and follicles in septal bile ducts affected by proliferative cholangitis. Most CD3+ cells also expressed CD4+ T cells but CD8+ T lymphocytes were scattered surrounding damaged septal bile ducts. Biliary intra-epithelial lymphocytes positive for CD3, CD4 and CD8 were frequently identifiable in the septal and interlobular bile ducts, suggesting the participation of these cells in the host's local defence against the parasite or its products. Numerous CD3+ lymphocytes were present around the interlobular bile ducts and in the hepatic sinusoids. Scarce WC1 γδ-T cells were observed throughout periductal areas in septal and interlobular bile ducts. CD79 αcy and CD45R positive B lymphocytes were frequently demonstrated in relation to lymphoid aggregates and follicles seen around the large bile ducts. Mainly at 180 days p.i. these bile ducts showed a central core of B lymphocytes CD79 αcy + surrounded by numerous CD45R and CD4+ lymphocytes. IgG + plasma cells, with a diffuse pattern or forming clusters near hyperplastic bile epithelium, were numerous in septal bile ducts (Fig. 12.5). These cells may be involved in the local humoral immune response, probably induced by the antigen load released by the parasites. The maximum serum IgG antibody level against excretory-secretory and somatic antigens of *D. dendriticum* in these lambs was reached on day 60 p.i. and remained high until the experiment ended 180 days p.i. [168]. In the portal tracts CD79 αcy, CD45R and IgG-positive cells were intermingled with C3+, CD4+ and CD8+ T cells. Macrophages positive for VPM32 and CD14 antibodies were observed scattered or in groups around septal bile ducts but also near the liver sinusoids and in the portal spaces. With the anti-MHC class IIβ monoclonal antibody, a large population of cells were positive (B and T cells and activate macrophages), especially around septal bile ducts but also in the portal tracts and liver parenchyma. There was no correlation between the parasitic burden and the cell distribution and intensity of labelling patterns observed.

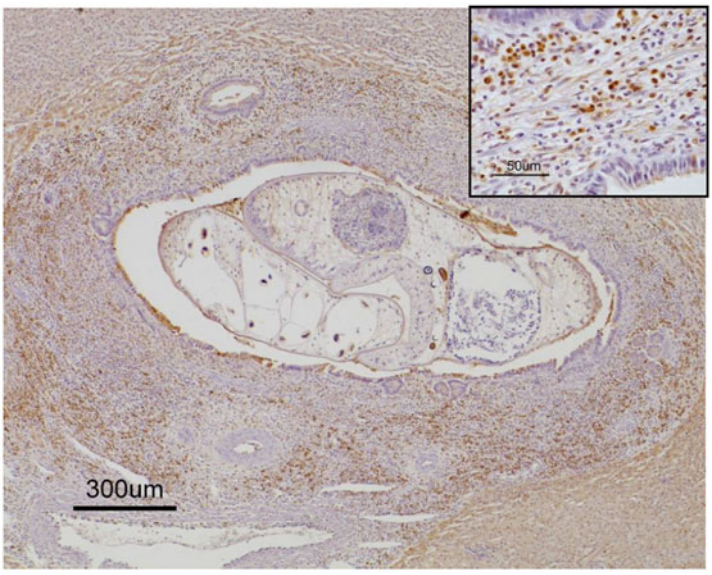

Fig. 12.5 Lamb liver. IgG-producing plasma cells with a diffuse pattern around a septal bile duct in which two worms of *D. dendriticum* are observed. Lamb infected with 1,000 metacercariae (72 worms recovered) slaughtered 60 days p.i. ABC system. Haematoxylin counterstain. Bar = 300 µm. *Inset*: higher magnification of positive IgG plasma cells. Bar = 50 µm

12.6 Diagnosis of Dicrocoeliosis

The diagnosis of ruminant dicrocoeliosis is mainly based on the post-mortem examination of the animal liver and gall bladder [169], on collecting the parasite found in these organs and detection of the eggs in the host faeces [40], as well as on immunological methods [168].

Dicrocoeliosis is usually diagnosed during post-mortem examination of the liver or by coproscopic techniques, although faecal examination has a low sensitivity when compared with those of liver necropsy. So the percentage of sheep positive to egg-count was only 26.9 % in an agricultural region of Sardinia (Italy); nevertheless, 50.3 % of the sheep livers examined had flukes of *D. dendriticum* [170].

Coprological examination is currently based on quantitative coprological techniques carried out by sedimentation and McMaster [13, 15, 40] and by flotation and McMaster, using in this case the high density solution as zinc sulphate (specific gravity 1.18) and potassium iodomercurate (at dilution 1:10), amongst others [13, 149, 171]. These coprological techniques allow the number of *D. dendriticum* eggs per gram (epg) of faeces from infected animals to be counted. The potassium iodomercurate solution gave the highest rate of egg recovery from faeces (91.2±9.4 %). The detection of the infection using this coprological technique has the disadvantage of the animals not having eliminated eggs of *D. dendriticum* until

the prepatent period is reached. This period varies in lambs from 48 to 79 days p.i. according to the different authors [73, 40, 50]. Due to this, most of the hepatic damage has already been done [147] and pasture contamination has started when the eggs of the parasite are detected. Moreover, false negative results concerning egg detection could be obtained because only a small sample of the animal faeces is examined with coprological techniques.

Because of this, serological methods such as enzyme-linked immunosorbent assays (ELISAs) have been used to detect anti-*D. dendriticum* antibodies, using somatic (SO) and excretory-secretory (ES) products as antigens [13, 146, 155, 168, 172–175]. Nevertheless, this indirect test is not specific [176], because it does not allow exposure to the parasite and active infection to be distinguished. Moreover, the antigens normally used to detect antibodies are highly complex mixtures of different protein compounds, which induce different immune responses and cause problems of cross reactivity. These circumstances make them practically inadequate for diagnosing the infection in hosts with multiple parasites, as occurs in ruminants kept on pasture.

On the other hand, SO antigen appears to stimulate a greater antibody response in cattle but this is of low specificity. Slightly less IgG response was detected using SO antigens rather than ES products in experimentally infected sheep [168]. Moreover, both antibody titres (peaked 60 days p.i.) were no correlated to the parasitic burden in this experiment. The antibody response in the bile of naturally infected cattle with worm burdens ranging from 120 to 280 was characterized using ELISA, and it was seen that IgA, IgM and IgG1 isotypes were predominant and there was little IgG2 reactivity [177]. Similarly not evident IgG2 reactivity was seen in Western blots of sera from the naturally infected cattle [178]. In this study immunoblots were positive for IgG1 and IgM antibodies and the IgG1 antibody response to proteins at 86 kDa was particularly prominent. The profile of reactive proteins seen in naturally infected sheep was in the range of 130 kDa [179]. Differences between cattle and sheep humoral responses to trematode infections provide a possible explanation.

As was reported 43.5 % of the 26 calves studied had *D. dendriticum* eggs present in their faeces and immunoblots performed with sera from these animals were all positive for antibodies [178]. All of these calves were serologically positive despite more than half being coprologically negative, showing the effectiveness of ELISA using E/S products for the diagnosis of dicrocoeliosis in cattle. Moreover, seropositivity in sheep was detected by indirect-ELISA in 86.2 % tested animals, whereas faecal prevalence was 6.7 %; all those faecal-positive were also ELISA-positive [146]. This fact (high percentage of sheep positive to the ELISA test and negative to egg-output) may indicate that the migratory phase of *D. dendriticum* was occurring in these sheep but could also indicate previous exposure to the parasite without current infection [146].

The indirect-ELISA using excretory-secretory antigens from *D. dendriticum* to evaluate humoral immune responses was accurate in detecting infected cattle and sheep [168, 178]. With this method *D. dendriticum* infection can be detected in experimental lambs as early as 30 days after infection, although the maximum level

of antibodies was obtained 60 days p.i., after which it decreased slightly but remained high until the end of the experiment 180 days p.i. [168].

To avoid the lack of indirect ELISA technique specificity, in order to be sure that the infection is active, it would be very important to develop a direct ELISA sandwich technique to detect *D. dendriticum* antigens in the faeces as well as the serum of infected animals. Nevertheless, to develop this technique first the *D. dendriticum* specific antigens must be identified, isolated, purified and characterized [179]. These antigens could also serve as vaccine if they can be shown to protect the animals against the parasite [180].

Recently, a new method to hatch *D. dendriticum* eggs, obtain the miracidium DNA and develop a PCR- molecular tool for detecting the presence of *D. dendriticum* eggs in the animal faeces was developed [41]. Moreover, some studies on morphological [18] and molecular detection by PCR [54] of *D. dendriticum* larval stages in molluscs and ants, first and second intermediate hosts of this parasite, have been carried out. On the other hand, studies on the adult parasite have been carried out concerning: isoenzymatic characterization [181], genetic variability [182, 183], molecular identification using the partial sequencing of 18S rDNA and the internal transcribed spacer nuclear (ITS-2) of ribosomal DNA [88], the 28S and ITS-2 [81] and the ITS-2 [184]. The interspecific variations comparing *D. dendriticum* and *D. hospes* [81] and *D. dendriticum* and *D. chinensis* [88] have been shown.

12.7 Treatment, Control and Prevention of Dicrocoeliosis

Applying prophylactic and control measures to dicrocoeliosis caused by *D. dendriticum* is difficult due to the complexity of its biological life cycle, the low specificity of the parasite with reference to the numerous species of mammals, molluscs and ants, definitive and intermediate hosts, as well as the complexity of its epidemiology.

As there is no vaccine against *D. dendriticum*, the most effective control method is to administer an efficacious anthelminthic to the ruminants, taking into account the dicrocoeliosis epidemiological model in a specific area [15, 18, 90].

Although various anthelminthics have been used against *D. dendriticum* (mainly in ovine) by different authors, like: Albendazole (ABZ), fenbendazole, luxabendazole, thiophanate, netobimin and diamphenethide, among others, nevertheless none are effective against the juvenile and immature stages of *D. dendriticum*, or if they are effective, as is diamphenethide, the dose must be high (240 mg/kg: 93–95 % efficacy) and serious side effects appear after administration [185]. This lack of effectiveness against juveniles must be taken into account when applying a strategic control [90].

One of the more common and important anthelminthics used to control *D. dendriticum* infection is ABZ [143]. This drug is a benzimidazole compound, possessing high activity against the lancet fluke. In the host ABZ is rapidly oxidized to ABZ sulphoxide (ABZ.SO), an active metabolite present in the blood stream. Passive transport is the main mechanism for the entry of benzimidazole anthelminthics

(ABZ and ABZ.SO) through the trematode tegument. However, active ABZ uptake and active efflux of ABZ and ABZ.SO also participate in the transport of these drugs in *D. dendriticum* [143]. In mouflons with dicrocoeliosis there is a significant increase in thiobenzamide-*S*-oxidase (TBSO) activity, mainly ascribed to flavine monooxygenases (FMO), and a significant decrease in glutathione-*S*-transferase (GST) has been documented [142]. Some substances of parasitic excreta or inflammation mediators may cause these changes in the expression of these liver biotransformation enzymes. In this study dicrocoeliosis in mouflons significantly increased ABZ.SO formation, probably because of the enhancement of FMO activity but only caused mild changes in ABZ hepatic biotransformation, so the use of ABZ in the therapy of dicrocoeliosis can be recommended.

ABZ administered at a single dose of 10 or 15 mg/kg per os decreased parasitic burden by 92.22 % [186] and by 99.6 % [187], respectively, in sheep naturally infected with *D. dendriticum*. A 94.05 % reduction in *D. dendriticum* egg shedding was obtained with two doses of 10 mg/kg, 7 days apart [186]. When the ABZ treatment was administered to sheep as intraruminal boluses (dose 42 mg/day), an efficacy of 91.89 % was obtained [188]. Moreover efficacies from 92.9 to 94 % were also obtained with doses of 15 and 20 mg/kg of albendazole in ovine in Germany [189]. Ewes and ewe lambs treated with ABZ at an oral dose rate of 15 mg/kg (three times that recommended for the treatment of gastrointestinal nematode parasites) achieved a 79.2 % reduction in *D. dendriticum* egg count 21 days post-treatment [149].

Orally administered thiophanate treatment in ovine produced 100 % efficacy with a 50 mg/kg [190] and a 200 mg/kg [191] dose. Other authors [192] recorded efficacies above 99 % using doses of 50, 100 and 200 mg/kg.

When a dose of 10 mg/kg of luxabendazole was administered to sheep, the worm reduction was 59 % and 99.1 % according to different authors [189, 193]. Moreover, doses of 10 and 12.5 mg/kg reduced 63.2 % and 83.3 % of worms, respectively [194].

The efficacy of netobimin administered at doses of 20 mg/kg was 98.8 %, 97.9 % and 89.1 %, respectively, according to some authors [189, 195, 196]. When a dose of 15 mg/kg was administered to sheep [197] the efficacy obtained was 91.9 %. On the other hand, the efficacy of mebendazole was 93–99.4 % when a dose of 40–80 mg/kg was administered [198]. The efficacy of fenbendazole varied from 99.9 to 100 % when a dose of 100 mg/kg was administered [185]. In an experiment carried out with a 50 mg/kg dose of praziquantel efficacies from 89 to 98 % were obtained [144].

Taking into account the approach to dicrocoeliosis epidemiology described in Sect. 12.4, as well as in previous studies carried out in the North West of Spain [15, 18, 118] together with an experiment on strategic control of *D. dendriticum* egg excretion in sheep carried out using an anthelminthic (ABZ) only effective against adult parasites [90], the following conclusion can be reached: In a zone with a continental climate within the Mediterranean Atlantic transition, the most effective control model consists of applying two treatments: One at the beginning of November (when ant hibernation starts) to eliminate the adult worms, and another treatment in January when, without reinfection (because ant hibernation does not finish until about the end of March), most of the metacercariae ingested by the

animals until November have become adults capable of shedding eggs. By applying these two treatments, the biggest reduction in egg excretion—mainly in the cold period when elimination is highest and egg survival greatest—is obtained [90]. Nevertheless, due to the complexity of dicrocoeliosis epidemiology, strategic treatments need to be repeated over several years to reduce the parasite load in ruminants, the contamination of pastures with viable parasite eggs, and the infection rate in the mollusc and ant intermediate hosts.

Other authors also carried out their studies on the efficacy of some benzimidazoles against *D. dendriticum* (in Germany) in winter, mainly to research their effect on mature worms [189].

Restrictive husbandry practices like no grazing early in the morning or late in the evening when the highest number of ants in tetany is present in the herbage may be is an option for dicrocoeliosis control. Methods against the intermediate host snails (molluscicide) and ants are not feasible due to their cost and for ecological reasons [14].

12.8 Economic Impact of Dicrocoeliosis

The economic and health significance of dicrocoeliosis is partly due to the direct looses occasioned by the confiscation of altered livers [21, 199, 200] and also the indirect ones caused by the digestive disorders derived from the hepatobiliary alterations caused by these parasites, such as decreased animal weight [40, 117], growth delay [59], reduced milk production [201], amongst others. Moreover, the additional costs incurred by the application of anthelminthic treatments, to which the animals must be subjected, have to be considered.

12.9 Other Genera of Interest

12.9.1 Genus Eurytrema: Eurytrematodosis

Dicrocoeliidae flukes of the genus *Eurytrema* Looss, 1907, responsible of mammal Eurytrematodosis, are common parasites which adults live in bile ducts, gall bladder, pancreatic ducts and intestine of cattle, buffaloes, camels, deer, goats, sheep, pigs and humans beings from Europe, Madagascar, Asia and South America [1, 11, 202, 203]. These parasites use in their life cycle land snails (genus *Bradybaena*) and grasshoppers (genus *Conocephalus*) as first and second intermediate hosts, respectively [11]. The most important species of the genus is the type-species *Eurytrema pancreaticum*. Moreover, *Eurytrema coelomaticum* is also common in Brazilian cattle [203] and in China ruminants [204], as well as *Eurytrema procyonis* in raccoons in the USA [204]. In the final host metacercariae de-encyst in the duodenum and migrate to the pancreas. Infection of the pancreatic ducts leads to chronic interstitial pancreatitis [204].

The infection prevalence by *E. coelomaticum* in cattle was 47.8 % in Brazil [205]. Progressive emaciation was the most common clinical sign and high plasma amylase concentration has been observed suggesting exocrine pancreatic insufficiency [203]. Grossly there were multiple dilated pancreatic ducts (mainly in the left pancreatic lobe for *E. pancreaticum*) with thickened whitish walls containing trematodes. Histologically extensive pancreatic parenchymal loss with replacement fibrosis, ectatic ducts with hyperplastic epithelium and fibrosis were found [204]. A granulomatous inflammatory reaction around the trematode eggs was also observed [203]. In sheep severe parasitism by *E. pancreaticum* was characterized by progressive weight loss, chronic pancreatitis, fibrosis and atrophy of the pancreatic parenchyma [202]. Marked glucosuria and diabetes mellitus have been described in cases of eurytrematosis in sheep [202].

12.9.2 Genus Platynosomum: Platynosomiosis

The adult parasites of genus *Platynosomum* Looss, 1907 (Dicrocoeliidae), responsible for platynosomiosis in birds and mammals in Europe, Africa, Asia, North, Central and South America, live in the liver, gall bladder and pancreas of these animals. Known life-cycles of *Platynosomum* type use land molluscs, arthropods as second and lizards as paratenic (or third intermediate) hosts [1].

Platynosomum fastosum has been detected in the liver and bile ducts of domestic cats in Malaysia, as well as in Central and South America, the Caribbean and southern USA [11]. This parasite evolves in snails (*Subulina octona*) and lizards (*Anolis cristatellus*). The cats become infected by eating metacercariae from naturally infected lizards. The metacercariae migrate from the common bile duct to the gall bladder and bile ducts, where they develop into adult trematodes [206]. The adults are characterized by: ellipsoidal, flattened and brownish bodies, 2–6 mm long and 0.5–2 mm wide, with the anterior end rounded and the posterior end tapered; thin smooth cuticle; oral sucker; acetabulum in the anterior half of the body; short oesophagus; and small intestinal caeca; testicles symmetrical; ovary located under the right testicle; uterus, in the posterior part of the body, contained small dark brown eggs; bilaterally vitelline glands located in the middle third of the body [207]. In cats the disease is generally asymptomatic but in some cases this trematode causes vomiting, diarrhoea and jaundice [11]. Dilation and thickening of the gall bladder wall, common bile duct and bile ducts were macroscopic findings associated with the presence of this parasite in cats [207, 208]. Histologically a non-suppurative cholangiohepatitis, characterized by bile duct fibrosis, hyperplasia of the ductal epithelium and infiltrate (mainly lymphocytes and some macrophages) has been observed [207, 208]. A relationship between the *P. fastosum* infection and the appearance of cholangiocarcinomas has been reported in cats [207]. The control measures include faecal screening, controlling the feral cat population and incorporating a trematode treatment drug into a parasite control programme [209]. This parasite has also been diagnosed in orangutans (*Pongo pygmaeus*) with clinical signs of inappetence and chronic diarrhoea and lesions in bile ducts and liver tissue [209].

The species *Platynosomum proxillicens* caused bile duct hyperplasia and fibrosis as well as hepatic inflammation in cockatoos (*Cacatua sulfurea*) [210]. Moreover, *P. ariestis* is a non-pathogenic Dicrocoeliidae found in the intestine of sheep.

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Chapter 13
Selected Wildlife Trematodiasis

Jane E. Huffman

13.1 Introduction

Many digenetic trematodes parasitize wild birds and mammals. The majority are not associated with significant disease conditions. The effects of trematodes on individual animals are well documented in the literature which increases substantially each year. The impact of trematodes on populations is, however, poorly understood. The effect adult trematodes might have upon their hosts is often difficult to predict. Considerable variation exists between genera and species occupying similar habitats in the host and even within a single host–parasite relationship. Many factors influence the final picture, but the precise location of the parasite within the host and its method of feeding are of particular importance. Chowdhury and Aguirre [1] compiled information on the most important helminth parasites and their diseases worldwide. Their book emphasizes the helminthological fauna of mammal species that are economically important and that represent a priority for conservation. Kumar [2] compiled information on the distribution, pathogenicity, diagnosis, treatment, and control of trematode infections and diseases in man and animals, including zoonotic trematodiasis. Toledo et al. [3] examined the significant literature on the immunology and pathology of intestinal trematodes in their definitive hosts. They emphasized information on selected species in six families for which the literature on these topics is extensive. The families were Brachylaimidae, Diplostomidae, Echinostomatidae, Gymnophallidae, Heterophyidae, and Paramphistomidae. Huffman [4] reported on the trematodes of birds in *Parasitic Diseases of Wild Birds*. Travassos et al. [5] published a landmark review article about Brazilian trematodes. Yamaguti [6] provided a synopsis of digenetic trematodes of vertebrates. Robinson and Dalton [7] reported

J.E. Huffman (✉)
Northeast Wildlife DNA Laboratory, Department of Biological Sciences,
East Stroudsburg University, East Stroudsburg, PA 18301, USA
e-mail: jhuffman@po-box.esu.edu

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on zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Acha and Szyfres [8] reported on *Zoonoses and Communicable Diseases Common to Man and Animals* in which they covered gastrodiscoidiasis, heterophysiasis, nanophyetiasis, opisthorchiasis, paragonimiasis, and schistosomiasis. The present chapter summarizes the information on trematodes of interest in some groups of mammals. Table 13.1 summarizes the principal animals involved, area of endemicity, location in host, and clinical manifestations from the trematode families Brachycladiidae, Brauninidae, Campulidae, Dicrocoeliidae, Diplostomatidae, Fascioloidea, Gastrothylacidae, Heterophyidae, Nasatrematidae, Neodiplostomidae, Notocotylidae, Opisthorchiidae, Paramphistomidae, Prouterinidae, and Troglotrematidae.

13.2 Cetaceans and Pinnipeds

The order Cetacea contains whales, dolphins, and porpoises. The order is divided into two suborders, Mysticeti (baleen whales) and Odontoceti (toothed whales, which includes dolphins and porpoises). Additionally, there are five species of freshwater dolphins. The pinnipeds comprise the families Odobenidae (walruses), Otariidae (sea lions, eared seals, and fur seals), and Phocidae (true seals). Trematode infections are common in cetaceans and pinnipeds. Forrester [9] summarized records of helminths and other parasites in 12 species of odontocetes from Florida, including dwarf sperm whales. Trematodes that infect the liver, biliary system, and pancreas in pinnipeds consist of two families (Campulidae, and Opistorchiidae) and the genera *Cyclorchis*, *Orthosplanchnus*, *Zalophotrema*, *Opistorchis*, *Metorchis*, and *Pseudamphistomum*.

Cyclorchis campula (*Campula oblonga*) primarily inhabits the bile and pancreatic ducts of cetaceans. This parasite causes extensive irritation of the ducts, with hyperplasia of the ductal epithelium and fibroplasia developing around the ducts. This chronic irritation may progress to a chronic fibrosing hepatitis and pancreatitis [10, 11]. Migaki et al. [11] described hepatic trematodiasis caused by *C. campula* in a juvenile Ganges River dolphin that had been in captivity at an aquarium for approximately 1 year. Histopathologic findings were severe chronic suppurative cholangitis, hyperplasia of the bile duct epithelium, and periductal fibrosis associated with fluke infection of the large bile ducts.

Cyclorchis campula has numerous prominent tegumental spines. This trematode has characteristic small yellow triangular eggs. Infection may lead to weight loss, decreased liver function, and predisposition to bacterial disease in chronic cases, or hepatic trauma due to migration of worms resulting in hepatitis, and death in acute cases [12].

Zalophotrema hepaticum from the California sea lion, harbor seal, and northern elephant seal is the most commonly reported trematode infecting the liver. Prevalence increases with age in California sea lions, and many older animals have thickened biliary ducts, probably as a consequence of infection [13, 14].

Table 13.1 The principal animals involved, area of endemicity, location in host, and clinical manifestations from some trematode families

Family	Trematode	Principal animals involved	Area of endemicity	Location in the host	Clinical manifestations
Brachycladiidae	*Hunterotrema caballeroi*	Amazon River dolphin	South America	Lungs	Bronchial obstruction, bronchiectasis, and atelectasis
Brauninidae	*Braunina cordiformis*	Atlantic bottlenose dolphin	Atlantic	Stomach	Irritation to the gastric mucosa
Campulidae	*Cyclorchis campula* (*Campula oblonga*)	Harbor and Dall's porpoises	Pacific	Bile ducts, liver	Fibrosis and hyperplasia of the epithelial lining of the bile ducts, small white nodules, less than 1 cm in diameter can be found on the surface of infected livers
	Orthosplanchnus arcticus	Sea otter	Pacific	Gall bladder	Mucosal hyperplasia and chronic inflammation
	Synthesium tursionis	Tucuxi dolphin	South and southeastern coast of Brazil	Intestine	Unknown
	Zalophotrema hepaticun	California sea lion, harbor seal, and northern elephant seal	Pacific and Atlantic Ocean	Liver	Causes biliary hypertrophy and fibrosis of the liver, signs are usually seen in adults and include icterus, lethargy, and anorexia, bilirubinemia, and increased serum hepatic enzymes are common
Dicrocoeliidae	*Eurytrema procyonis*	Raccoons, red and gray foxes	North America	Pancreas, pancreatic ducts	Usually none, it has been noted that in some cats with hundreds of worms, the pancreatic duct becomes hardened and thickened and this may lead to pancreatic insufficiency
	Eurytrema pancreaticum	Sika deer, red deer, tufted deer	China, Japan	Pancreatic duct	Pancreatitis
	Platynosomum concinnum	Felids	North and South America	Liver	Enlargement of the gallbladder and biliary ducts, with leukocyte infiltration, adenomatous hyperplasia, and fibrosis of the ductal areas
	Dicrocoelium dendriticum	Deer, moose, woodchucks, rabbits	North America	Biliary passages	Periportal fibrosis

(continued)

Table 13.1 (continued)

Family	Trematode	Principal animals involved	Area of endemicity	Location in the host	Clinical manifestations
Diplostomatidae	*Alaria arisaemoides*	Red fox	Western North America	Small intestine	Mucosal inflammation
	Alaria marcianae	Foxes, wolverine, wolf, coyote, lynx, puma, bobcat	Alaska, Canada	Lungs, small intestine	Catarrhal duodenitis
Fascioloidea	*Fasciola hepatica*	Deer, squirrel, beaver, nutria, marsupials	North America, cosmopolitan, Australia	Liver, bile ducts and gallbladder	Weakness, anemia, internal hemorrhage, diarrhea, muscular edema, emaciation, prostration, severe liver damage, cirrhosis, calcification of bile ducts, fibrinous pleuritis, toxemia
	Fascioloides magna	White-tailed deer, native and exotic cervids, elk, moose	North America	Liver, bile ducts and gall bladder, lungs	Not pathogenic for white-tailed deer, in other species hepatic fibrosis and destruction; massive hemorrhage, fibrinous peritonitis; thick-walled cysts and abscesses with brown to black fluid; black pigment in mesentery
	Fasciola jacksoni (*Fascioloides jacksoni*)	Elephants, wild boar	Pakistan, India, Sri Lanka, Burma	Bile ducts	Hemorrhagic tracks, fibrosis/cirrhosis of liver; pseudolobulation, proliferative/hyperplastic changes in the bile duct; desquamated cells in bronchial lumen mixed with fibro-hemorrhagic exudate; clinically resembles ovine fascioliasis with dropsy and mud-eating sometimes; death in heavy infections
	Fasciolopsis buski	Wild boar	China	Small intestine	Enteritis, intestinal obstruction
	Parafasciolopsis fasciolaemorpha	Elk, moose, roe deer	Russia, eastern and southern Europe	Liver, bile ducts	Thickening and hardening of the walls of bile ducts, walls turn yellow-white; ducts are blocked and strained with the formation of cavities filled with the trematode, their eggs, and disintegrated products of hepatic tissue; a capsule of connective tissue is formed around the cavities
Gastrothylacidae	*Fischoederuius sp.*	Elk, moose, carobou, western roe deer	Russia, India	Rumen and reticulum	Edema, anorexia, and diarrhea

Heterophyidae	*Ascocotyle patagoniensis*	South American sea lion	Patagonia, Argentina	Intestine	Unknown
	Cryptocotyle lingua *C. jejuna*	Mink, seals	North America	Small intestine	Enteritis, degeneration of epithelium, hemorrhagic erosions
	Nanophyetus salmincola synonym *Troglotrema salmincola*	Raccoons, foxes, skunks, and other fish-eating mammals	Pacific northwest of the USA, Siberia	Small intestine	Mild gastroenteritis
	Pholeter gastrophilus	Harbor porpoises		Pyloric stomach, small intestine	Trematode buries deep in the mucosa, forming small, black, cavity nodules that can be identified on palpation
Nasitrematidae	*Nasitrema globicephalae*	Cetaceans		Air sinuses, brain	*Nasitrema* sp. normally reside in the air sinuses; aberrant migration of *Nasitrema* occurs directly from the air sinus–inner ear complex to the subdural space found in the nasal passages and sinuses of cetaceans. Ova of these trematodes have been associated with necrotic foci in the brains of animals showing behavioral aberrations and have been incriminated as a cause of localized pneumonia in cetaceans. Infections are often accompanied by halitosis and brown mucus around the blowhole and occasionally by coughing
Neodiplostomidae	*Pharyngostomum cordatum*	Tiger, wild cat	India	Small intestine	Destruction of mucous membrane and muscular coat
Notocotylidae	*Quinqueserialis floridensis*	Florida	Round-tailed muskrat	Not reported	Not reported
	Ogmocotyle indica	Lesser panda	China	Small intestine, bile duct	Enteritis

(continued)

Table 13.1 (continued)

Family	Trematode	Principal animals involved	Area of endemicity	Location in the host	Clinical manifestations
Opisthorchiidae	*Metorchis conjunctus* *M. albidus*	Foxes, raccoons, muskrats, mink, other fish-eating mammals	North temperate regions	Liver, gallbladder, bile ducts	Seldom cause any recognizable clinical signs
	Opisthorchis felineus	Foxes, seals, other fish-eating mammals are definitive hosts	Europe	Bile duct, gall bladder, liver	Acute febrile illness with arthralgia Lymphadenopathy, skin rash; suppurative Cholangitis and liver abscess in subacute, chronic stages; possible increased risk of cholangiocarcinoma
	O. viverrini	Fish-eating mammals	Southeast Asia	Bile ducts	Upper abdominal pain, diarrhea, fever, jaundice possible acutely; chronic infections with cirrhosis, pancreatitis, high incidence of cholangiocarcinoma
	Amphimerus pseudofelineus	Coyotes, opossums	The USA, Canada, Central and South America	Liver	Pancreatitis
	Pseudamphistomum truncata	American mink, wolf, red fox, raccoon dog, ferret, otter, shrews, Caspian seal	Europe, Ireland	Liver, gallbladder, bile ducts	

Paramphistomidae	*Zygocotyle lunata*	North America	Deer, moose	Small intestine	Unknown
	Gastrodiscoides hominis	India, Malaya, Java	Wild boar, Napu mouse deer, field rat, rhesus monkey	colon	Inflammation, mucoid diarrhea
	Paramphistomium cervi	North America, Australia, Asia, Africa, eastern Europe, and Russia	Deer, moose, caribou	Abomasums, rumen, small intestine	Profuse diarrhea, anemia, and lethargy
Prouterinidae	*Prouterina wescotti*	Black bear	Idaho	Nasal sinuses, brain, lungs	Emaciation, weakness, nasal discharge
Troglotrematidae	*Paragonimus kellicotti*	Mink, muskrat, raccoon, fox, bobcat, weasel, wolf, muskrat	Southern Canada, eastern US	Lungs, peritoneal/ pleural cavities	Intermittent cough, lethargy, chronic bronchiolitis, chronic eosinophilic granulomatous pneumonia
	P. peruvianus (*mexicanus*)	American opossum, four-eyed opossum, Jaguarundi, ocelot, opossum, puma, tayra	Mexico, Central America, Peru, Brazil	Lungs	Intermittent cough, lethargy, chronic bronchiolitis, chronic eosinophilic granulomatous pneumonia

Meningoencephalitis caused by aberrant trematode migration was described in two California sea lions (*Zalophus californianus*) admitted to a rehabilitation hospital. Both animals displayed seizure activity and were euthanized due to poor response to therapy. Gross abnormal findings included liver flukes (*Zalophotrema hepaticum*) in the bile ducts and areas of swelling and necrosis in the cerebrum, cerebellum, and brain stem. Histopathology revealed meningoencephalitis with necrosis, hemorrhage, and many trematode eggs within the brain. In one sea lion, an adult trematode was found on the surface of the cerebrum [15].

Braunina cordiformis is usually observed in the second chamber of the stomach of Atlantic bottle-nosed dolphins. It causes minimal damage and irritation to the gastric mucosa. The fluke, when attached to the gastric mucosa, has a characteristic urn-shaped appearance in the lumen of the stomach [16].

Hunterotrema caballeroi is a rather large fluke (up to 25 cm long) observed in the Amazon River dolphin. This parasite causes a mild to moderate mucoid exudate in the bronchi of these animals. However, it has been incriminated in bronchial obstruction, bronchiectasis, and lung closure resulting in reduced or absent gas exchange [17]. *Cetitrema meadi* sp. n. a liver fluke from Gervais' beaked whale (*Mesoplodon europaeus*) was reported [18].

Pholeter gastrophilus is observed in the second chamber of the stomach of dolphins. These flukes have prominent spines on the tegument and yellow, single operculated eggs. This parasite buries deep in the submucosa, forming prominent small black cavitary nodules that can be identified on palpation. The mucosa usually remains intact over these parasitic nodules. The nodules have abundant fibrous connective tissue surrounding the parasite with a variable granulomatous and eosinophilic inflammatory reaction [14]. Jaber et al. [19] reported on the pathological and immunohistochemical study of gastrointestinal lesions in dolphins stranded in the Canary Islands. The most common lesion was chronic granulomatous gastritis of the glandular stomach, associated with the parasite *Pholeter gastrophilus*, and characterized by the parasites, their eggs, or parasite debris in the mucosa, submucosa, or tunica muscularis, surrounded by numerous lysozyme-positive macrophages and neutrophils, and more peripherally by abundant fibrous tissue containing variable numbers of immunoglobulin (Ig) G+ plasma cells, and small numbers of CD3+ T lymphocytes and IgM+ and IgA+ plasma cells.

Nasitrema species are common flukes located in the head sinuses of many porpoises, dolphins, and toothed whales. These flukes range in size from 9 to 12 mm (*Nasitrema stenosomum*) to 28 to 35 mm (*Nasitrema gondo*). This parasite is normally found in the submucosal glands of these sinuses. Occasionally this fluke is observed in the middle ear. This parasite rarely causes problems; however, these flukes will occasionally migrate to the brain and cause serious central nervous system damage. Lesions caused by this fluke usually contain numerous small yellow triangulated fluke eggs. Numerous strandings have been associated with parasitic migration of this parasite into the brain. The life cycle is unknown [20].

*Nasitrema globicephala*e and other species of that genus inhabit the head and air sinuses of small odontocetes. Eggs of *Nasitrema* have been associated with necrotic foci in the brains of animals showing behavioral aberrations (loss of equilibrium,

and generalized central nervous signs) and have been incriminated as a cause of localized pneumonia in cetaceans. Infections are often accompanied by halitosis and brown mucus around the blowhole and occasionally by coughing. Morimitsu et al. [21] reported that *Nasitrema* was the cause of parasitogenic eighth cranial neuropathy in a stranding of Risso's dolphins. *Nasitrema* has also been reported to cause encephalitis in a striped dolphin [22], and a cerebral necrosis in stranded common dolphins (*Delphinus delphis*) [23, 24]. It should be noted that *Nasitrema* is not responsible for all or even most strandings. While species of *Nasitrema* are frequently found associated with stranded dolphins and porpoises, the actual role they play in contributing to that outcome is still uncertain within the context of other factors.

Diagnosis of *N. globicephalae* is based on demonstration of typical operculated trematode eggs in blowhole swabs or feces. The adults of the trematode in the nasal sinuses and posterior nasal passage of the dolphins are considered harmless for the host, but their eggs, aspirated deep into the bronchial tree, may initiate a foreign-body inflammatory reaction in the lungs, and continuous aspiration of such eggs may provoke a chronic pneumonia condition [25].

Dailey et al. [26] reported a new species of eye fluke *Philophthalmus zalophi* from the Galápagos sea lion (*Zalophus wollebaeki*). Infections with *P. zalophi* were found only in sea lions from 3 to 8 months of age. Young sea lions are very oral during this period of their development, and they were observed mouthing rocks and shells from the bottom of the pools that contained infected sea lions.

Hernandez-Orts et al. [27] described a new heterophyid species, *Ascocotyle patagoniensis* n. sp., based on specimens collected from the intestines of the South American sea lion *Otaria flavescens* from Patagonia (Argentina). *Ascocotyle* usually infect fish-eating birds or mammals in freshwater or brackish habitats. *Ascocotyle patagoniensis* n. sp. is the first species of the subgenus described from a marine mammal. The life cycle of the marine species from the *Ascocotyle*-complex infecting pinnipeds is unknown [27].

Bishop [28] reported on parasite-related lesions in a bearded seal, *Erignathus barbatus*. Significant findings included diffuse intrahepatic bile duct fibrosis and chronic cholangitis; multiple nodules of chronic fibrosing pancreatitis, and gastric ulcers. The pancreas was firm and contained numerous firm, gray, finely lobulated nodules up to 1 cm in diameter. Many nodules contained large numbers of yellow-walled trematode eggs with a triangular or pyriform outline and a single operculum. The eggs were surrounded by fibrous tissue and acute and chronic inflammation; many degenerate eggs were infiltrated by multinucleated giant cells. The nodules also contained atrophic acini and exocrine ducts with hyperplastic epithelium. Although specific identification of the cause of the pancreatic and liver lesions in the seal was not determined, Bishop [28] reported that the lesions are most likely the result of parasitism by either *Orthosplanchmus fraterculus* or *O. arcticus*. Although no lesions have been associated with these parasites in the bearded seal, *O. fraterculus* produces mucosal hyperplasia and chronic inflammation of the gall bladder of the sea otter. The same species has been found in both gall bladder and pancreatic ducts of bearded seals [29–31].

13.3 Sirenia

This order is composed of manatees and dugongs. These species are fully aquatic, herbivorous mammals that can adapt to freshwater, brackish, and marine environments. The predominant parasites of sirenians are monostome trematodes (except for one species, *Nudacotyle undicola*), and they are exclusive to sirenian [32]. Nine genera (*Chiorchis*, *Indosolenorchis*, *Lankatrema*, *Lankatremoides*, *Rhabdiopoeus*, *Schizamphistoma*, *Solenorchis*, *Taprobonella*, and *Zygocotyle*) may inhabit the stomach, pyloric cecum, and intestine in large numbers. *Lankatrema* can produce lesions in the stomach and form cystic cavities in the mucosa. *Opisthotrema*, *Chocleotrema*, and *Pulmonicola* inhabit the nasal passages, Eustachian tubes, airways, and lungs, and a fourth genus *Labicola* occurs in the upper lip. Diagnosis is by detection of eggs with polar filaments in feces or sputum.

13.4 Herbivores (Cervids/Elephants/Bovids)

The giant liver fluke, *Fascioloides magna*, is of interest to wildlife managers, veterinarians, and researchers, due to its unusual body size (3–10 cm), high pathogenic potential and because it is continuously spreading to new areas. The trematode is frequently found in the liver of deer and is known as the large American liver fluke. They are purple-gray in color, and when found while cutting open or slicing deer liver, they resemble a blood clot. They are frequently surrounded by a fibrous capsule, bathed in a dark, muddy-appearing fluid [33]. Deer will encapsulate mature flukes in the liver restricting their migration and hence damage. In deer, as with other cervidae, there is a favorable balance between the host and the parasite, resulting in minimal evidence of disease [34, 35].

Foreyt [36] infected mule deer (*Odocoileus hemionus*) and elk (*Cervus elaphus*) to determine if they could serve as experimental definitive hosts for *Fascioloides magna*. Three mule deer each inoculated with 50 survived the infection and shed eggs in feces; thus, mule deer can function as definitive hosts for *F. magna*. The other three mule deer inoculated with 50 ($n=1$) or 250 ($n=2$) metacercariae died from the trematode infection One elk calf inoculated with 2,000 metacercariae died from the infection 44 days after inoculation. The remaining three elk, each inoculated with 250 metacercariae, survived infection, and two of the three shed eggs in the feces.

The natural host of *F. magna* is the deer, which is also a reservoir host; it is enzootic in five major areas: (1) the Great Lakes; (2) Gulf coast, lower Mississippi, and southern Atlantic seaboard; (3) northern Pacific coast; (4) Rocky Mountain trench; and (5) northern Quebec and Labrador. Liver flukes have been found in moose in the Upper Peninsula of Michigan and in captive elk, but have not been reported in free-ranging elk. Some other states and Canadian provinces report the parasite in moose, elk, white-tailed deer, mule deer, bison, and yak. It has also been reported in red deer, fallow deer, and sambar deer in Europe. The fluke may infect cattle, sheep, and llama [37, 38].

Wobeser et al. [39] diagnosed *F. magna*, in two moose (*Alces alces*) and six wapiti (*Cervus elaphus*) from central Saskatchewan. Fecal samples collected from wild wapiti at five sites in the commercial forest zone in Saskatchewan contained eggs believed to be those of *F. magna*. Recently, *Fascioloides magna* infections have been considered to be the greatest source of mortality in a declining moose population in northwestern Minnesota [40]. The presence of the American liver fluke (*Fascioloides magna*) in Croatian wild ruminant species was detected by Janicki et al. [41].

Detection of antibodies provides a very useful tool to gain more knowledge about the distribution of liver trematodes. Nonlethal methods are strongly encouraged for the analysis of the risk of infection among wild ruminants. A seroepidemiological survey was conducted to analyze exposure to hepatic trematodes (*Fasciola hepatica* and *Dicrocoelium dendriticum*) in wild ruminants from southern Spain. The samples were analyzed using the excretory/secretory antigens of each trematode to determine the IgG response. The IgG-seroprevalence against *F. hepatica* was significantly higher in the cervids. Statistical differences according to gender were observed [42].

13.5 Elephants

Fasciola jacksoni (Cobbold, 1869) is a highly prevalent trematode colonizing the liver (less frequently the lungs, kidneys, pericardia, and intestines) of *Elephas maximus indicus* and *Elephas maximus maximus* in the Indomalayan region, causing cirrhosis, hemorrhages, and connective tissue proliferation. Severe submandibular and ventral abdominal edema was observed in an Asian elephant (*Elephas maximus maximus*) in which liver flukes (*Fasciola jacksoni*) were recovered from the bile ducts at postmortem examination. Examination of blood samples and serum from this elephant and another eight elephants showed that most had anemia and hypoproteinemia [43]. Heneberg [44] assessed the phylogenetic relationships of *Fasciola jacksoni* in relation to representative species of the superfamily Echinostomatoidea using four independent DNA regions. Reflecting the combined data, reclassification of *Fasciola jacksoni* as *Fascioloides jacksoni* comb. nov. was suggested by Henneberg [44].

13.6 Bovids

The water buffalo or domestic Asian water buffalo (*Bubalus bubalis*) is a large buffalo found on the Indian subcontinent to Vietnam and Peninsular Malaysia, and in Sri Lanka and Borneo. Although *Explanatum explanatum* (Creplin, 1847) has not been reported from the wild water buffalo (*Bubalus arnee*), it is important to consider the pathology of this parasite in the domestic water buffalo.

Haque et al. [45] reported on the infection of *Explanatum explanatum* (Creplin, 1847) in the liver of water buffalo (*Bubalus bubalis*). Macroscopic examination revealed massive infection of adult fluke in bile ducts and intrahepatic ductules in 131 (18 %) cases. The predominant features were multifocal granulomatous nodules throughout the luminal surface of the bile ducts. Histopathological study of tissue sections cut adjacent to and through the site of attachment of individual worm revealed intense infiltration of inflammatory cells such as lymphocytes, macrophages, plasma cells, eosinophils as well as fibrocytes. This was associated with fibrosis and thickening of the bile ducts. Due to high level of prevalence and intensity of natural infection, amphistomiasis appears to be endemic in this geographical region and probably represent one of the most important animal health problems. It is hoped that the study may draw attention to the need for educating farmers, regarding the economic importance of infection of these amphistome parasites and also for the development of control strategies to prevent the spread of infection to ruminants.

In 1924, *Fascioloides magna* was reported from two of an unspecified number of bison examined from Buffalo Park at Wainwright, Alberta, Canada [46]. *Fasciola hepatica* has been reported from one bison in Montana [47] and one bison in Wyoming [48].

Bison (*Bison bison*) were infected with known numbers of the metacercariae of *Fasciola hepatica* and *Fascioloides magna* to evaluate susceptibility and to document potential pathogenicity [49]. Foreyt and Drew [49] reported that bison are highly susceptible hosts for *Fasciola hepatica*. None of the bison inoculated with *Fascioloides magna* had flukes or lesions characteristic of fluke infection at necropsy. Bison inoculated with *Fasciola hepatica* had characteristic liver fluke lesions at necropsy. Results from the study by Foreyt and Drew [49] indicated that bison are susceptible to infection with *Fasciola hepatica* and are efficient definitive hosts. Because no *Fascioloides magna* were recovered in the study, bison may have a decreased susceptibility or innate resistance to *Fascioloides magna* infection, which may account for a lack of reported infections in this host.

13.7 Carnivores (Felids/Mustelids/Canids)

In the USA, the ocelot (*Leopardus pardalis*) is an endangered felid found only in a few remaining vestiges of native thornshrub brushland in the Lower Rio Grande Valley (LRGV) of extreme southern Texas. *Alaria marcianae* and *Brachylaima* sp. were identified from the ocelot in the LRGV. The helminth fauna of ocelots in the LRGV is reflective of that from wild felids in general; all have been reported previously from the bobcat (*Lynx rufus*) and mountain lion (*Puma concolor*) [50].

The genus *Paragonimus* is the only trematode genus with flukes that reside as adults in the lungs of marsupials, carnivores, and primates. *Paragonimus* species are extremely successful parasites and are widely geographically distributed, with *Paragonimus* species being endemic in Asia, the Americas, and Africa. *Paragonimus*

species have yet to be reported from Europe, Australia, and Antarctica [51]. Bech-Nielsen et al. [52] found *P. kellicotti* in 4.6 % (3/65) of cats with respiratory tract disease in Louisiana. Certain species have a limited geographic distribution, whereas others, such as *P. westermani*, are more widely distributed. The distribution of *P. westermani* ranges from Japan throughout Southeast Asia to India. For example, *P. miyazakii* is endemic in Japan and *P. heterotremus* is endemic in Thailand, but *P. westermani* occurs in both of these locations. *Paragonimus kellicotti* is the only *Paragonimus* species that is endemic to North America. *Paragonimus mexicanus* occurs in Central and South America. The distribution of *Paragonimus* species in part reflects the distribution of permissive animals that support infection. The major fauna of North America that support the life cycle of *Paragonimus kellicotti* include domestic animals such as dogs and cats, the wild animal hosts that support infection include skunks (*Mephitis mephitis*), red foxes (*Vulpes vulpes*), coyotes (*Canis latrans*), mink, (*Neovison vison*), and bobcats (*Felis rufus*). The disease has been found to be rare in raccoons (*Procyon lotor*) [53].

The occurrence of several spherical cyst-like structures approximately 1 cm in diameter and firm on palpation were described by Snyder et al. [54] on gross examination of the lungs of two bobcats. Blunt dissection of several cysts revealed two mature *Paragonimus kellicotti* per cyst. The pulmonary pleura overlying the cysts was smooth, opaque, and white to gray in color. The necrotic and cystic lesions appeared similar in both animals and were limited to the bronchi and peribronchial tissue. These lesions consisted of moderate to severe dilatations of the lumina with marked hypertrophy of mucosal epithelial cells in all the air passages. Within the lumina there were variable amounts of exudate that contained moderate numbers of inflammatory cells (predominantly neutrophils with lesser numbers of macrophages), copious amounts of mucus and red blood cells. Occasionally thick-shelled embryonated fluke eggs were seen within the lumina of air passages and in the pulmonary parenchyma. In a number of areas, the lumina of air passages were extremely dilated and distorted. In such areas the mucosal lining revealed moderate to severe diffuse squamous metaplasia. Within a few of such severely dilated lumina, cross sections of flukes (sometimes seen in pairs) were present. There were multifocal ulcerations of the metaplastic squamous mucosa in these areas. The peribronchiolar tissue associated with the more severely affected air passages was moderately to severely infiltrated with inflammatory cells in which macrophages and lymphocytes predominated. The latter also formed prominent lymphoid follicles around the air passages. The arteries around most of the airways revealed moderate to severe medial hypertrophy. Elsewhere in the lung the alveolar tissue appeared moderately thickened, and there was marked hypertrophy of the smooth muscle.

Descriptions of gross and histologic lesions in other wildlife infected with *P. kellicotti* [53, 55] indicate that the host reaction associated with the presence of this parasite in the pulmonary system of different definitive hosts is quite variable. In addition, differences in the numbers of flukes recovered and the average size of flukes vary with the species of definitive host that is infected [56].

Gross and histopathologic lesions have been described in the domestic cat (*Felis catus*) infected with *P. kellicotti* and are similar to those found in the bobcats [57].

Based on the prevalence, size of flukes recovered, and the minimal host reaction in the pulmonary tissue, it is thought that mink are the normal definitive hosts for *P. kellicotti* in North America.

Previous reports in the literature have not indicated any compromised pulmonary function or clinical signs associated with the presence of these parasites in bobcats. Most animals with paragonimiasis are asymptomatic; however, there may be a variety of clinical signs referable to the respiratory tract. Reichard et al. [58] reported on the lung parasites of bobcats from Alabama, Kansas, New Mexico, Oklahoma, and Virginia.

Platynosomum concinnum is a small fluke (6×2 mm) found in the bile and pancreatic ducts of Felidae in southeastern USA, Puerto Rico and other Caribbean Islands, South America, some of the Pacific islands, and parts of Africa. Its life cycle includes the snail *Sublima octona* and a crustacean (wood louse) as intermediate hosts and certain lizards as paratenic hosts. Cats acquire the parasite by feeding on infected lizards. In mild cases, nonspecific chronic signs of unthriftiness may be seen. Severe infections, however, may cause the "lizard poisoning" syndrome, which is characterized by anorexia, persistent vomiting, diarrhea, and jaundice, leading to death [59–61]. *Pharyngostomum cordatum* is a parasitic intestinal fluke of domestic and wild cats throughout Asia. The clinical presentation and pathogenesis is thought to be asymptomatic [62].

In sea otters, (also bearded seals) the gallbladder fluke, *Orthosplanchnus fraterculus*, causes cystic hyperplasia of the gallbladder mucosa. It also is believed to be responsible for causing a chronic fibrosing cholecystitis which is sometimes characterized by nodular thickening of the periductal and gallbladder connective tissue. This parasite does not appear to adversely affect the host. These flukes are very small, 1–3 mm in length. The eggs have the characteristic yellow color of fluke eggs and are triangular in appearance [63].

Mayer et al. [64] reported on the helminth parasites of the southern sea otter *Enhydra lutris nereis* in central California, the abundance, distribution and pathology. Three species of *Digenea* (*Microphallus pirum*, *M. nicolli*, *Plenosoma minimum*) were found in 47 % of the otters examined, at times in massive numbers (>3,000 per cm^2). Margolis et al. [65] reported on the helminths of sea otters from Prince William Sound.

All opisthorchiid species are thought to cause symptoms associated with obstruction of the biliary system [66]. *Opisthorchis viverrini* causes several hepatobiliary diseases in mammals [67]. *Pseudamphistomum truncatum* is a gall bladder digenean native to Eastern Europe and common in a range of wild carnivores. It was first reported in Britain following examination of otter carcasses. Otters examined postmortem showed abnormal gall bladders which were thickened, fibrous or inflamed. Simpson et al. [68] concluded that cholecystitis resulted from the presence of *P. truncatum* in mink and otters in southwest England. Sherrard-Smith et al. [69] reported two biliary parasites in UK otters. Infection with *P. truncatum* or *Metorchis albidus* appears to significantly damage the biliary system in these mustelids. Both *P. truncatum* and *M. albidus* are generalists, potential parasites of a wide range of carnivores, including the red fox [70], mink, and otters [68].

Lesions caused by the hepatic trematode, *Metorchis conjunctus* Cobbold, 1860, in five species of Carnivora, the dog, cat, raccoon, mink, and gray fox were compared by Mills and Hirth [71]. Marked hyperplasia of the mucosa of the intrahepatic bile ducts and gall bladder was found. The inflammatory reaction consisted principally of lymphocytes and plasma cells with a few eosinophils. Verminous granulomas were also present and induced a local eosinophilic response. With heavy infections, the raccoon developed severe cirrhosis, and osseous metaplasia was found in the bile ducts of one mink.

Troglotrema acutum inhabits the frontal and ethmoidal sinuses of foxes and mustelids in Europe. Flukes live in pairs in cysts in these sinuses. These parasites cause decalcification and atrophy of the bony walls of the sinuses and eventually result in perforation into the cranial cavity. Microorganisms enter the cranial vault, leading to fatal, purulent meningitis. No treatment is available. Ribas et al. [72] reported *Troglotrema acutum* from badger *Meles meles* skulls from Catalonia (northeastern Iberian Peninsula). They described the damage caused in the affected skulls, along with details regarding the use of computed tomography to detect hyperostosis, leakage in the sinus structure, and bone surface erosion in the affected skulls.

The reservoirs for *Nanophytes salmincola* are raccoons, mink, and skunks [73]. Because these flukes embed deeply between the villi of the intestine, infection with a large number may cause enteritis. Raccoons can naturally spread *N. salmincola* [74]. *Neorickettsia helmintheoca* is the etiological agent for salmon poisoning disease, found to be present in all stages of the trematode. It is 0.3 μm in size and Gram negative. Thus far, only canids are susceptible to disease by rickettsia and it is still uncertain how the rickettsia leaves the trematode vector and reaches the host tissues. Experiments do show that the bacteria lead to necrosis of lymph follicles, ulceration, and severe hemorrhage in its host [75].

Alaria alata, *A canis*, *A. americanum*, and other *Alaria* spp. are small (2–6 mm) flukes usually found in the small intestine of foxes, mink, and wild carnivores in the western hemisphere, as well as in Europe, Australia, and Japan. The anterior part of the body is flat, and the posterior part is conical. The eggs are oval, light brown, and fairly large (98–134 × 62–68 μm). Mesocercariae of *Alaria* are found in the tissues of frogs, and snakes. Canids become infected by feeding on these intermediate hosts. The young flukes migrate through various organs of the definitive host, including the diaphragm and lungs, before reaching the small intestine. Although the flukes are generally considered to be nonpathogenic, large numbers may cause pulmonary hemorrhages during migration or enteritis when they mature in the small intestine [76]. Shoop and Corkum [77] demonstrated that *A. marcianae* can be transmitted to the young mammals by drinking their mothers' infected milk.

Alaria alata is widely distributed in canids in Europe. The mesocercarial life cycle stage has recently been found in the paratenic wild boar host [78]. Over 500 foxes were examined during a wildlife survey for zoonotic diseases in 2009 and 2010. The prevalence of *A. alata* ranged from 21 to 26 % in 2009 and 2010. Spatial analysis suggests that *A. alata* may have a limited distribution being confined mainly to areas of pasture especially in the central plain and north Munster. Elevated prevalence indicated a clustering and that the level of parasitism was greatest in foxes from those areas where the prevalence of infection was highest [79].

Other species of flukes, usually not pathogenic, have been found occasionally in the intestine of carnivores; these include *Heterophyes heterophyes* in some north African and Asian countries; *Metagonimus yokogawai* in Asia; *Cryptocotyle lingua* in the USA, Canada, Japan, Siberia, and Europe; and *Apophallus donicum* in North America and eastern Europe. Their life cycles include snails as first intermediate hosts and fish as second intermediate hosts.

Eurytrema procyonis is a small fluke (2.1 × 1.0 mm) that commonly is seen in the pancreatic duct of raccoons in the USA. The eggs are medium sized (45–53 × 29–36 μm), and the life cycle involves a land snail and a second intermediate host that is thought to be an arthropod. It was first described in the raccoon pancreas by Denton [80] in Houston. Since then, reports of its occurrence in the pancreatic duct of foxes, cats, and other species have appeared. The primary habitat of these flukes is the medium pancreatic ducts, although they may simultaneously infect the biliary tract. Infections of the pancreatic duct causes distention, thickening of the ducts, and chronic interstitial pancreatitis, leading to periductal and acinar fibrosis. A fox infected with canine distemper virus had multiple *Eurytrema procyonis* trematodes within the major pancreatic duct. The ductal epithelium was slightly hyperplastic, and mild periductal fibrosis was present. There was dilatation of the pancreatic duct containing the parasites. Numerous eosinophilic intracytoplasmic inclusions were present in the epithelium of multiple organs, including the pancreatic ducts [81].

13.8 Ursids

Forrester [9] reported *Heterobilharzia americana* and *Pharyngostomoides procyonis*, from the American black bear. In each report, a single parasite of each species was detected in one bear. Millemann and Knapp [73] experimentally infected black bears with the salmon poisoning fluke, *Nanophyetus salmincola*, but infections in free ranging black bears have not been reported. *Nanophyetus salmonica* has been found in the Alaskan brown bear and the Asiatic black bear *Selenarctos tibetanus* [82].

Foster et al. [83] examined 22 Florida black bear (*Ursus americanus floridanus*) cubs (≤12 months-old) for endoparasites between 1998 and 2003. *Brachylaima virginianum* were found (11 flukes) in the small intestines of 1 of the 22 bears during the survey. *Alaria marcinae* was found in the small intestines of 2 of the 22 bears examined. Addison et al. [84] found *Alaria americana* (nine flukes) in one bear (1.2 % of 83 bears examined) during a survey of black bears in central Ontario, Canada, 1975–1977. This was probably an incidental infection due to ingestion of an infected mouse, frog, or snake. It was noted that *Alaria alata* had been reported once in a Yugoslavian brown bear. During a study of grizzly bears (*Ursus arctos*), Pence et al. [85] found *Echinostoma revolutum* in 2 of 66 bears (3 %) from Montana and Wyoming.

Foreyt et al. [86] described *Prouterina wescotti* from a free-ranging black bear (*Ursus americanus*) which died in May 1995 in northern Idaho (the USA).

The trematodes were detected in the brain, lungs, and nasal sinuses, and were likely responsible for the emaciated condition, copious nasal discharge, neurological signs, and death of the bear. *Prouterina wescotti* has been found only once, and its normal definitive host remains unknown [87].

A description is given of *Ogmocotyle ailuri* (Price, 1954) based on material recovered from a lesser panda, *Ailurus fulgens*, which died in the Zoological Park, Washington, DC *Ogmocotyle ailuri* closely resembles *O. indica* (Bhalerao, 1948), and the two species may eventually be found to be synonymous.

13.9 Marsupials

Trematode infections of most macropods are uncommon, no doubt being related to habitat and presence of the required intermediate hosts. Except for fascioliasis, reports of their causing disease are rare. Information on helminth parasites of macropods is found in reviews and papers cited in Ladds [88] as well as in other reviews and checklists [89–92]. Nine families of trematodes have been reported from Australian marsupials, Echinostomatidae, Paramphistomidae, Cathaemasiidae, Dicrocoeliidae, Fasciolidae, Diplostomidae, Brachylaimidae, Prosthogonimidae, and Hasstilesidae.

Macropods infected with *Fasciola hepatica* include kangaroos, wallabies, and pademelons. Infected macropods may have no clinical signs or may show severe cachexia and anemia. Even when large numbers of flukes are present, signs of illness may not be apparent. At necropsy, up to 80 flukes have been counted in red-necked wallabies.

Changes associated with these flukes have included severe distortion of the liver with excessive fibrosis, and cystic swelling of the bile ducts. Microscopic changes in fascioliasis clearly vary with host susceptibility or resistance. Mild to severe cholangiohepatitis is the essential lesion. Widespread multi-focal hemorrhage and necrosis of hepatic parenchyma due to migrating immature flukes may occur, along with subsequent scarring, and eosinophil infiltration around the bile ducts.

Cholangiohepatitis associated with the introduced common liver fluke (*Fasciola hepatica*) has been reported from numerous species of macropods in southeastern Australia and considered one of the most pathogenic parasites of marsupials [92, 93]. Cases of infection in macropods appear limited to the range of the intermediate host *Lymnaea tomentosa*. Infections have been reported in eastern gray kangaroos, red-necked wallibies, swamp wallabies, and Tasmanian pademelons [93, 94]. Species-specific differences in pathological responses to infection have been reported. Eastern gray kangaroos are considered tolerant of moderate infections although fatalities attributed to fascioliasis have been observed [95]. Hepatic fibrosis and severe anemia occur in Tasmanian pademelons and red-necked wallabies, with some infections being fatal. Pathological changes include fibrosis, bile duct hyperplasia and eosinophilic cholangiohepatitis [96].

Blair et al. [97] described *Macropotrema pertinax* gen. et sp. nov. (Digenea: Paramphistomidae) from the cecum of *Macropus agilis* (Gould, 1842) from northern Australia. At the point where the worm attaches to the caecal wall of the host, the entire mucosa is destroyed and there is an inflammatory cell infiltration in the intact mucosa surrounding the attachment site. Two paramphistomes, *Macroptrema pertinax*, and *Gemellicotyle wallabicola* may cause gastrointestinal lesions in the agile wallaby, but these are of a localized nature and seem to be of little consequence to health of the host [88]. Sandars [98] reported on the pancreatic fluke, *Zonorchis australiensis* sp. nov. (Trematoda), from Australian marsupials.

13.10 Rodents

The avian eye trematode *Philophthalmus lachrymosus* Braun, 1902 was reported from the Brazilian capybara. Previously, natural infections with *P. lachrymosus* and other species of *Philophthalmus* have been occasionally reported from man, with few data on experimental infections of nonhuman mammals. Clinical signs in the capybara were represented by ocular secretion, blindness, and emaciation. Gross lesions consisted of opacity of the cornea, anemic mucosa with adhered parasites; other lesions of the palpebral conjunctiva were represented by diffuse and multiple millimetric (about 3 mm) nodular bright-whitish formations together with a discrete congestive process. Parasites were generally located in the conjunctival inner and outer ocular canthus and in the inferior conjunctiva. Low worm burdens were mostly observed in the ocular canthus. The microscopic lesions consisted of papillar projections with squamous epithelial cells. In the center of the papillae, feeble vessels were observed. Frequently, trematodes were found attached to these projections by the ventral sucker; this attachment provoked a remarkable constriction of the papillae. Caliciform cells, although absent in the areas of cellular proliferation, were well observed around these sites. The stroma presented a discrete diffuse mononuclear inflammatory infiltrate and hyperemia [99].

Gastrodiscoides hominis, the agent of gastrodiscoidiasis, primarily an intestinal fluke of the pig, also infects other vertebrates including humans. Another definitive host species in which this trematode has been found is the wild boar, in the Thekaddy forest area in Kerala, India [100]. This parasite has also been found in Napu mouse deer (*Tragulus napu*) from Malaya, as well as in the field rat (*Rattus brevicaudatus*) from Java, and rhesus monkeys (*Macaca mulatta*) in India [101, 102]. There are also records in other monkeys, *Macaca fascicularis*, *M. irus*, *M. philippinensis*, and *M. cynomolgus* [103], and in the orangutan, *Pongo pygmaeus* [104]. In Thailand, it was found in 7.4 % of rats studied [105], as well as in a spectacle monkey *Presbytis molalophos* from Chumphon Province [106]. A survey of trematode infections in introduced fur-bearing mammals in the Volga Delta, Russia was carried out between 1993 and 1997. *G. hominis*, which locally infects wild boars, has recently been found in introduced American muskrats (*Ondatra zibethica*) [107]. The parasite is usually asymptomatic and affects the small intestine in animals with mild symptoms.

In pigs, pathological symptoms include infiltrations with eosinophils, lymphocytes, and plasma cells. The submucosa can show edema and thickening, resulting in subacute inflammation of the cecum and mucoid diarrhea.

Presidente and Ramsden [53] reported that a muskrat at necropsy had four dark nodules on the lungs and from these ten *P. kellicotti* were recovered. Five intact flukes measured 13–15 × 8–9 mm in size and contained eggs. Although tissues were severely autolyzed, the nodules were identified as dilated bronchi with thick walls. The epithelium was eroded in some areas, and there was squamous metaplasia in others. Most of the wall consisted of bronchial glands that were hyperplastic and hypertrophic; dilated glands contained an excessive quantity of mucus. Collagen and small numbers of mononuclear cells were also seen. A zone of compression surrounded these altered bronchi, and there were aggregations of eggs in adjacent alveoli. Chronic pneumonitis characterized by interstitial thickening, leukocyte infiltration, and many macrophages containing a black pigment were associated with some fluke eggs. The changes in the bronchial wall were most similar to those seen in some mink but the reaction was more extensive and severe. The presence of black pigment in the pulmonary parenchyma was like that described in the coyote [53].

13.11 Bats

Bats receive increased attention in infectious disease studies, because of their well-recognized status as a reservoir species for various infectious agents. This is even more important, as bats with their capability of long distance dispersal and complex social structures are unique in the way microbes could be spread by these mammalian species. Nevertheless, infection studies in bats are predominantly limited to the identification of specific pathogens presenting a potential health threat to humans. But the impact of infectious agents on the individual host and their importance on bat mortality is largely unknown and has been neglected in most studies published to date.

13.12 Insectivores

Definitive hosts of brachylaimids are homeothermic mammals and birds normally feeding on snails or accidentally ingesting them with other food. Many brachylaimid species show a clearly restricted spectrum of closely related definitive host species (or sometimes even a single one), suggesting a phylogenetic specificity, as is the case of species infecting shrews (Soricidae) and moles (Talpidae). Within mammals, insectivores, rodents, and marsupials are the main definitive hosts. Artiodactyls (pigs, Suidae), carnivores, and lagomorphs are only secondarily hosts.

The adult stage of brachylaimids is intestinal, including species which are hematophagous [108]. Only a very few species present an adult stage infecting other parts

of the digestive tract or organs directly related or connected to the digestive tract: *Brachylaima oesophagei* and *B. fulvus* in the esophagus and stomach [109, 110]. *Scaphiostomum* species in ducts of liver and pancreas [108], and *Dollfusinus frontalis* in nasal and frontal sinuses [111, 112]. Exceptions are the lumen of the kidney sac as final microhabitat for *Parabrachylaima* [113], and the urinary system for species of *Zeylanurotrema* [114, 115], although a species of the latter, *Z. sphenomorphi*, has been described from the host's intestine [116].

Recently, a new brachylaimid species has been described based from adult trematode specimens found in the kidneys and ureters of the forest shrew *Myosorex varius* (Smuts, 1832) (Insectivora: Soricidae: Crocidosoricinae) from a restricted, very damp area of the Hottentots Holland Mountain range, near Cape town, South Africa [117].

13.13 Nonhuman Primates

Strait et al. [118] reviewed in detail those trematodes most likely to produce lesions in the alimentary tract and pancreas of the nonhuman primate host. Pathogenic and nonpathogenic trematodes in the alimentary tract and pancreas of nonhuman primates are listed in Table 13.2.

Trematodiasis in nonhuman primates can be caused by infection with a number of species of trematodes. *Gastrodiscoides hominis* is a small, orange-red fluke that attaches to the mucosa of the cecum and colon. Infection usually is asymptomatic when the parasites are present in small numbers. Heavy infections produce mucoid diarrhea and mild chronic colitis. The parasite is distributed throughout the tropical orient and has been described in various *Mucacu* species that range throughout this geographic area [103, 119, 120]. The life cycle is indirect, with a snail serving as the intermediate host [121]. Diagnosis can be made by identifying the characteristic egg in the feces or by finding the typical adult flukes in the lumen of the cecum or colon at necropsy. Infection usually is asymptomatic when the parasites are present in small numbers. Heavy infections produce a mucoid diarrhea and mild chronic colitis. Attachment of the flukes to the intestinal mucosa results in focal lesions characterized by hyperemia, loss of surface epithelium, and necrosis. Neutrophilic infiltrates may be associated with these lesions. The submucosa may be sclerotic because of proliferation of fibrous connective tissue and a lymphoplasmacytic cell infiltrate [121]. This parasite has been reported to cause a mild diarrhea in man, but because of the obligatory snail intermediate host in the life cycle, infected captive monkeys are not a direct health hazard for man [120].

Watsonius watsoni, *W. deschieni*, and *W. macaci* have been reported to inhabit the intestinal tract of several Old World primate species (guenons, baboons, and cynomolgus monkeys). Adult trematodes of this genus are translucent, orange, and pear shaped. The complete life cycle is not known but probably involves a snail intermediate host and is thought to be similar to that of *Fasciola hepatica* [122]. *Watsonius watsoni* and *W. deschieni* have been reported to be associated with

Table 13.2 Trematodes described from nonhuman primates (adapted from [118, 125])

	Location in host	Host
Brachylaimidae		
Brachylaima sp.	I	Prosimians
Plagiorchiidae		
Plagiorchis multiglandularis	I	Old World Monkeys
Lecithodendriidae		
Novetrema nycticebi	I	Prosimians
Odeningotrema apidion	I	Prosimians
O. bivesicularis	I	Prosimians
Odeningotrema sp.	I	Prosimians
Phaneropsolus bonnei	I	Prosimians, Old World Monkeys
P. lakdivensis	I	Prosimians
P. longipenis	I	Prosimians, Apes
P. perodictici	I	Prosimians
P. orbicularis	I	New World Monkeys
P. oviforme	I	Prosimians, Old World Monkeys
P. simiae	I	Old World Monkeys
P. aspinosus	I	Old World Monkeys
Primatotrema macacae	I	Old World Monkeys
Pithecotrema kelloggi	I	Old World Monkeys
Dicrocolidae		
Athesmiafoxi	Bd	New World Monkeys
A. heterolecithodes	Bd	New World Monkeys
Brodenia laciniata	Bd, P	Old World Monkeys
B. serrata	P	Old World Monkeys
Concinnum brumpti (syn. *Eurytrema brumpti*)	Bd, P	Apes
Controrchis biliophilus	Gb, Bd	New World Monkeys
Dicrocoelium colobusicola	Bd	Old World Monkeys
D. lanceatum	Bd	Old World Monkeys, Apes
D. macaci	Bd	Old World Monkeys, Apes
Euparadistomum cercopitheci	Gb	Old World Monkeys
Euparadistomum sp.	Gb	Prosimians
Eurytrema pancreaticum	Pd	Old World Monkeys
E. satoi	Bd, P	Old World Monkeys, Apes
Leipertrema rewelli	P	Apes
Leipertrema sp.	SI	Prosimians
Platynosomum amazonesis (syn. *Conspicuum conspicuum*)	Gb, Bd	New World Monkeys
P. marmoseti (syn. *Conspicuum conspicuum*)	Gb, Bd	New World Monkeys
P. fastosum	Gb, Bd	New World Monkeys
P. minutum	Gb, Bd	New World Monkeys
Skrjabinus sp.	Gb, Bd	Prosimians
Zonorchis goliath	Bd	New World Monkeys

(continued)

Table 13.2 (continued)

	Location in host	Host
Z. microcebi	Bd	New World Monkeys
Zonorchis sp.	Gb, Bd	Prosimians
Fasciolidae		
Fasciola hepatica	L	Old World Monkeys
Fasciolopsis buski	D, S	Old World Monkeys
Opisthorchiidae		
Clonorchis sinensis	Bd	Old World Monkeys
Opisthorchis felineus	Bd, Pd	Old World Monkeys
Heterophyidae		
Haplorchis pumilio	I	Old World Monkeys
H. yokogawai	I	Old World Monkeys
Metagonimus yokogawai	I	Old World Monkeys
Pygdiopsis summa	I	Old World Monkeys
Microphallidae		
Spelotrema brevicaeca	I	Old World Monkeys
Echinostomatidae		
Artyfechinostomum sp.	I	Old World Monkeys
Echinostoma aphilactum	SI	New World Monkeys
E. ilocanum	I	Old World Monkeys
Reptiliotrema primate	I	Old World Monkeys
Notoctylidae		
Ogmocotyle ailuri	SI	Old World Monkeys
O. indica	SI, S	Old World Monkeys
Paragonimidae		
Paragonimus westermani	Lungs, pleural cavity, diaphragm, body cavity, brain	Old World Monkeys
P. africanus	Lungs	Old World Monkeys
Achillurbainiidae		
Achillurbania sp.	Parotid gland	Prosimians
Troglotrematidae		
Beaveria sp.	I, L	Prosimians
Schistosomatidae		
Schistosoma bovis	Mesenteric and abdominal veins	Old World Monkeys
S. haematobium	Mesenteric, visceral, and abdominal veins	Old World Monkeys, Apes
S. japonicum	Mesenteric and portal veins	Old World Monkeys, Apes
S. mansoni	Mesenteric and abdominal veins	New and Old World Monkeys, Apes
S. mattheei	Mesenteric and abdominal veins	Old World Monkeys
Schistosoma sp.	Mesenteric and abdominal veins	Prosimians, Old World Monkeys, Apes

(continued)

Table 13.2 (continued)

	Location in host	Host
Diplostomidae		
Diplostomid mesocercariae	Visceral and pulmonary cysts	New and Old World Monkeys
Neodiplostomum tamarini	I	Old World Monkeys
Paramphistomatidae		
Chiorchis noci	I	Old World Monkeys
Gastrodiscoides hominis	Cecum, colon	Old World Monkeys
Watsonium deschiensi	I	Old World Monkeys
W. watsoni	I	Old World Monkeys
W. macaci	I	Old World Monkeys

Gb gall bladder, *Bd* bile duct, *Pd* pancreatic duct, *P* pancreas, *FS* frontal sinuses, *I* intestine, *L* liver, *S* stomach

diarrhea, severe enteritis, and death in monkeys. Little else is known about the anatomic effects of these species, and diagnosis can be made from the characteristic egg in the feces or adults in the intestine at necropsy [122, 123].

The public health considerations for these flukes are the same as described for *G. horninis. Paragonimus africanus* was recovered from a free-ranging drill (*Mandrillus leucophaeus*) in Cameroon. This was the first record on the natural infection of a nonhuman primate with lung flukes in Africa [124].

13.14 Concluding Remarks

This chapter reviews the trematodes of representative mammalian fauna. The pathogenesis of trematodes depends to a great extent on the intensity with which the host is infected, the location in the host, and if it is the normal definitive host. Our knowledge of losses caused by trematodes is difficult to estimate. Losses due to stunting and general unthriftiness are even more difficult to determine. Further knowledge on pathogenicity and the effect of trematodes on wildlife populations is needed in wildlife parasitology.

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