Neglected Tropical Diseases

Sunit K. Singh *Editor*

Neglected Tropical Diseases – South Asia

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Neglected Tropical Diseases

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Preface

Neglected Tropical Diseases (NTDs) constitute a significant part of the total disease burden in South Asia. A high burden of NTDs in South Asia has affected economic progress of countries in this region. The content in this book has been decided to give an overall view of the NTDs in South Asia. The NTDs of South Asia include the soil-borne helminth infections, vector-borne viral infections, parasitic infections and bacterial infections. The major NTDs are restricted to a particular area of the developing or underdeveloped nations, and a few of them infect globally in certain climatic conditions. The neglected viral infections like Kyasanur Forest Disease (KFD) were earlier reported to be restricted to Karnataka in India, but this infection has also been recently reported in Kerala and Maharashtra in India. This is the one example suggesting the spread of NTDs. The table of content of the NTDs of South Asia includes the most common neglected viral, bacterial and protozoan infections, which results in the compromise of the health and well-being of any society. The investors do not see a big market for the medicines related to the NTDs from a profit point of view. Therefore, the NTDs rarely attract the attention of investors for NTD drug development, compared to other infectious diseases. South Asian countries must make efforts on developing the next generation of drugs, diagnostics and vaccines for NTDs. There is a need for public–private partnerships to develop new drugs and diagnostics for NTDs to serve the population. The proper management of NTDs can reduce the mortality and disability-adjusted life year (DALY) of the persons suffering from NTDs. There is a need to strengthen the public health systems in the areas/communities suffering from NTDs by utilising the need based on affordable and sustainable control measures.

NTDs contribute to social and economic burden due to the social stigma, physical disabilities, discrimination and malnutrition associated with NTDs, which lead to unemployment and poverty and affect the community's social and economic development adversely.

There is need of incremental advance in efforts being made in controlling, eliminating or eradicating NTDs. More efficient and proactive healthcare systems with easy access to affordable medicines are required for proper management of NTDs in South Asia.

Varanasi, India Sunit K. Singh

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About the Editor

Dr. Sunit Kumar Singh completed his bachelor's degree programme from GB Pant University of Agriculture and Technology, Pantnagar, India, and master's degree programme from the CIFE, Mumbai, India. After receiving his master's degree, Dr. Singh joined the Department of Pediatric Rheumatology, Immunology, and Infectious Diseases, Children's Hospital, University of Wuerzburg, Wuerzburg, Germany, as a biologist. Dr. Singh completed his PhD from the University of Wuerzburg in the area of molecular infection biology.

Dr. Singh has completed his postdoctoral training at the Department of Internal Medicine, Yale University, School of Medicine, New Haven, Connecticut, USA, and the Department of Neurology, University of California Davis Medical Center, Sacramento, California, USA, in the areas of vector-borne infectious diseases and neuroinflammation, respectively. He has also worked as visiting scientist at the Department of Pathology, Albert Einstein College of Medicine, New York, USA; Department of Microbiology, College of Veterinary Medicine, Chonbuk National University, Republic of Korea; Department of Arbovirology, Institute of Parasitology, Ceske Budejovice, Czech Republic; and Department of Genetics and Laboratory Medicine, University of Geneva, Switzerland. He is having extensive experience in the area of virology and immunology. Dr. Singh also served as a scientist and led a research group in the area of molecular neurovirology and inflammation biology at the prestigious CSIR—Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. Presently, he is working as Professor of Molecular Immunology and leading a research group in the area of Human molecular Virology and Immunology, in the Department of Molecular Biology, Faculty of Medicine, Institute of Medical

Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India. His main areas of research interest are molecular neurovirology and immunology. There are several awards to his credit, including the Skinner Memorial Award, Travel Grant Award, NIH-Fogarty Fellowship and Young Scientist Award. Dr. Singh has published many research papers in the areas of molecular virology and inflammation biology in various peer-reviewed journals. Dr. Singh has edited several books such as "Neuroviral Infections Vol-I & Vol-II", "Viral Hemorrhagic Fevers", "Human Respiratory Viral Infections", Human Emerging and Re-emerging Infections Vol-I & Vol-II and "Viral Infections and Global Change". Dr. Singh is associated with several international journals of repute as an associate editor and editorial board member.

Overview of Leishmaniasis with Special Emphasis on Kala-azar in South Asia

Kwang Poo Chang, Bala K. Kolli and Collaborators

Contents

For collaborator details please see the list provided at the end.

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Abbreviations

AVL	Anthroponotic visceral leishmaniasis
CL	Cutaneous leishmaniasis
DALY	Disability-adjusted life year
IRS	Insecticide residue spread
MCL	Mucocutaneous leishmaniasis
NTD	Neglected tropical diseases
PKDL	Post-kala-azar dermal leishmaniasis
PV	Parasite-containing vacuole
rK39	Recombinant kinesin 39 amino acid repeats
rKE16	Recombinant kinesin antigen from *L. donovani*
VL	Visceral leishmaniasis
ZCL	Zoonotic cutaneous leishmaniasis

1 Global Overview of Leishmaniasis

1.1 Disease Types

Leishmaniasis is a complex disease caused by *Leishmania* infection, producing variable clinical symptoms, e.g., cutaneous, mucocutaneous, and visceral leishmaniases [1–3]. Cutaneous leishmaniasis (CL) caused, for example, by *Leishmania major/L. tropica* is marked by the appearance of skin lesion in various forms, which are often innocuous and self-healing, while mucocutaneous leishmaniasis (MCL) caused, for example, by *L. braziliensis* is a protracted disease, resulting sometimes in facial disfigurement of the ear, mouth, and nose. Neither CL nor MCL is life-threatening per se. Only in non-healing case has death of these patients been reported due to secondary infections or other causes, e.g., suicide as a result of unbearable psychological stress. Visceral leishmaniasis (VL) caused by *L. donovani/L. infantum* is far more severe. It is often fatal, if untreated, resulting from systemic and progressive infection of macrophages by *Leishmania* in the reticuloendothelial systems or lymphoid organs, chiefly the spleen, liver, and bone marrow. Disorders of hematological and hepatosplenic functions are thus the clinical manifestations of VL, including hepatosplenomegaly, fever, anemia, leucopenia, hypergammaglobulinemia, and cachexia. The development of all leishmaniases follows a chronic course lasting for months and sometimes years.

1.2 Disease Incidence/Distribution

Leishmaniasis is very widespread, currently putting a world population of >350 million at risk with up to ~1.2 million cases at a death rate in the tens of thousands per year [4]. Of the 16 categories of neglected tropical diseases (NTD) assessed for

the period from 2005 to 2013, leishmaniasis ranks next only to malaria as the second worst in the age-standardized DALYs (disability-adjusted life years) and second only to dengue fever in the rate of DALY increase from 5.7 to 5.9 million [5]. In 1985, historical, parasitological, clinical, epidemiological, and control program information was compiled for the endemic areas in the Indian subcontinent, the Middle East, Central Asia, North and East Africa, China, Europe, and Central and South America [6]. Recent efforts published in 2012 have yielded bionomic data of leishmaniasis with more details for each of the ~100 countries or territories included [4]. There are still endemic areas, e.g., West Africa, where information is not readily available in any detail, indicating that leishmaniasis is still more pervasive and entrenched than is known.

Leishmaniasis is a disease of poverty and often flares up in areas of low endemicity into epidemic proportion due to natural or man-made disasters, including famine, drought, flood, earthquakes, and wars. This is currently most evident in Sudan, Iraq, Syria, and Afghanistan where military conflicts further trigger refugee migration in droves, thereby bringing the disease into neighboring countries and beyond.

1.3 Transmission

Leishmaniasis is a vector-borne disease, which is transmitted by the blood-feeding female sand fly of different species in various locations (Fig. 1, Lower). There are hundreds of different sand fly species, of which dozens serve as the vector of leishmaniasis in different endemic sites [7]. Sand flies are inconspicuous, fragile, and hairy winged dipterans, similar to, but smaller than, mosquitoes in size. For epidemiological surveys and other studies, these flies are captured in the field by CDC-light trap, suction pump, and sticky paper at dawn and at dusk when they are active. The distribution of the vector species coincides well globally with that of the disease. The disease is largely a zoonosis and is considered as an anthroponosis in few places where reservoir animals have not been found, e.g., Indian VL. The animals, which are recognized as reservoirs, include rodents; domestic and wild dogs or canids, such as fox; and other wild animals, like sloths in South America and possibly hares in Eurasia (Fig. 1, Upper). Humans acquire infection when stepping into the sylvatic cycle of ongoing transmission by vectors among the reservoir animals. The most well-established and best-studied reservoir for human VL is dog in the Mediterranean basin, Brazil, and many other places of low endemicity where this animal suffers from canine leishmaniasis with clinical manifestations akin to human CL and VL. Transmission of leishmaniasis has been reported on rare occasions via blood transfusion, coitus and accidental inoculation via contaminated needles, but not by oral or respiratory route. Risk factors for natural transmission include exposure to infected sand flies in the endemic areas, human genetic factors, malnutrition (Cf. Appendix—Box 1), immunosuppression

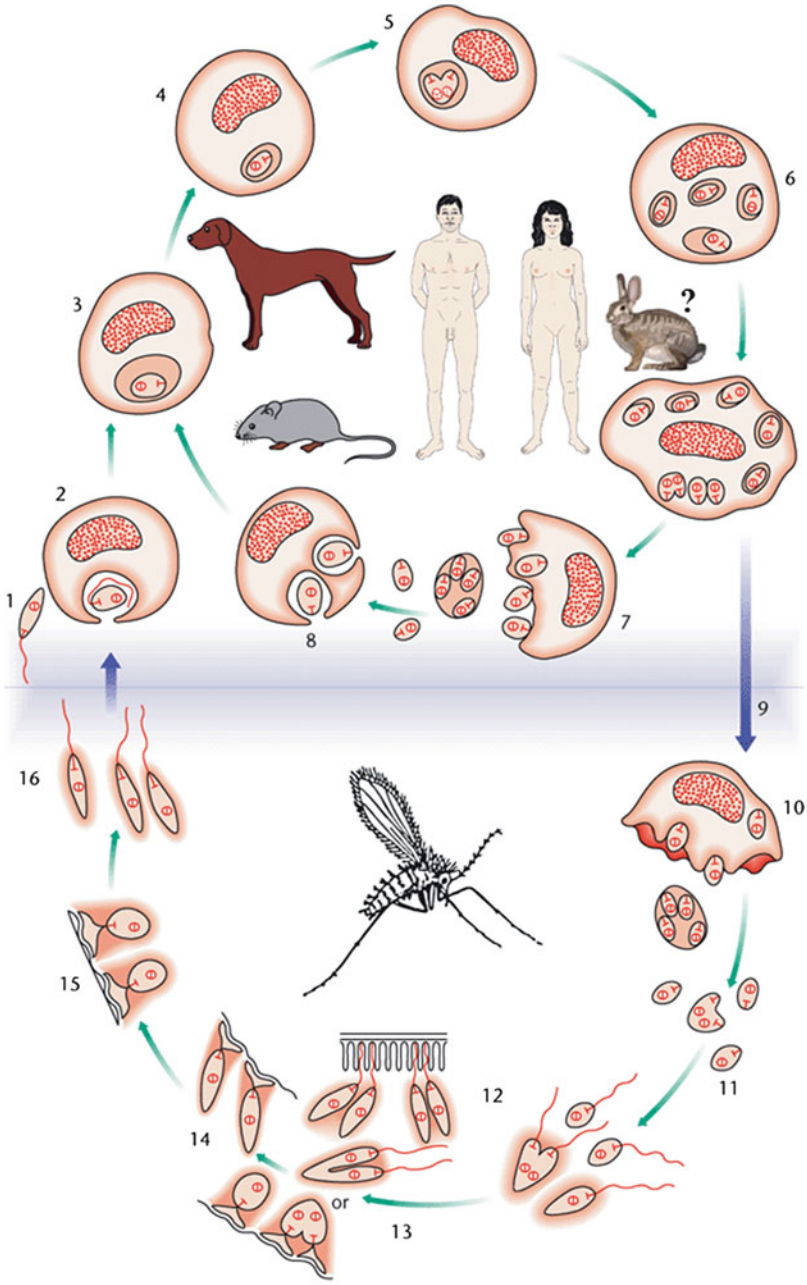

Fig. 1 Diagrammatic depiction of *Leishmania* transmission cycle. Upper, Amastigote stage, which lives intracellularly as non-motile entities in macrophages of infected mammalian hosts, i.e., human/animal reservoirs. "?", Hare as a potential reservoir. Lower, Promastigote stage, which lives as motile flagellated forms each with an anterior flagellum in the gut lumen of female sand fly. Modified from: Chang KP 2012 Leishmaniasis. Encyclopedia Life Science, John Wiley & Sons, Ltd. www.els.net

of individuals after organ transplantation or due to AIDS/other infectious diseases, and needle sharing among drug addicts.

Life Cycle Stages The life stages of *Leishmania* in the transmission cycle are depicted in Fig. 1. The mammalian stage is known as amastigotes—nonmotile round to oval bodies, which live and replicate in the macrophages of infected mammalian hosts (Fig. 1, Upper). Infected cells provide the source of amastigotes for infecting additional host cells, accounting for metastasis of the infection. As the female vectors take blood meals from infected hosts, they pick up infected cells. The amastigotes are released from these cells and differentiate into the insect stage or motile promastigotes, which replicate extracellularly in the alimentary canal of the vector, giving rise to different morphological forms (Fig. 1, Lower). Some promastigotes differentiate into infective or metacyclic forms, which migrate to the proboscis or mouth part and are delivered into the skin of the mammalian host as the vector takes blood meal. During this cyclic transmission, *Leishmania* undergo differentiation in the two different hosts, thereby remaining parasitic throughout their life. In each cycle of transmission, *Leishmania* remain much longer as intra-macrophage amastigotes in the mammalian host than as extracellular promastigotes in the vector. This is made obvious by the disparity between insects and mammals in their respective life span.

1.4 Diagnosis

Detailed accounts for this are available in a number of recent papers [8–11], which is summarized very briefly as follows. Visualization of parasites in the clinical samples from symptomatic patients constitutes the time-honored gold standard for definitive diagnosis of leishmaniasis. The routine procedures for this include microscopic examinations of fresh or Giemsa-stained smears of lesion aspirates for the presence of amastigotes and/or cultivation of the samples in suitable media for their differentiation into and/or replication as promastigotes. These century-old practices have been gradually replaced by less cumbersome and more sensitive and specific methods, i.e., serodiagnosis for the presence of *Leishmania*-specific antibodies or circulating antigens and by PCR amplification of *Leishmania*-specific DNAs. The pros and cons of these diagnostic methodologies have been thoroughly reviewed for their relative merits with reference to the gold standard of parasite visualization. Worthy of special mention is the chromatographic strip of amastigote-specific recombinant antigens (rK39) that was developed first by SG Reed and his colleagues, as it is highly sensitive and specific for the diagnosis of Eurasian VL [12]. The strips have been successfully field deployed in dipstick format for reaction with a drop of blood to facilitate on-the-spot diagnosis. Under development are more sensitive and

specific serological and molecular diagnostics suitable for use with samples that are readily available for collection by noninvasive means, e.g., saliva and urine [13].

Adequate methodology is thus available for definitive diagnosis of leishmaniasis—a prerequisite not only for treatment decision but also for assessing incidence of the disease relevant to designing control strategies. However, the current measures for both disease prevention and treatment are inadequate and require urgent attention, as noted from the descriptions in the following sections.

1.5 Prevention

This is the most desirable measure for any disease control but has not been fully exploited for deployment to control leishmaniasis. At least some of the preventive approaches are amenable to or favorably disposed for implementation in principle. Summarized below are the available approaches and the status of their application to the prevention of leishmaniasis.

1.5.1 Vector Control

Vector control to disrupt the transmission cycle is routinely practiced for curbing all vector-borne diseases. To control sand flies needs to target their adults, since the breeding site for the larval stage remains obscure. This approach has been put into practice in most control programs for leishmaniasis, including the use of insect repellents, insect repellent-/insecticide-impregnated bed net, insecticide residue spray (IRS) to kill domiciliary sand flies [14–16], and/or insecticide-impregnated dog collars [17]. Applicability of these approaches varies widely with the conditions in different endemic areas as much as the effectiveness of their execution. Vector control has inherent limitations to prevent leishmaniasis. For example, such approaches are impractical for controlling wild species of sand flies, which are too widely distributed often in vast areas of open field to control. Toxicity of insecticides in use and the development of insecticide resistance by the vectors present additional obstacles. Better approaches are clearly needed to control sand flies safely and effectively, for example, by developing biotechnology for genetically modified vectors (GMO) [18], such as those under study for mosquito control, and by exploring the relatively safe and resistance-averting photodynamic insecticides [19]. These new approaches however await further studies to assess their feasibility in conjunction with a better understanding of the vector biology.

1.5.2 Reservoir Control

Reservoir control is inherently difficult, especially in endemic areas where transmission is zoonotic via wild animals. Control of reservoirs is impractical in many places, e.g., Central and South America, where a diverse group of different wild

animals with complex ecology appears to serve in that capacity [20]. In Central Asian steppes, mechanical destruction of rodent burrows and the use of poisoned baits were applied to control great gerbil as the known reservoir for simple CL caused by *L. major* [21]. Similar measures were contemplated for the control of hyrax as the reservoir of CL caused by *L. tropica* in East Africa. Implementation of such measures is, however, impractical beyond the immediate surrounding of human habitats, thereby leaving the sylvatic cycle of transmission unchecked in the vast uninhabited area. In endemic areas where dog is the proven reservoir, reduction or elimination of its population has been shown to reduce the incidence of human VL [17, 22]. This approach also has the limitation of being unsustainable for several reasons. One is the difficulty to permanently eliminate both stray and owned dogs due to their mobility from one location to another. Another reason is the difficulty of controlling wild canine species from serving as additional reservoirs. Also, objection has been raised against dog culling based on humanitarian ground. Nevertheless, control of dog population is accepted as a preventive measure of the control programs in endemic sites where canine VL coexists with human VL. When human is the only known reservoir in the case of anthroponotic leishmaniasis, stringent observation of the operational stipulations to identify patients for treatment is expected to break the transmission cycle effectively. Anthroponotic VL (AVL) has been indeed eliminated from east and north China by this approach [22]. In many endemic areas, infected, but nonclinical, cases exist, raising the possibility that these healthy carriers may play a role as a potential reservoir in addition to post-kala-azar dermal leishmaniasis (PKDL). This issue is significant in Indian kala-azar where many asymptomatic cases have been reported [23–26].

1.5.3 Vaccination

Vaccination is the best preventive measure, but vaccines are not available to protect human population against leishmaniasis. This approach has long been thought as very feasible, considering that patients invariably acquire lasting or lifelong immunity after natural or chemotherapeutic cure. The recent explosion of reviews written on this topic is indicative of the intense interests in the development of vaccines [27–33]. Readers are referred to these reviews for the history of past successes and failures and for details of the current attempts. It suffices to mention here the earliest form of vaccination and the latest development in the field. Inoculation of healthy individuals with lesion-derived live parasites in a hidden place is the crudest form of vaccination for simple CL. This is known as "leishmanization" that has been practiced for millennia to protect individuals from the potentially facial disfiguring CL in the Middle East and Central Asia. "Leishmanization" is effective but unacceptable unless accomplished without a full-blown disease. Clearly, vaccines need to be developed with optimization of not only their safety and ease of production but also efficacy for both human and canine leishmaniasis. In a recent US NIH-sponsored workshop, candidate vaccines for VL are listed, including recombinant peptides and adjuvants, cDNA, and whole-parasite vaccines [30].

Of particular interest is the target product profile (TPP) analysis by modeling various relevant parameters available, for example, in the VL-endemic Bihar, India. The ideal vaccine based on such analysis is expected to cost $5 or less per dose with 70% efficacy, regardless of the duration of protection [30]. The current course to evaluate the safety and efficacy of vaccine candidates in the pipelines is too arduous to expect rapid progress. Adaptation of fast-track preclinical and clinical trials will ease the constraints to expedite the development of an ideal vaccine.

1.6 Treatment

This subject also requires significant attention, especially for the potentially fatal VL, as indicated in recent reviews [34–37]. The mainstay of treatment for leishmaniasis is chemotherapy, but none of the drugs in use was specifically designed and developed for treating this disease, i.e., antimonials (meglumine antimoniate or Glucantime®, sodium stibogluconate or Pentostam®), miltefosine, pentamidine, amphotericin B, ketoconazole, and paromomycin [38, 39]. The antimonials remain to be the first-line drug of choice for VL treatment, even though they are decades old, and the mode of action remains basically unknown. In addition, the development of antimonial resistance necessitates their prolonged use at extremely high dosages, resulting in significant side effects of death from kidney and/or heart failures. The antimonial dosages for treating VL have increased from 20 mg/kg daily for 1–2 weekly courses (still in use in the nondrug-resistance areas) to 30 mg/kg daily for 30 days or longer continuously in places like Bihar, India. Although antimonials have not been used for more than a decade in this hyperendemic area, 78% of the recent clinical isolates are still antimonial resistant to a variable extent [40]. The other anti-leishmanials listed are mostly repurposed anticancer, antifungal, or antibacterial drugs (e.g., [41]). Resistance of VL patients to the treatment with these drugs is also emerging (e.g., [42, 43]). Amphotericin B-liposome (AmBisome®) is reported as curative by a single dose administration, but it is still too expensive for general use in endemic areas of poverty. The current strategy to alleviate the problems of drug resistance and toxicity is a combination use of two different drugs each at a lower dosage. Of particular relevance to mention is thus the recent advance in identifying proteosomal protease of *Leishmania* and other protozoan parasites as a specific drug target by screening ~1 million small molecules [44]. This would be the first specific anti-*Leishmania* drug, should it be successfully developed into a product for clinical use.

Chemotherapy of simple CL faces the dilemma of its necessity, considering the tendency of its spontaneous self-resolution. Treatment is given to hasten the healing, thereby minimizing the scar formation and promoting herd immunity, i.e., reducing the infected population as a potential source of parasites for spreading. Treatment of protracted or non-healing CL/MCL also faces the problems of patients' unresponsiveness to the medication. Alternative approaches for treating CL are available by using physical means, e.g., cryotherapy, thermotherapy by

using radio wave-generated heat [45], and photodynamic therapy. These types of physical therapy for CL are still under trial for efficacy evaluation.

1.7 Epidemiology Mathematical Modeling

Advances have been made to develop algorithms for analysis of clinico-epidemiological data of leishmaniasis, including remote sensing and geographic information system or GIS technologies, to estimate or predict risk factors, disease burdens and spreading, and efficacies of various control measures [46–51]. Applications of mathematics to the analysis of available information are expected to contribute significantly to the development of more effective control programs. It is important to provide numerical measurements for all the variables of the disease as guidelines to facilitate policy, budgetary, and management decision making. Effective collections of demographic, environmental, vector, reservoir, and clinical information are essential to construct a robust database for such analysis to improve the evaluative and predictive potentials of the available software programs.

1.8 Control Programs

Programs have often been developed under the aegis of WHO to control leishmaniasis in endemic countries [52]. Expert committees are organized by the responsible agencies to strategize detailed plans for operational managements and assignment of responsibilities. Budgets are drawn for appropriation by the governments of concern and sometimes supplemented by programs from not-for-profit domestic and/or international organizations. Control programs usually include the following components: (1) screening endemic populations for leishmaniasis to identify patients (active case detection); (2) treatment of the identified patients; (3) vector surveillance and control; (4) where applicable, dog control; and (5) annual surveillance of patient, vector, and reservoir populations for program evaluation. The success of the control programs has been shown by a reduction in the annual incidences of leishmaniasis in some countries. Only in China has elimination of VL been reported with some measure of success by the implementation of an integrated program that was launched in ~1950 and ended in ~1960 [22]. Components of the program include a combination of the aforementioned measures: diagnosis of the endemic populations to identify patients for antimonial chemotherapy, vector control, and dog elimination. As a result, the anthroponotic VL was eliminated in the east and north areas of high endemicity, but AVL and zoonotic VL of the mountain type (dog as the reservoir) and desert type (hare as the possible reservoir) have persisted until today in the western regions. It is apparent from the experiences of this control program that anthroponotic VL can be eliminated even by using antiquated methodology, while zoonotic VL is difficult to control.

2 Leishmaniasis in South Asia

2.1 Clinico-epidemiological Types

Figure 2 is a sketch map to delineate the general endemic areas of both CL and VL reported in South Asia. Briefly summarized below are the epidemiological and other specific information reported for seven different disease types (color coded):

1. **Anthroponotic VL** (**red**) caused by *L. donovani* and transmitted by *P. argentipes* is the most severe, representing bulk of the incidence and mortality in the world record estimated for this disease [4]. The endemic region covers a large area, consisting of the northeastern part of India, including Bihar, Jharkhand, Uttar Pradesh, and West Bengal, the southern portion of Nepal and Bhutan, and the western part of Bangladesh [10, 39, 53–56]. Indian AVL, also known as kala-azar (black fever), has a long history going back to the British colonial period [57]. The causative agent for AVL was first discovered in India and named after the British military commanding officers: Lieutenant General Sir William Boog Leishman and Major Charles Donovan [58]. The AVL in the adjacent countries has not attracted much attention until recently.
2. **Zoonotic VL** (**orange**) caused by *L. infantum* with dog as the reservoir has long been reported sporadically in the sub-Himalayan Kashmir, northern Pakistan [59], and almost certainly also in the bordering northwestern India. The occurrence of canine leishmaniasis in this area is reminiscent of Mediterranean infantile VL, although the vector has not been fully identified.
3. **Anthroponotic CL** (**green**) caused by *L. donovani* s.l. and also transmitted by *P. argentipes* was discovered more recently to occur throughout the inhabited area in Sri Lanka [60–63]. Lesion appears in various forms, as described for all CL, and responds poorly to antimony therapy but heals eventually with the expected Th2 to Th1 switch [64]. Reservoir animals are unknown, although rK39 seropositive dogs were reported. The CL previously reported in Kerala in the southwest tip of India probably has an epidemiology [65] similar to that in Sri Lanka. In both locations, VL has been reported in small numbers and was thought to result from the infection by the same parasite that causes CL in Sri Lanka [66]. Genomic sequence comparison between *L. donovani* s.l. and Indian *L. donovani* revealed numerous single base substitutions but also a difference in the copy number of A2 genes, being slightly higher in the latter [67], consistent with previous findings for the involvement of these genes in viscerotropism [68].
4. **Anthroponotic CL** (**pale yellow**) caused by *P. sergenti*-transmitted *L. tropica* has been reported in Rajasthan in northwestern India [69, 70]. The ACL extends into the adjacent region of Pakistan [71] and has a similar epidemiology to that straddling the border between Pakistan and Afghanistan [72].
5. **Zoonotic CL** (**purple**) caused by *P. saheli*-/*P. papatasi*-transmitted *L. major* has long been reported as widespread in the central, west, and southwest of Pakistan [73] extending in continuum with the endemic sites in eastern Iran and perhaps southern Afghanistan. Few incidences of this ZCL have also been reported in Rajasthan.

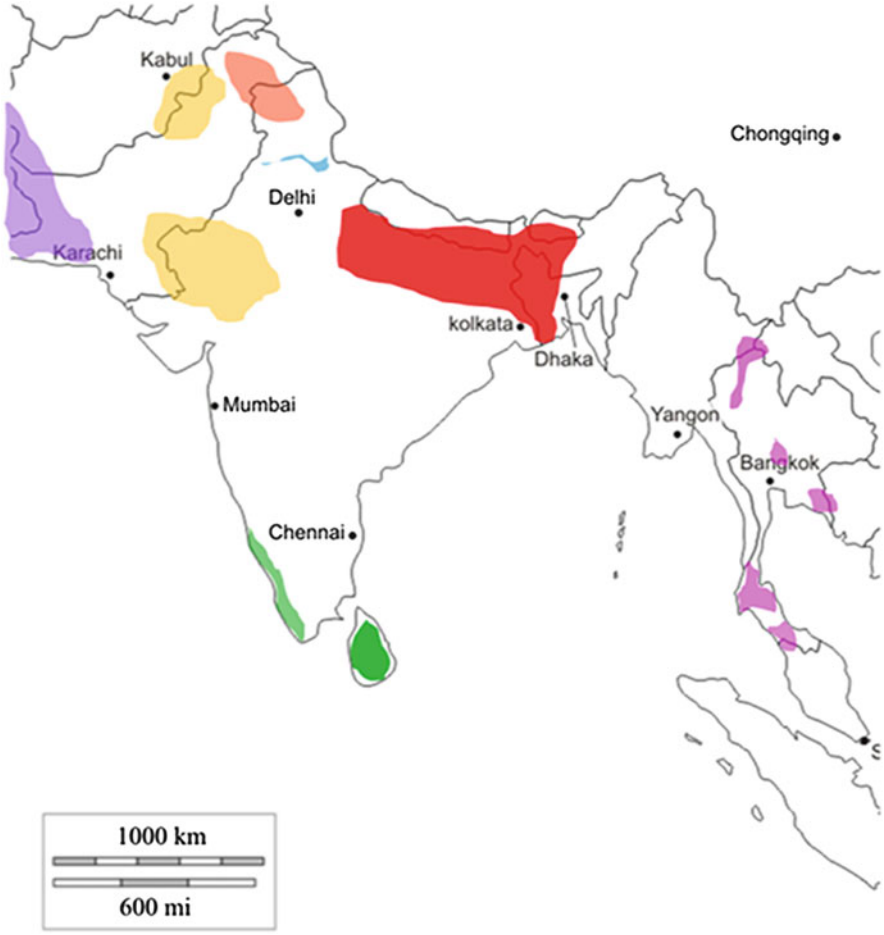

Fig. 2 Distribution of different types of leishmaniasis in South Asia. A template of the map (http://www.d-maps.com/carte.php?&num_car=32159&lang=en) is colored to approximate the endemic areas of different leishmaniasis. Red, VL and PKDL due to *L donovani* in India, Bangladesh, Nepal and Bhutan; Orange, sporadic infantile VL in northwest India, Kashmir and Pakistan; Blue, CL due to *L. infantum* and/or *L. tropica* in the Satluj river valley; Light brown, CL due to *L. tropica* in Rajasthan, India, adjacent Pakistan and at its border with Afghanistan; Purple, CL due to *L major* in coastal Pakistan and neighboring Iran; Green, CL due to *L donovani* s.l. in Sri Lanka and Kerala, India; Cherry, CL/VL due to *L enriettii* in Thailand and its border with adjacent countries. Note: Colors mark the general areas of leishmaniasis

6. **Zoonotic CL** (**blue**) caused likely by *L. infantum* and/or *L. tropica* with dog as the possible reservoir appears in the Satluj river valley of Himachal Pradesh, India [74, 75]. The CL cases are quite numerous in the villages along the river. Cultivation of *Leishmania* from the cutaneous samples of these subjects proved difficult, yielding few promastigotes refractory to subculture. Analysis of PCR products from such materials revealed sequences, indicative of *L. infantum* or

L. tropica or a mixture of the two [76]. Dog serum samples are rK39 positive, suggestive of its reservoir potential. Few cases of typical Indian AVL have been reported among the migrant workers from Bihar in the area. From these VL patients, *L. donovani* was readily grown in contrast to those from the CL cases.

7. **CL** (**cherry**) caused by members of the *L. enriettii* complex (*L. siamensis* s.l. and *L. martiniquensis* s.l.) has occasionally been reported in Thailand [77]. There is evidence of *Leishmania*-HIV coinfection [78]. The existence of similar leishmaniasis is expected in the neighboring countries, judging from the reports of its incidences along the border of Thailand with Myanmar [79], Cambodia, and Malaysia. Investigation is ongoing to confirm the suspected vectors and reservoir [80]. Imported cases of VL and CL have also been reported from the region.

The brief introduction of leishmaniasis in South Asia serves to illustrate the complexity of leishmaniasis in this region. There are clearly very different epidemiological types, almost like a microcosm of this disease complex in the world, although most of them have not received sufficient attention for detailed investigation. Readers will find available information in the literature cited.

2.2 Indian Kala-azar or visceral leishmaniasis

Indian Kala-azar or AVL commands the greatest national and international attention because of its high incidence and mortality, as already mentioned, particularly in Bihar as the epicenter at present. Indian subcontinent accounts for nearly 70% of world's AVL cases, amounting to several hundred thousand annual cases. India has the world's highest national incidence, Nepal and Bangladesh being the next. Together, at risk of acquiring AVL is ~200 million of the population in these three countries. In Bhutan, AVL is sporadic and widely dispersed.

During the long period from the discovery of AVL in the early twentieth century until now [57, 58], an enormous amount of observations and experiences has been garnered for all aspects of AVL in India. Information derived from this rich history of investigation has thus provided the foundation knowledge invaluable for the study of AVL worldwide. Readers are referred to the extensive reviews cited in this article for different aspects of Indian kala-azar. Given below are very brief accounts of some specific points of interest.

2.2.1 Clinical Features

Figure 3 illustrates the salient features of Indian kala-azar: palpable or marked splenomegaly as the most noticeable symptom of life-threatening VL (Fig. 3a, b) and perioral macules and papules as the rather innocuous manifestation of PKDL

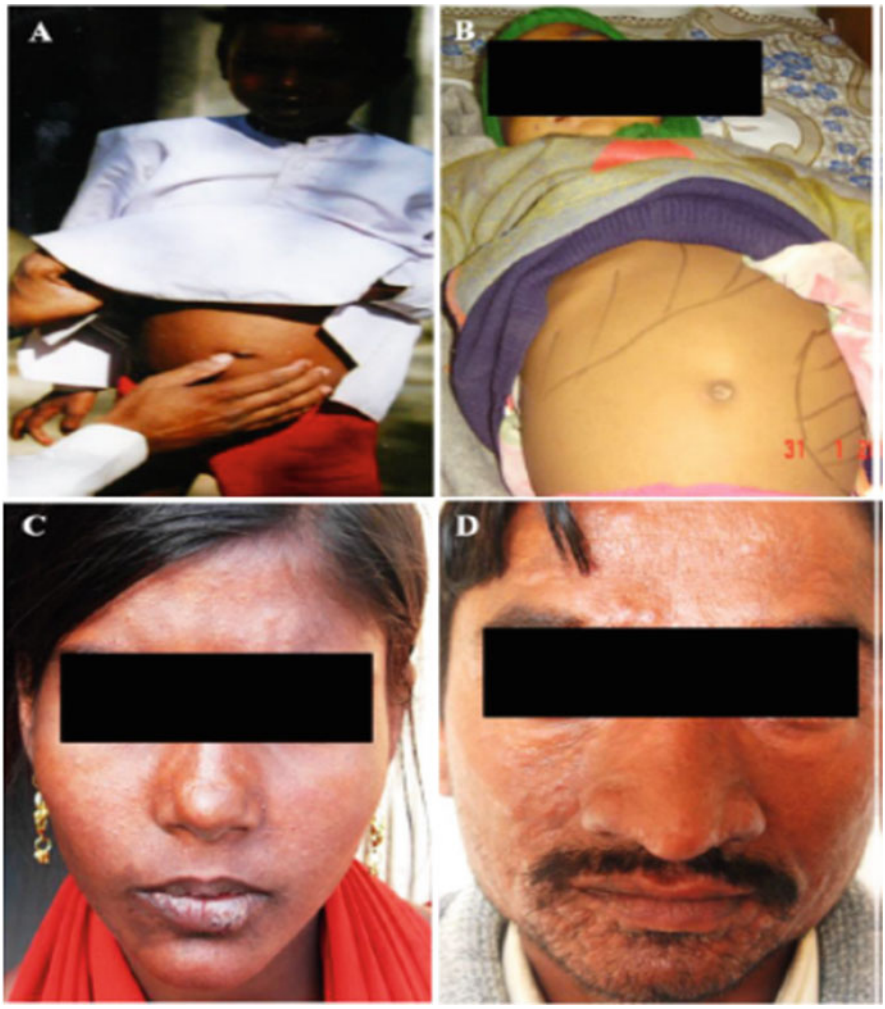

Fig. 3 Patients of kala azar with splenomegaly (**a**, **b**) and post-kala azar dermal leishmaniasis with macular rash around the mouth (**c**, **d**) in Bihar, India. Photos taken in 1995 during visits to kala azar clinics courtesy of Sarman Singh (**a**) and supplied by Shyam Sundar (**b**–**d**)

(c, d). PKDL has been reported to occur in 5–10% of the Indian VL patients after chemotherapeutic cure but also independently of VL. Whether PKDL is the carrier or reservoir of *L. donovani* for AVL is a matter of some controversy [81]. Whether VL and PKDL are caused by the same parasite strain has not been firmly established, resulting mainly from the small sample size of PKDL examined. This is due in part to the difficulty of growing promastigotes from PKDL samples. In addition, the relative innocuity of PKDL provides little incentive for patients to visit the clinics and, when they do, to grant consent for facial sample biopsies.

2.2.2 Splenic Aspiration, Bone Marrow Puncture, and Serodiagnosis

Biopsied samples of infected tissues are used for direct visualization of amastigotes by microscopy for diagnosis when other means are not available, fail, or require confirmation. Infected tissues were collected from Indian patients by splenic aspiration (Fig. 4a, b) or bone marrow puncture (c, d), both requiring skills acquired

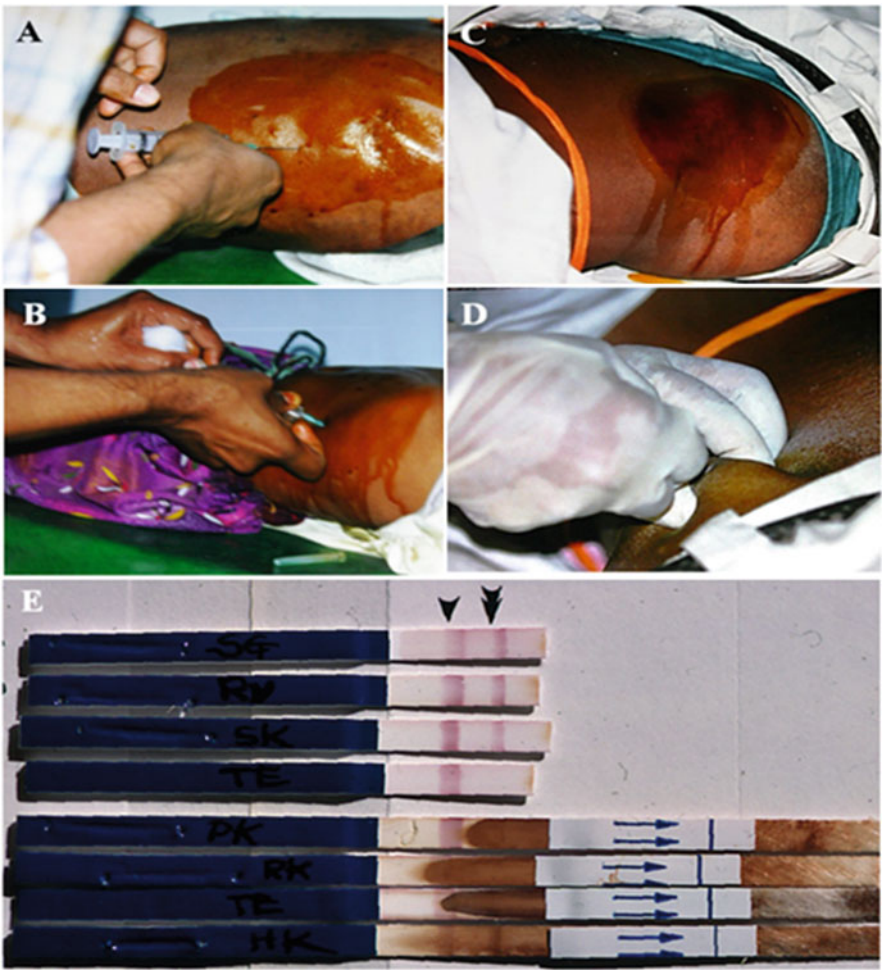

Fig. 4 Diagnosis of kala azar. Splenic aspiration (**a**, **b**) and bone marrow puncture (**c**, **d**) samples for cultivation in NNN medium and/or by microscopy of smears for the presence of amastigotes. Photos taken in 1995 during visits to kala azar clinics courtesy of Shyam Sundar (**a**, **b**) and Sarman Singh (**c**, **d**). (**e**) Serodiagnosis of kala azar with rK39 dipsticks. A drop of blood was placed in the absorbing pad at the bottom for upward migration. Positive diagnosis of kala azar is indicated by the appearance of double reaction bands (single and double arrows) and negative by the appearance of a single band (single arrow in sample four from the top). The developed bands can be read more clearly by removing the bottom of the dipsticks along with the slow migrating hemoglobin, which tends to obscure the reading (bottom four samples)

through considerable clinical experience to minimize potential complications and pains. Collection of these samples is indispensable for laboratory research. They are needed for direct use in clinical pathology/immunology investigation and for preserving the isolates in culture. For the latter purpose, infected tissues are inoculated into susceptible animals and/or into culture media for *Leishmania* replication. Successful passages of the isolates successively in vivo or in vitro facilitate their preservation and amplification to provide the large sample size often needed for many types of laboratory research.

Diagnosis of Indian kala-azar has been based on less invasive serology, such as the use of rK39 dipsticks (Fig. 4e), which requires only a drop of blood. Diluted blood is deposited on the bottom absorbing pad, whence serum immunoglobulins, including anti-K39 antibodies, migrate upward to react with incorporated reagents, producing the color products visible for reading in minutes. The emergence of two bands is indicative of the presence of anti-K39 IgG, hence positive diagnosis for kala-azar (upper three strips); the appearance of a single band validates the dipstick used but shows no detectable anti-K39 IgG and thus kala-azar negative (fourth strip from the top). The reaction bands remain readable after storage for >10 years when the bottom portion of the developed dipsticks is cut off to prevent their obscuration by the slower migrating hemoglobin (bottom four strips). The rK39 and similar rKE16 dipsticks are the principal method in clinical use for diagnosis of kala-azar with excellent specificity and sensitivity in India, as found elsewhere in Eurasian continent. Recently cured VL patients and asymptomatic subjects are also seropositive for rK39, precluding its use for prognosis of kala-azar in these cases.

2.2.3 Kala-azar Elimination Initiatives

Some of the measures described in Sect. 1.8 have been deployed in an attempt to control AVL over the decades in India. Epidemic kala-azar has however persisted with cyclic outbreaks at places in the Indian subcontinent, calling attention to an urgent need to tackle this tenacious problem with renewed vigor. In 2005, AVL elimination strategic plans were unveiled for implementation, as declared in the memorandum of understanding that was jointly signed initially by India, Bangladesh, and Nepal and later also by Bhutan and Thailand [52]. The declaration pledged to implement the programs with a targeted goal of reducing the incidence to <1 per 10,000 in 10 years. Financial, technical, and manpower resources were mobilized by national and international agencies of both public and private sectors in support of the efforts. As a result, the incidences have been substantially reduced, especially in Nepal and Bangladesh. This initial success is highly significant, providing the impetus to continue the efforts for completing the unmet goal.

The best-documented plan that followed is the "National Road Map for Kala-azar Elimination" [82] that was published by the National Vector Borne Disease Control Programme (NVBDCP), Minister of Health and Family Welfare, India, in 2014. The document (55 pages) was thoughtfully prepared with meticulous details in collaboration with stakeholders, consisting of those in the endemic states of India, e.g., ICMR- and CSIR-affiliated institutes (see below), and the regional

health/public health offices and disease centers in Patna, Kolkata, and Lucknow as well as international/foreign agencies, e.g., WHO [52], Drugs for Neglected Diseases Initiative (DNDi) [83], Médecins sans Frontières (MSF) [84], and others. Description of the plan is very thorough and in good order by covering every imaginable action needed to take at the time, including HIV coinfection. Most commendable is the inclusion of previous flaws for corrections, such as incomplete or tardy execution of prescribed plans due to late arrival or reduction of the budgeted funds. The vector control with DDT, long banned in many countries, is a glaring surprise, but it also indicates that cost-effective insecticide is not available as a suitable alternative. Replacement of DDT with pyrethroids has been indicated. The "National Road Map" does not address the issue of coordination for concerted actions with neighboring countries (but see [85]). Nor does it cover other types of existing leishmaniasis summarized in Sect. 2.1. Overall, this is a comprehensive strategic plan, which is expected to fulfill the stated missions toward its targeted goal, if implemented in full. This assessment is consistent with the evaluation of disease burdens, transmission dynamics, and other epidemiology/public health modeling by internationally renowned experts [53–56, 86].

3 Experimental Leishmaniasis

Leishmania infection to cause leishmaniasis as described has not been examined in a natural setting from parasite delivery by the sand fly bites to the subsequent evolution of the disease at cellular and molecular levels. The difficulty of examining such events in human or animal leishmaniasis in the field is obvious, necessitating laboratory investigation of such host-parasite interactions. Pioneer investigators have used human volunteers, often themselves, to verify *Leishmania* infection as the etiology of leishmaniasis, its transmission by sand fly as the vector, and its zoonosis in animals, e.g., dog as the reservoir. Further investigation of human leishmaniasis has been constrained by logistic difficulties and regulatory compliance, limiting it to clinical observations of already diseased patients, laboratory studies of their cell/tissue samples, and clinical trials of anti-leishmanial drugs and vaccines. Attempts to fill the knowledge gaps have relied on laboratory studies of cultured parasites and their interactions with the laboratory-reared vectors and animal models. For decades, such experimental studies of *Leishmania*/leishmaniasis have produced voluminous publications. Given below are a brief summary of investigation on *Leishmania* and host-parasite interactions in in vitro and in vivo experimental models.

3.1 Causative Agents

The ease of isolating *Leishmania* from field-collected samples in culture varies widely, ranging from very difficult or rarely successful in some endemic sites to

highly successful at a high rate (albeit rarely 100%) elsewhere. Information given below is based on the work with cultured parasites.

3.1.1 Cell/Molecular Biology

Leishmania are microscopic single-cell eukaryotes or trypanosomatid protozoa, each containing a full complement of the typical eukaryotic cell organelles: nucleus, nucleolus, mitochondrion, Golgi, lysosomes, and endoplasmic reticulum. Unusual cell organelles found in *Leishmania* include glycosomes [87] and acidocalcisomes [88], responsible for partitioning of the glycolytic pathway and calcium storage/mobilization, respectively. Unique to this group of protozoa are also the subpellicular microtubules as cytoskeleton and the anterior flagellar pocket equivalent to the food vacuole in other eukaryotic protists. A single flagellum originates in the flagellar pocket from kinetosome or centriole, which is located in the cytoplasm just above the kinetoplast that is packed with mitochondrial circular DNAs concatenated in large copy number. Stainability of the DNA-rich kinetoplast by polychromatic dye (Giemsa) facilitates the identification of amastigotes in tissue samples for diagnosis by microscopy. As amastigotes differentiate into promastigotes, the flagellum extends beyond the flagellar pocket as the cell body increases in length and in width. The principle function of the flagellum is to propel promastigotes forward, responsible for their mobility in the fly gut and for interactions with mammalian host cells to facilitate infection. The flagellum may also serve as a sensor for nutrients, such as glucose, considering the presence of flagellum-specific glucose transporter [89]. The lining membrane of the flagellar pocket is endowed with endocytic activities and transport mechanisms, responsible for the uptake of nutrients and drugs [90]. *Leishmania* replicate as diploid cells by mitosis and produce no morphologically distinct or identifiable sexual stages, although genetic recombination has been shown experimentally to occur as a rare event [91].

Leishmania are aerobic cells with many biochemical peculiarities. Most prominent is their inability of de novo purine and heme biosynthesis, rendering them dependent on the uptake of these or their precursor molecules as essential nutrients apparently from exogenous sources in their natural habitats (Cf. Appendix—Box 2), i.e., fly gut and the phagolysosomes of the mammalian macrophages. The metabolic pathways unique to *Leishmania* include the biosynthesis of unusual glycans, phosphoglycans, proteophosphoglycans, and lipophosphoglycans (LPG), which together with glycoproteins (leishmanolysin, gp63 protease) form the cell surface glycoconjugates. Bulk of these molecules is downregulated during promastigote-to-amastigote differentiation, suggestive of their functional significance in the insect stage and in its early interactions with host cells in the mammalian hosts. *Leishmania*-unique molecules or pathways are ideal targets to develop specific and effective drugs against leishmaniasis, but such rationale approach has never been seriously contemplated for lack of resources.

In general, the haploid genome of each *Leishmania* is ~30 megabase pairs in size. There are ~35 paired chromosomes, variable in size from several hundred base

pairs to ~2 megabase pairs each. The genome of *L. major* was first sequenced to completion in 2005. It consists of ~33 million base pairs, including >8000 putative protein-coding genes, of which ~3000 are clustered into >600 gene families, all in tandem repeats. Syntenic conservation of these repetitive gene clusters is evident as complete genome sequences became available subsequently from additional species for comparison (see [92]). *Leishmania* and related trypanosomatid protozoa regulate gene expression differently. The evolution of highly repetitive genomes in existence suggests that gene dosage effects provide a mechanism to constitutively regulate gene expression. Rapid changes of the genes in copy number via polyploidy, episomal, and chromosomal amplification are also likely to regulate their expression, judging from the presence of transposon elements scattered among the >2000 repetitive sequences per genome. Operationally during *Leishmania* growth cycle and differentiation, pre-mRNAs are Pol II transcribed polycistronically followed by their spliceosome-mediated trans-splicing into 5′-capped monocistronic polyadenylated mRNAs. In *Leishmania*, the splicing events together with UTR-mediated mRNA stability regulate the expression of >8000 protein-coding genes, while the transcription of rDNAs is Pol I mediated and promoter driven. Genomic, transcriptomic, and proteomic analyses have been completed for a number of cultured species [93–102]. Rapid advances in the next-generation sequencing and related technology are expected to further strengthen the genetic and protein databases to facilitate the identification of drug and vaccine targets.

3.1.2 Phylogenetic Taxonomy, Population Genetics, and Evolution

Leishmania is divided taxonomically into subgenus *Leishmania* and *Viannia*, consisting of some 20 different named pathogenic species, including those already mentioned in the foregoing sections. Members of the *Leishmania* subgenus exist in all continents, while those of the *Viannia* subgenus are limited to Central and South America. Some species can cause diseases as described or those of intermediate clinical symptoms. Several species are nonhuman pathogens but are normally the parasites of gerbils (*L. turanica*), guinea pig (*L. enriettii*), or lizards (*L. tarentolae*).

Phylogenetic analysis of *Leishmania* sequences or isoenzymes from cultured isolates segregates them into separate clades often corresponding to different named species, regardless of the markers or probes used [103, 104]. Specific *Leishmania* species/strains so identified in different endemic sites, however, do not always produce the same disease phenotypes, independent of subspecies sequence heterogeneity. For example, *Leishmania* cultured from CL patients in some places were identified as *L. infantum*/*L. donovani*, which are associated largely with VL in most places. Similarly, *Leishmania* grown from VL patients were typed as *L. tropica*/*L. major*—the normally CL-causing species. This is also the case often for members of the *Viannia* subgenus, causing either CL or MCL. At least for those in the *Leishmania* subgenus, this genotype-phenotype incongruence has been noted repeatedly, excluding inadvertent sample mixed-ups or other frivolous causes. Whether the selection of cultivable variants during isolation is

associated with this incongruence is unknown, pending further investigation (see Leishmaniome, Concluding remarks).

Subpopulation heterogeneity of individual *Leishmania* species collected and grown from given endemic areas has been characterized by microsatellite and other DNA analyses, predictive of their abundance, mobility, dispersal bottlenecks, and evolutionary interrelationships [105]. Similar predictions have been advanced for isolates cultured from Indian subcontinent based on whole-genome phylogenetic analysis [106]. The predictions from such studies are of interest and valuable but remain tentative, since the sample size examined is relatively small and derived from cultured promastigotes isolated from limited geographic regions. When *Leishmania* DNAs were PCR amplified directly from clinical specimens, their RFLP and sequence analysis sometimes yielded unexpected outcome, suggestive of the presence of multiple species [107] or even other nonpathogenic trypanosomatid protozoa, such as *Leptomonas* [108]. These findings raise the possibility that the sequence database from cultured isolates may not be fully representative of the causative agents in the patients, calling attention for a need to examine field-collected samples directly. Parasites grown in culture may represent cultivable geographic stocks or cultivable clones from a given clinical sample, leaving those non-cultivable ones unavailable for laboratory investigation.

3.2 Host-Parasite Interactions

Many in vitro and in vivo experimental models have been established for detailed examinations of host-parasite interactions. Here, attention will be focused on few examples of relevance to the main theme of this chapter.

3.2.1 Animal Models

All reservoir animals of zoonotic leishmaniasis, except domestic dog, are wild species, which are difficult to breed in the laboratory. Animal models used for experimental leishmaniasis thus have been limited to few laboratory animals: largely various strains of mice, sometimes hamsters and dogs, and occasionally primates [109]. The best animal model for VL or kala-azar caused by *L. donovani* is the Syrian golden hamster. This parasite can be successively passaged as amastigotes in these animals every month or two via i.v. inoculation, producing heavy splenic parasite loads and clinical signs and symptoms closely mimicking human VL (Fig. 5). However, PKDL does not develop in this or any other animal model. Human CL also can be duplicated by needle inoculation of the footpad, ear dermis, or tail base of various mouse strains, e.g., BALB/c with cultured promastigotes of some strains/species, e.g., *L. major*, *L. tropica*, *L. braziliensis*, *L. mexicana*, and *L. amazonensis*. The lesions produced can be self-healing or non-healing and become protractedly necrotic.

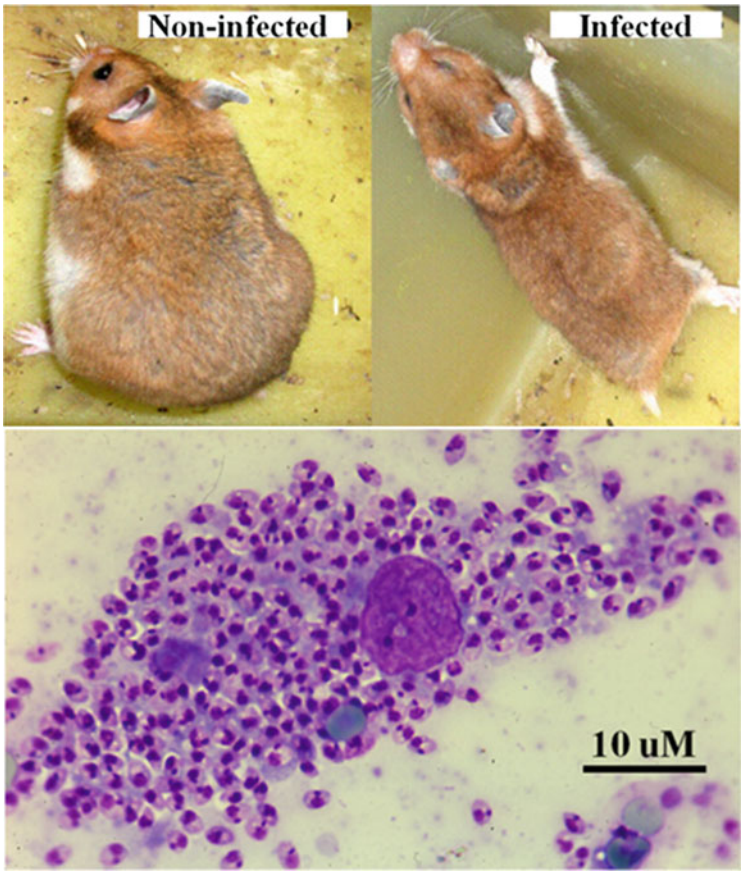

Fig. 5 Syrian Golden hamster in vivo model for experimental visceral leishmaniasis. Upper, Control (non-infected) and hamster infected with *Leishmania donovani* (infected). The infection in this model mimics the clinical signs and symptoms of chronic human kala-azar, e.g., anemia, lethargy, cachexia, weight loss and death. Bottom, Splenic impression smear from a hamster infected for >1 month, showing a macrophage replete with numerous amastigotes. Giemsa stain. Upper panel photos courtesy of Dr. Anuradha Dube, CDRI, Lucknow, India

Mice are often used as a preferred model for studying mammalian immunobiology in infectious and noninfectious diseases, including leishmaniasis (e.g., [110]). This is chiefly due to the ready availability of immunological reagents needed for qualitative and quantitative analysis of their immune mediators and cell molecules. Equally important is the availability of knockout and knock-in mutants of immunity-related genes that have been made easy and precise in mice, especially with the recently developed CRYSPR/Cas9 technology. Experimental leishmaniasis has been thus extensively studied in mice, contributing significantly to our general understanding of the immune regulatory mechanisms. Examples include the original discovery of Th1 and Th2 dichotomy and the immune mediators and

cells, responsible for mouse resistance and susceptibility to *L. major*. This line of investigation has now been expanded to multiple paradigms involving additional T-cell subsets. The outcome of such investigation is expected to further advance our understanding on the regulatory mechanisms of both innate and adaptive immunity [111], irrespective of their immediate relevance to human leishmaniasis.

Animal models for experimental leishmaniasis are needed for screening potential anti-leishmanial drugs and vaccines. These models, including mice, will be of value also for the laboratory maintenance of *Leishmania* infection and amastigote procurement.

3.2.2 Vector Models

A number of vector species, e.g., *Phlebotomus papatasi*, *P. dubosqi*, *P. sergenti*, and *Lutzomyia longipalpis*, have been successfully reared in the laboratory, providing sand fly colonies for the study of vector biology, vector-parasite interactions, and experimental transmission of leishmaniasis [7, 112]. Of considerable interest are the immunological activities of the sand fly saliva proteins as vaccines/adjuvants in experimental leishmaniasis [113–115]. Vaccination of animal models with saliva antigens protects them from challenges with *Leishmania* delivered by the bites of infected vectors [29, 116]. Such observations of fly saliva are novel, but unique to experimental leishmaniasis, since there have been little or no similar findings with other vector-borne diseases, e.g., mosquito-transmitted malaria.

3.2.3 *Leishmania*-Macrophage In Vitro Models

It has long been known that *Leishmania* parasitized patients by taking residence exclusively in their macrophages—mononuclear phagocytes that normally ingest and digest invading pathogens. *Leishmania* infection of primary cultures or cell lines of human and animal macrophages thus has been extensively studied in vitro. Figure 6 presents some essential elements and events of the infection in such in vitro models, i.e., in vitro cultured promastigotes (a) and their transfectants with green fluorescent protein (b), the attachment of promastigotes to macrophages (c), and intracellular entry of promastigotes and their differentiation into amastigotes in a parasitophorous vacuole (PV) (d, e). The intracellular amastigotes of some species are amenable to isolation from heavily infected macrophages of the J774 cell lines (Fig. 6f).

***Leishmania* infection of macrophages** with different species shows common features but also differences among different host-parasite combinations used. Receptor-mediated endocytosis is generally accepted as the mechanism for the entry of promastigotes into macrophages, involving the binding of promastigote surface molecules, e.g., LPG and gp63, as the ligands with multiple receptors of the macrophages examined [117–119]. *Leishmania* surface protease gp63 has been proposed to suppress the induction of noncoding Alu RNA and 7SL

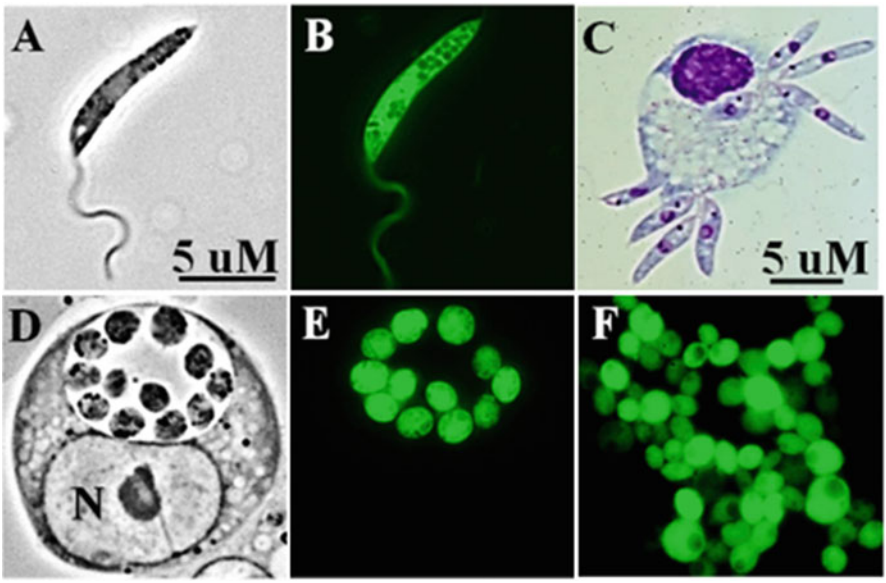

Fig. 6 *Leishmania*-macrophage in vitro model for studying host-parasite cellular interactions. (**a**, **b**) In vitro cultured promastigotes and their GFP (green fluorescence protein) transfectants, respectively; (**c**) Attachment of promastigotes to a hamster macrophage; (**d**, **e**) Differentiation of endocytosed promastigotes into amastigotes and replication of the latter in a communal parasitophorous vacuole of a macrophage; (**f**) Amastogotes isolated from infected culture of J774 macrophage line. (**a**, **d**): Phase contrast; (**b**, **e**, **f**): GFP fluorescence; (**c**) Giemsa-stained methanol-fixed sample

RNA-mediated inflammatory and cytotoxic activities of macrophages, thus favoring the establishment of *Leishmania* infection in these otherwise hostile immune cells [120, 121]. The endocytosed promastigotes end up in the endosome-lysosome vacuolar system [122–124] (Cf. Appendix—Box 3 and Commentary) where they differentiate into amastigotes and replicate in this acidic hydrolytic environments. Indeed, under the conditions of lysosomal acidic pHs and mammalian body temperatures, some *Leishmania* spp. do differentiate from promastigotes into amastigotes and replicate continuously as axenic amastigotes in the absence of macrophages. Recent evidence indicates that ferrous reductase/iron transporter plays a role in *Leishmania* differentiation for successful intracellular parasitism [125] and that a unique glycan with terminal N-acetylgalactosamine of *L. donovani* plays a role in its infectivity to macrophages and antimonial resistance [126]. *Leishmania* infection suppresses the functions of macrophages, as indicated by the unresponsiveness of infected cells to signals for immunity-eliciting events, i.e., respiratory burst, IFN-γ production, inflammasome activation, and antigen presentation due to sequestration of MHC molecules to the parasitophorous vacuolar membrane. Comparative analysis of the transcriptome profiles between infected and noninfected macrophages has revealed these and other differences.

The parasitophorous vacuoles (PVs) where *Leishmania* take residence for replication are unique. They are not an exclusive or secluded intracellular site but in continuum with the extracellular milieu of the infected macrophages via their endocytic pathway. There are different types of PVs (Fig. 7): (1) fluid-filled large PVs, each containing amastigotes in variable numbers (a); (2) small PVs, each containing one to several amastigotes (b); and (3) tight-fitting PVs, each with a single amastigote without visible vacuolar space (not shown). Fluorescent macromolecules, e.g., FITC-dextran endocytosed by infected macrophages, emerge in the PVs, regardless of whether they are the large ones produced by *L. amazonensis* (Fig. 7a–c) or the small ones by *L. tropica* (Fig. 7d, e). Similarly, infected macrophages may endocytose other substances, like hemoglobin or erythrocytes, and shuttle them into the PV, thereby providing heme and other essential nutrients to the *Leishmania* therein (Cf. Appendix—Box 2). Intra-PV amastigotes are thus nutritionally less host cell dependent than obligate intracellular pathogens. This is supported by the successful cultivation of some *Leishmania* species as axenic amastigotes under host cell-free conditions.

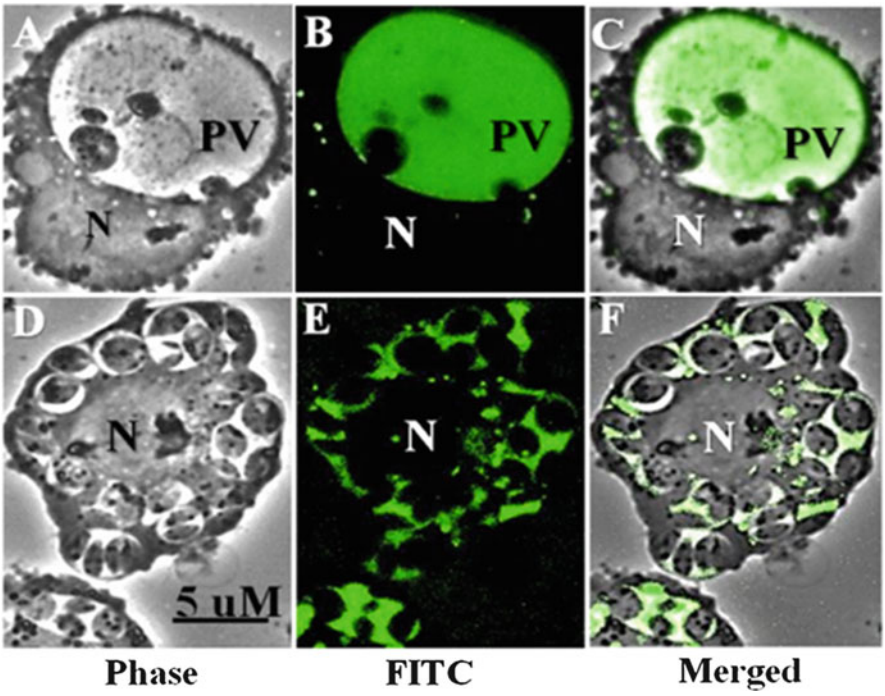

Fig. 7 Accessibility of the endosome/lysosome-dwelling amastigotes to the extracellular milieu of *Leishmania*-infected macrophages. (**a** and **d**) Large and small parasitophorous vacuoles (PV) produced by *Leishmaia* infection of J774 macrophages, respectively. Addition of FITC-dextran (MW = ~185,000) to the infected culture resulted in fluorescent PVs (**b**, **c** and **e**, **f**). Such intra-phagolysosomal parasitism is rare and has significance in nutritional requirements of intracellular *Leishmania*, chemotherapy of leishmaniasis and host immunity

***Leishmania* metastasis** is typically depicted to result from the infection of previously noninfected macrophages by amastigotes that are set free after the disintegration of heavily parasitized cells (Fig. 1, Upper). An additional or alternative mode of metastasis has been proposed. Heavily parasitized, albeit still intact macrophages may be recognized as degenerating or damaged cells by the immune system and thus marked for clearance by "scavenging phagocytosis." Metastasis of amastigotes in this way has the potential advantages of allowing them to avoid leishmanolytic factors present in the extracellular milieu and to infect multiple host cells simultaneously.

3.2.4 Future In Vitro and In Vivo Models

The in vitro studies of host-parasite cellular and molecular interactions have made considerable head way with the advent of new molecular, immunological, and imaging tools. Together with the in vivo animal models, the use of these tools has created a rich source of imaginative ideas in conjunction with the advances in immunobiology. The early host-parasite interactions are clearly a critical phase in natural infection to set the stage for the development of leishmaniasis. Exactly how the parasite, vector, and host molecules interact for *Leishmania* to achieve a successful parasitism still remains enigmatic (Cf. Appendix—Box 3 and Commentary). Attention has been devoted to many different areas of investigation, especially the signal pathways of toll-like/nod-like receptors in innate immunity. The intricacy of such pattern/danger recognition systems in relation to the adaptive immunity to *Leishmania* has been under intensive investigation [127]. Two additional areas of relevance are worthy of mention. One is the intervention of other nonimmune and immune cells in the initial host-parasite interactions, including the early responding neutrophils [128]. Another is the discovery of exosomes, which were reported to originate from *Leishmania* and/or *Leishmania*-infected cells for delivery of mediators to regulate the activities of distant immune cells [129]. These interesting laboratory observations await further investigation for extrapolation to natural infection. Laboratory findings like these have the potential to provide explanations for and may lead to clinically relevant applications. One example is the therapeutic effectiveness of amphotericin B that is dramatically enhanced when encapsulated in liposomes. This is due in large part to their increased endocytic uptake by infected macrophages, thereby targeting the drug specifically to the amastigotes in their PV, like FITC-dextran as shown in Fig. 7.

Extrapolation of most laboratory findings to the real world of clinical leishmaniasis still requires our additional efforts to develop effective tools, for example, in vitro 3D human tissue/organ models, realistic or virtual systems, and humanized animals. Infection of a hematopoietically humanized mouse model with *L. major* produced encouraging outcome, showing its potential utility for assessing human immune responses and for drug screening [130]. Further improvement of this and other models is expected to facilitate the verification of laboratory findings more readily for their clinical relevance.

3.3 Leishmania *Model for Microbial Virulence [131, 132]*

Figure 8 presents a hypothetical scenario to explain how *Leishmania* causes leishmaniasis and its resolution. The progression of leishmaniasis can be divided into three phases: (1) incubation period, the early stage of infection by vector-delivered promastigotes before any noticeable disease signs or symptoms; (2) disease phase, subsequent development of the amastigote-induced clinical symptoms and signs; and (3) resolution, spontaneous or chemotherapeutic cure followed by lasting immunity (Fig. 8, steps I–III). The manifestation of these three phases presumably results from the host immune response (or the lack of it) to the three different sets of *Leishmania* molecular determinants.

The first set consists of *Leishmania* invasive/evasive determinants, which allow promastigotes to overcome the innate immunity of the host for successful parasitism of its macrophages. This set includes mainly the promastigote surface and secretory products, frequently referred to as "virulence factors" in the literature. Their functional significance in the sequential order of infection is listed as follows: (1) resistance to antimicrobial cellular and soluble factors in the host body fluids, as first encountered by promastigotes after entry, (2) promotion of their phagocytosis by macrophages for residence in the phagosome-lysosome vacuolar compartment of these phagocytes, (3) neutralization of the antimicrobial factors in this compartment for the survival of promastigotes and their differentiation into amastigotes, and (4) modifications of the infected cells to avoid immune surveillance and facilitate amastigote replication (Cf. Appendix—Box 3 Commentary). The

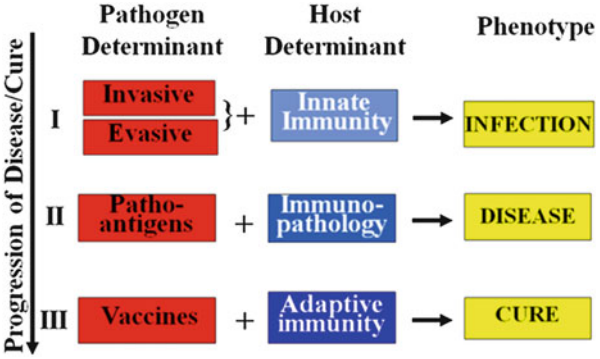

Fig. 8 Diagrammatic depiction of host-parasite interactions in leishmaniasis. The progression of leishmaniasis is depicted to follow sequential events (**infection, disease and cure**), resulting from the interactions of multiple sets of pathogen molecule and host immune determinants. **Invasive/evasive determinants and innate immunity**, *Leishmania* molecules responsible for invasion of host/host cells and evasion of host innate immunity; **Pathoantigens and disease**, Putative parasite molecules responsible for immunopathology manifestation of the disease; **Vaccines and cure,** *Leishmania* antigens responsible for activation of adaptive immunity to effect a cure with lasting immunity that occurs spontaneously or after chemotherapy. Independent regulation of these determinants is proposed to account for different clinical outcomes observed (see text)

invasive/evasive determinants are often downregulated as promastigotes differentiate into amastigotes and are not known to cause pathology in the host. However, intra-macrophage parasitism due to the activities of these determinants is a prerequisite for the subsequent manifestation of virulence in leishmaniasis.

The second set of determinants is of amastigote origin and referred to as the disease-causing "pathoantigens." Their interaction with the immune system is envisioned to result in immunopathology, responsible for the main clinical manifestations in leishmaniasis. The best evidence for this is the hyperimmunoglobulinemia that is a well-known clinical sign of VL, marked by a reversal of the normal albumin: IgG ratio in patients' sera. The excessive IgGs produced include anti-*Leishmania* antibodies, which are specific largely to amastigote intracellular antigens and are thus non-protective. Instead, these and other antibodies form aggregates and immune complexes, producing kidney, hematological, and other disorders. The B-cell epitopes for some of these *Leishmania* molecules are known, as they have been categorized and identified for use as the antigens for serodiagnosis, e.g., the 39 aa repeats of kinesin (rK39) [12]. Other clinical symptoms of leishmaniasis may be associated with the same or different *Leishmania* "pathoantigens," which may be identified as pathological T-cell epitopes. Inference of their existence finds examples in the immunopathology models for microbial, viral, and autoimmune diseases.

The third set of *Leishmania* determinants is the naturally occurring immunity-inducing vaccines. Their existence is indicated by the clearance of the disease followed by the development of lasting immunity in patients after spontaneous or therapeutic cure of leishmaniasis. Leishmanization is the crudest form of effective immunization that makes use of the full complement of all-natural vaccines present in live *Leishmania*, as does the suicidal mutants genetically or chemically modified in vitro. The natural vaccines may include the peptides, which have been shown to confer prophylactic activity in vaccination trials. Ongoing efforts to produce more effective vaccines are expected to identify additional molecules of these *Leishmania* determinants.

The proposed model makes it possible to explain the spectrum of *Leishmania* virulence (as defined by the severity of the clinical outcomes) solely on the basis of up- and downregulations of specific parasite determinants. For example, asymptomatic infection and non-healing disease may result from downregulation of pathoantigens and vaccine molecules, respectively. The proposed model is applicable to the general population, consisting of mostly immune-competent individuals. The roles of the parasite determinants in regulating *Leishmania* virulence become more complicated and less predictable for the immunocompromised subpopulations. Immunosuppression is known to result from human genetic defects, malnutrition (Cf. Appendix—Box 1), HIV coinfection, or other causes. These immunocompromised subpopulations suffer from systemic immune dysfunction and paralysis, rendering them generally more susceptible to many different infectious diseases. Immunopathology produced under the circumstances is less predictable based on the model presented.

4 Basic and Applied Kala-azar Research in India

A vibrant and active biomedical research community has long existed in India, under the aegis of both national and international organizations, for basic, clinical, and other researches of kala-azar. There are internationally sought-after kala-azar clinics in the well-established endemic sites, drug/vaccine production facilities, and many kala-azar research laboratories. Nowhere else in the world can one find another country, except perhaps Brazil, to match India in the scale of dedication, devotion, and contribution to kala-azar research. Given below are examples of some Indian institutions and recent kala-azar research to illustrate the points.

4.1 Indian Institutions with Kala-azar Research Components

Some Indian government agencies, which provide administrative and financial support:

CSIR: Council of Scientific and Industrial Research http://www.csir.res.in/
DBT: Department of Biotechnology http://www.dbtindia.nic.in/
ICMR: Indian Council of Medical Research http://www.icmr.nic.in/
DST: Department of Science and Technology http://www.dst/gov.in

Some institutions with active kala-azar research laboratories:

All India Institute of Medical Science (AIIMS), New Delhi, http://www.aiims.edu/en/component/search
Banaras Hindu University Institute of Medical Sciences (BHU-IMS), Varanasi, http://www.bhu.ac.in/ims/
Central Drug Research Institute (CSIR-CDRI), Lucknow, http://www.cdriindia.org/home.asp
Indian Institute of Chemical Biology (CSIR-IICB), Kolkata, http://www.iicb.res.in/
Jawaharlal Nehru University (JNU), New Delhi, http://www.jnu.ac.in/
Kala-Azar Medical Research Center (KAMRC), Muzaffarpur, http://www.tuugo.in/Companies/kala-azar-medical-research-center/0150003454869#!
National Institute of Immunology (DBT-NII), New Delhi, http://www.nii.res.in/
National Institute of Pathology (ICMR-NIP), New Delhi, http://instpath.gov.in/
National Center for Cell Science (DBT-NCCR), Pune, http://www.nccs.res.in/
Rajendra Memorial Research Institute of Medical Sciences (ICMR-RMRI), Patna, http://www.rmrims.org.in/
Jamia Hamdard (University), New Delhi, http://www.jamiahamdard.edu

Not listed are also many other additional public and private laboratories and clinics in India.

4.2 Indian Kala-azar Research

The work that originated from Indian institutions covers a full range of disciplinary areas. This can be illustrated by a brief summary of randomly selected examples after scanning recent publications by PubMed search. Epidemiology studies have long been undertaken for both CL [69, 75] and VL [55, 56, 133] with the continuation by the local health stations expected in all endemic areas. There are publications pertinent to the origin, epidemiological significance, and treatment of Indian PKDL [81, 134]. Of epidemiological interest are the work, which questions the IRS efficacy of DDT for vector control [135], and the preliminary observation of promastigote growth from goat, implying its reservoir potential (S. Singh, Personal communication [136]). Confirmation of both will figure significantly in kala-azar elimination initiatives. Asymptomatic human infection has been well documented clinically by multiple groups with the recognition of its significant implication in epidemiology, posing challenges to the control programs [23–26]. For serodiagnosis, a variant version (rKE16) of rK39 was developed commercially, showing excellent specificity and sensitivity in rapid test formats for Indian kala-azar [11] and for *Leishmania*-HIV coinfected cases [137]. Most exciting is the recent report of dipsticks using urine for noninvasive diagnosis of AVL and PKDL [138]. In clinical immunology, T-cell regulatory cytokines were analyzed [139–141] that have relevance to immunotherapy of VL patients [142]. In experimental immunology, anti-*Leishmania* immune response was reviewed with a focus on TLR-CD40 cross talk [143]. Of relevance are two laboratory findings: *Leishmania* tyrosyl tRNA-synthase mimicry of host chemokine [144] and the therapeutic implication of cholesterol-mediated MHC conformational changes in relation to CTL activities [145]. Further translational research of such findings will be of interest. Of significance are the experimental studies on the apoptosis of *Leishmania* (Cf. Appendix—Box 4) and autophagy of infected macrophages (Cf. Appendix—Box 1), both having biological and immunological implications in regulating host-parasite interactions. Clinical trials for the efficacy of anti-leishmanials, e.g., AmBisome and paromomycin [38, 39], have been completed and alternative treatment options developed [35]. Chemotherapy of kala-azar was comprehensively reviewed [34–36] and studied by screening compounds such as enzyme inhibitors, e.g., pyrimidine analogues [146], and for identification of drug targets, e.g., pteridine reductase [147], lipid antigen delivery [148], Aurora kinase [149], nucleoside diphosphate kinase [150], and screening natural and other products and development of delivery strategies [151, 152] for anti-leishmanial activities. Vaccine development has received perhaps the greatest attention, as indicated by the publication of many reviews on this subject (e.g., [30–33]) and half a dozen potential vaccine candidates under investigation, e.g., centrin gene knockouts [153, 154], KMP-11 [155], kinesin motor domain [156], ORF-F DNA [157], hemoglobin receptor [158, 159], and NAD-dependent SIR-2 protein [160]. A multivalent vaccine, consisting of all these candidates, may be of interest to develop. Application of -omic biotechnology has been accomplished by different

laboratories for genotype and phenotype analysis of *L. donovani* [94–101] and vector [161]. A surprise finding is *Leptomonas* grown from VL patients based on the genomic analysis [108].

The summary provided above is not an exhaustive literature review but exemplifies the depth and breadth of kala-azar research in India.

5 Concluding Remarks

5.1 "Leishmaniome" and Diversity of Leishmaniasis

The study of leishmaniasis in South Asia has identified two issues, which are of importance to consider for their relevance to the control of this disease complex at large.

One issue is related to the findings that *Leishmania* are taxonomically more divergent than expected in some endemic sites. Phylogenetic analysis of sequence data [104] showed such divergence in the CL samples from the Satluj river valley (Fig. 2, blue), suggesting that *L. infantum* and *L. tropica* are present in different CL patients and even coexist in a single patient lesion [76]. Lending credence to this unexpected finding is a similar conclusion that is also based on sequence analysis of the CL samples, albeit from a different endemic site, i.e., the hilly southeast Turkey [107]. Notable in both studies is the use of limited materials from the original lesions instead of promastigotes grown from the amastigotes therein, since they are difficult to culture in vitro. These inadvertent observations raise the question of whether promastigotes grown from infected tissues are indeed, as often assumed, representatives of all *Leishmania* in given endemic sites. The same issue was also raised by the findings that *Leptomonas* instead of *L. donovani* emerged in culture from the splenic aspirates of kala-azar patients [108] and in association with PKDL isolates [162] based on genomic sequencing. While further confirmation of this *Leishmania-Leptomonas* coexistence is desirable, the cross-taxon sequence heterogeneity seen in infected tissues is suggestive of "leishmaniome," akin to "microbiome." While leishmaniome is not expected to be as divergent as microbiome of our gut flora, its prevalence in other endemic sites warrants investigation by examining amastigotes directly in infected tissues in addition to cultured promastigotes. This is doable by laser microdissection microscopy of infected tissues for isolating individual amastigotes followed by single-cell whole genomic sequencing. Leishmaniome, if verified to exist in many endemic sites, would have significant implications in considering all aspects of leishmaniasis, including the identification of the true culprits of the disease as the right target for investigation.

Another important issue is the necessity of studying the different epidemiological types of leishmaniasis in India and its neighboring countries (Fig. 2). The current efforts focus only on kala-azar and PKDL. This inattention to the remaining disease types will increase the risk of their persistence and spread. In all endemic areas with different disease types, VL patients have been reported and often

considered as imported from the AVL/PKDL endemic areas. Investigation of these cases is needed to rule out the possibility that they may be in fact transmitted locally by indigenous vector and *Leishmania* species. Importation of leishmaniasis from one endemic area to another is expected to increase with increasing population mobility, and clinical manifestations of the patients are unreliable to distinguish different epidemiological types, e.g., ACL/ZCL versus PKDL and AVL versus ZVL. A full-fledged, long-term investment is needed to investigate all epidemiological types in different endemic sites—an indispensable element in the road map toward the success of the kala-azar elimination programs.

5.2 *Issues Emerged from South Asia Kala-azar Elimination Initiatives*

Section "Kala-azar Elimination Initiatives" briefly summarizes the current road map to kala-azar elimination in South Asia. Unforeseen roadblocks are expected to emerge, requiring timely attention during the appropriate phases of program implementation.

Policy and management issues are more amenable to rectification as they emerge, such as the need:

1. To develop consensus parameters for assessing progress and endpoint of kala-azar elimination jointly by the pledged countries.
2. To expand vector studies and unify the control measures, i.e., the use of DDT in India versus pyrethroids in Bangladesh and Nepal. It is understood that India has phased out DDT and is now using pyrethroids.
3. To formulate a uniform policy for developing vaccines by Indian companies, i.e., Zydus and Gennova.
4. To consolidate the strategies for effective chemotherapy. These include the use of DNDi miltefosine and paromomycin combination, the availability of clinics needed for delivering one-shot-to-cure AmBisome, and mitigation of patients' cross-resistance to both miltefosine and pentamidine.

Recognition of these emerging issues by the authority offers the opportunity for policy adjustments, thereby making these problems more manageable. Long-term investment will be needed for in-depth laboratory studies and field work of sand fly vectors and drug-resistance mechanisms.

Development of "biomarkers" is crucial for successful implementation of kala-azar elimination programs, such as differentiation of cured from relapse cases and diagnosis of drug-resistant, PKDL, and asymptomatic cases. Asymptomatics account for as much as 4% of the "Musahars" subpopulation in Bihar, but this needs to be ascertained. "Reliable" biomarkers to specifically determine all these different clinical and subclinical cases are inherently difficult to develop. "Leishmaniome" approach may offer some hope for finding unique *Leishmania*

sequences for evaluation. Methodology is available to detect such sequences in the infected tissues directly by PCR or patients' antibodies to their products. Identification of phenotype-specific antigens will facilitate assays of the host response to them by DTH and cytokine release.

5.3 Indian Leadership in One-Health Approach to Research Collaboration

Sections 4.1 and 4.2 briefly introduced the extent of Indian administrative, clinical, and research institutions, research laboratories, and diversity. The depth and breadth of this enterprise are substantial, putting India, with or without outside input [163], as a de facto leader in kala-azar research. Provision of additional incentives will further strengthen the already ongoing interlaboratory, interinstitutional, and international collaborative research activities as well as to foster One-Medicine, One-Health [164], and One-World [19] approach for closer integration of different disciplinary areas (Cf. Appendix—Box 5). "*Leishmania* without border" is evident from the existence of different clinico-epidemiological types of leishmaniasis that spread in different countries with contiguous areas of similar geographic landscape and topology in South Asia (Fig. 2). Cross-border collaboration to elucidate the relationship of various leishmaniasis types is desirable to hasten the goal toward kala-azar elimination.

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Appendix

Box 1: Malnutrition, Autophagy, and Susceptibility to Kala-azar

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There is an interesting relationship between nutritional deficiency and aggravation of kala-azar. Kala-azar patients in Bihar are malnourished. Starvation induces autophagy. When autophagy is triggered in macrophages in vitro either by pharmacological mediators or by starvation, infection of these cells with SAG-resistant *Leishmania donovani* (LD) results in its exuberant intracellular replication (1). Interestingly, this was not seen when these macrophages were infected with SAG-sensitive parasites (1). The autophagic cells after infection undergo apoptosis, which then may favor parasites to egress and accelerate cell-to-cell transmission and dissemination (1), as shown in the cases of a wide variety of bacteria and apicomplexan parasites (2). In our earlier work, GP63 was shown to cleave dicer that inhibits maturation of miR 122, which constitutes ~80% of the hepatic microRNAs and is important for lipid metabolism (3). This is known to cause hypocholesterolemia, as generally noted to be severe among kala-azar patients (4). The cholesterol level in some patients is lowered to one-tenth of the normal level. It is well known that cholesterol is important in maintaining the conformation of membrane proteins like acetylcholine receptor and serotonin receptor (5), MHC-II

(continued)

Box 1 (continued)

protein (6, 7), and also for the lateral mobility of membrane protein (8). *Leishmania* infection of antigen-presenting cells, like macrophages and dendritic cells, has been shown to significantly alter the kinetic parameters of peptide-MHC-II stability (K_{on} and K_{off} kinetics), resulting in immune dysfunction (9). This is perceived as part of the mechanisms (10), coupled with decrease in membrane cholesterol (11, 12) by which intracellular LD manipulates host metabolic pathways and contributes to the aggravated pathogenesis. Thus, autophagy pathway may contribute to aggressive infection in the mammalian host by the antimony resistant LD as compared to the sensitive ones. Metabolic dysfunction induced by the LD infection may contribute to the establishment of the infection in the mammalian host.

References

1. Mukherjee B, Mukhopadhyay R, Sundar S, Dujardin J-C, Roy S. Probing the strategies of antimony resistant *Leishmania donovani* to induce aggressive infection. Session "Molecular basis of virulence & Tropism", 6th World Congress of Leishmaniasis, May 16–20 Toledo, Spain. 2017.
2. Friedrich N, Hagedorn M, Soldati-Favre D, Soldati T. Prison break: pathogens' strategies to egress from host cells. Microbiol Mol Biol Rev. 2012;76(4):707–20.
3. Ghosh J, Bose M, Roy S, Bhattacharyya SN. Cell Host Microb. 2013;13:277–88.
4. Ghosh J, Lal CS, Pandey K, Das VNR, Das P, Roy Choudhuri K, Roy S. Ann Trop Med Hyg. 2011;105:267–71.
5. Gimpl G. Cholesterol protein interaction: methods and cholesterol reporter molecules. Subcell Biochem. 2010;51:1–45.
6. Roy K, Ghosh M, Pal TK, Chakrabarti S, Roy S. J Lipid Res. 2013;54:3106–15.
7. Roy K, Mandloi S, Chakrabarti S, Roy S. PLoS Neg Trop Dis. 2016;10: e0004710.
8. Ghosh M, Roy K, Das Mukherjee D, Chakrabarti G, Roy Choudhury K, Roy S. PLoS Negl Trop Dis. 2014;4(8):e3367.
9. Roy K, Naskar K, Ghosh M, Roy S. J Immunol. 2014;192:5873–80.
10. Chakraborty D, Banerjee S, Sen A, Banerjee KK, Das P, Roy S. J Immunol. 2005;175:3214–24.
11. Sen S, Roy K, Mukherjee S, Mukhopadhyay R, Roy S. PLoS Pathog. 2011;7:e1002229.
12. Benerjee S, Ghosh J, Sen S, Guha R, Dhar R, Ghosh M, Datta S, Raychaudhury B, Naskar K, Holder AK, Las CS, Pandey K, Das VNR, Das P, Roy S. Infect Immun. 2009;77:2330–42.

Box 2: *Leishmania* Acquires Heme from Host Hemoglobin

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A rational approach to search for a novel drug/vaccine target against intracellular pathogens is the exploitation of biochemical differences between the parasite and its mammalian host. *Leishmania* is auxotroph for heme, as the parasites lack complete heme biosynthetic pathway (1). Heme is a critical prosthetic group required by the parasites for several metabolic pathways. Thus, heme acquisition process in *Leishmania* could be a potential target (2). However, how parasites acquire heme is not well depicted. Interestingly, it has been shown that *Leishmania* expresses a high-affinity receptor for hemoglobin (Hb) in the flagellar pocket of the parasites (3). Hemoglobin first binds to this high-affinity receptor (HbR) and endocytosed via a clathrin-mediated process (4). Subsequently, Hb is internalized into early endosomal compartment in the parasite via Rab5-regulated process (5, 6). Finally, internalized Hb is targeted to the parasite lysosomes by Rab7-dependent process where it is degraded to generate intracellular heme, which parasites use for their survival (7). Interestingly, it has been shown that HbR is a surface-localized hexokinase, a glycolytic protein (8). Thus, HbR regulates two major functions in parasite: (a) it acts as Hb receptor on cell surface to acquire heme and (b) it also regulates glycolysis. Moreover, it has been shown that blocking the Hb uptake by anti-receptor antibody or inhibiting the targeting of internalized Hb to the lysosomes is detrimental for the parasites, rendering them unable to acquire heme from Hb degradation. In addition, it has been shown that newly synthesized HbR exit the endoplasmic reticulum (ER) via COPII-regulated process and targeted to the cell surface by Rab1-independent unconventional secretory pathway (9, 10). Interestingly, knocking down of these regulatory proteins by specific siRNA inhibits parasites' growth. These results unequivocally prove that parasites acquire heme from Hb.

As HbR is found to regulate two major functions in parasite, therefore HbR could be a potential new target. Consequently, HbR is evaluated as potential vaccine candidate against visceral leishmaniasis. It has been shown that vaccination of mice and hamsters with HbR-DNA constructs inhibits more than 99% splenic and hepatic parasite burden in comparison to infected and vector control animals. It has been shown that impaired T-cell response and inhibition of IL-2 production are associated with VL. Interestingly, it has been shown that HbR vaccination can reverse the impaired T-cell response with higher production of IL-2 and induce Th1 protective response (11). These results demonstrate that HbR-DNA immunization offers major advantages over other vaccine candidates against VL because it is functionally important in the parasite life cycle, conserved across various *Leishmania* species, and naturally immunogenic in VL patients.

(continued)

Box 2 (continued)

References

1. Sah JF, Ito H, Kolli BK, Peterson DA, Sassa S, Chang KP. Genetic rescue of *Leishmania* deficiency in porphyrin biosynthesis creates mutants suitable for analysis of cellular events in uroporphyria and for photodynamic therapy. J Biol Chem. 2002;277:14902–9.
2. Kelly JX, Ignatushchenko MV, Bouwer HG, Peyton DH, Hinrichs DJ, Winter RW, Riscoe M. Antileishmanial drug development: exploitation of parasite heme dependency. Mol Biochem Parasitol. 2003;126:43–9.
3. Sengupta S, Tripathi J, Tandon R, Raje M, Roy RP, Basu SK, Mukhopadhyay A. Hemoglobin endocytosis in Leishmania is mediated through a 46 kD protein located in the flagelar pocket. J Biol Chem. 1999;274:2758–65.
4. Agarwal S, Rastogi R, Gupta D, Patel N, Raje M, Mukhopadhyay A. -Clathrin-mediated hemoglobin endocytosis is essential for survival of Leishmania. BBA Mol Cell Res. 2013;1833:1065–77.
5. Singh SB, Tandon R, Krishnamurthy G, Vikram R, Sharma N, Basu SK, Mukhopadhyay A. Rab5 mediated endosome-endosome fusion regulates hemoglobin endocytosis in Leishmania donovani. EMBO J. 2003;22:5712–22.
6. Rastogi R, Kapoor JKV, Langsley G, Mukhopadhyay A. Rab5 isoforms specifically regulate different modes of endocytosis in Leishmania. J Biol Chem. 2016;291:14732–46.
7. Patel N, Singh SB, Basu SK, Mukhopadhyay A. Leishmania requires Rab7-mediated degradation of endocytosed hemoglobin for their growth. Proc Natl Acad Sci USA.2008;105:3980–5.
8. Krishnamurthy G, Vikram R, Singh SB, Patel N, Agarwal S, Mukhopadhyay G, Basu SK, Mukhopadhyay A. Hemoglobin receptor in leishmania is a hexokinase located in the flagellar pocket. J Biol Chem. 2005;280:5884–91.
9. Bahl S, Parashar S, Malhotra H, Raje M, Mukhopadhyay A. Functional characterization of monomeric GTPase Rab1 in the secretory pathway of Leishmania. J Biol Chem. 2015;290:29993–30005.
10. Parashar S, Mukhopadhyay A. GTPase Sar1 regulates the trafficking and secretion of the virulence factor gp63 in Leishmania. J Biol Chem. 2017. doi:https://doi.org/10.1074/jbc.M117.784033 (in press).
11. Guha R, Gupta D, Rastogi R, Vikram R, Krishnamurthy G, Bimal S, Roy S, Mukhopadhyay A. Vaccination with Leishmania hemoglobin-receptor-encoding-DNA protects against visceral Leishmaniasis. Science Transl Med. 2013;5:202ra121.

Box 3: *Leishmania* Survive in Phagolysosomes (Misnomer)

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Ref: Verma JK, Rastogi A, Mukhopadhyay A. Leishmania donovani resides in modified early endosomes by upregulating Rab5a expression via the downregulation of miR-494. Plos Pathog. 2017;13:e1006459.

Several intracellular pathogens block the phagosome maturation to lysosomes in the host cells for their survival. Rab GTPases are the central regulators of membrane trafficking pathways; therefore, most of the intracellular pathogens modulate the function of host endocytic Rab GTPase specially the Rab5 to inhibit their lysosomal targeting. In contrast, *Leishmania* are thought to reside in phagolysosomal compartment in mouse macrophages as the *Leishmania*-containing parasitophorous vacuole (PV) recruits lysosomal markers such as Lamp1, Lamp2, and cathepsin D. However, how parasites survive in such a detrimental compartment in a cell is not well demonstrated. Recently, we have shown that *Leishmania donovani* specifically upregulates the expression of Rab5a by inhibiting the synthesis of miR-494 in human macrophages which negatively regulates the expression of Rab5. *Leishmania* downregulates the expression of miR-494 by degrading c-Jun via their metalloprotease gp63. Subsequently, *L. donovani* recruits and retains these overexpressed Rab5a along with early endosome-associated antigen (EEA1) on PV to reside in early endosomes. Recruitment of Rab5a on *Leishmania*-containing PV promotes fusion with early endosomes to inhibit transport to the lysosomes. Finally, we have found that the parasite also modulates the early endosome by recruiting Lamp1 and inactive pro-cathepsin D on PV via the overexpression of Rab5a in human macrophages. Thus, *Leishmania* resides in early endosomes not in phagolysosomes as thought earlier. But PV also recruits lysosomal enzymes in immature and inactive form in human macrophages which help the parasites to survive in human macrophages.

Interestingly, overexpression of Rab5 by downregulating the synthesis of miR-494 happens only in human and hamster macrophages, but not in mouse macrophages as miR-494 target site is absent in the 3'-UTR of mouse Rab5a. Thus, our results unequivocally prove that *Leishmania* resides in modified early endosomes in human macrophages but also resolve the controversy why it was thought that *Leishmania* resides in phagolysosomal compartment using mainly mouse macrophages. Thus, these results also indicate why among the two animal models of leishmaniasis, hamster model mimics human infection, whereas *Leishmania* infection is self-healing in mouse.

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Box 3 (continued)

Commentary on "*Leishmania* Survive in Phagolysosomes: Misnomer" by KP Chang

Since the 1970s, *Leishmania* have been recognized as a phagolysosomal parasite of the macrophages—its exclusive host cells in susceptible animals. This conclusion was drawn by a number of early investigators from their work on *Leishmania* infection of macrophages in vitro and in vivo in animal models. In infected macrophages, *Leishmania*-containing vacuoles (PVs) and phagolysosomes are congruent in their physical and chemical properties, as shown by multiple experimental approaches, i.e., (a) particulate or fluorescent tags of the secondary lysosomes emerge in the endosomes, which contain *Leishmania*, e.g., *L. donovani*, in human peripheral blood monocyte-derived macrophages (1); (b) acidity of the PVs, as measured under living conditions of *L. donovani*-infected macrophages based on pH-dependent changes in the fluorescence intensity of FITC-dextran (2); (c) cytochemical localization of lysosomal enzyme activities in the PV, e.g., alkaline phosphatase and myeloperoxidase reaction products deposited in the PV of *L. donovani*-infected human primary phagocytes-monocytes, neutrophils, and eosinophils (3); and (d) co-localization of *L. donovani* with phagolysosomes in the liver from infected animals after subcellular fractionation (Andre Trouet; 4). Together, all these lines of evidence indicate that *L. donovani* does reside in phagolysosomes shortly after in vitro infection of macrophages from human and other mammalian hosts and after in vivo infection of animals to a steady state.

Inconsistent with the previous conclusion are more recent work based chiefly on the "Rab cascade model" to explain the regulation of directional and orderly trafficking of vacuoles/vesicles for the transport of their cargoes along the mammalian endocytic and secretory pathways. There are dozens of Rabs or GTPase isoforms and other membrane proteins, which tether to the cytoplasmic side of the vacuoles. Some RabGTPases are thought to be the master regulators, which order the events of membrane trafficking and regulate the localization of the subsequent Rabs, thereby determining indirectly the identity of vacuoles/endosomes and their functional status. These and other membrane-associated proteins are regulated by a network of signal pathways and indirectly by microRNAs. The readout of these and related vacuolar membrane molecules is based invariably on immunofluorescent microscopy of fixed cell samples and Western blot analysis in conjunction with the use of inhibitors and cutting edge, albeit globally affecting genetic approaches: specific gene knockdown/knock-in, transcriptome/miRNA analysis, etc. This powerful combination of cellular and molecular tools allows one to scrutinize the PV membrane proteins and, more importantly, to

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Box 3 (continued)

manipulate them for predicting the intracellular location and fate of intracellular pathogens. The burgeoning literature in this field includes excellent work on the PV-associated membrane molecules after endocytosis of *Leishmania* by macrophages. Interested readers are referred to the publications in this area of investigation for details. It suffices to briefly mention a couple of examples: Albert Descoteaux and his colleagues have long reported inhibition/modulation of phagosome maturation by *Leishmania* lipophosphoglycans (LPG) and Zn-metalloprotease (gp63) in macrophages after infection in vitro with metacyclic promastigotes of *L. major* (5, 6); Peter Kima and his colleagues described the association of ER markers with *Leishmania*-containing endosomes, thereby considering them as chimeric (7, 8). The most recent paper described above by Amitaba Mukhopadhyay and his colleagues presents an excellent piece of work to further advance our understanding on the molecular events of the PV membrane proteins during the early infection of human macrophages in vitro by *L. donovani*. Key points of relevance are recapitulated very briefly as follows: the parasite-secreted gp63 apparently downregulates c-Jun in the pathway necessary for the expression of miR494, which regulates Rab5a negatively. The resulting upregulation of Rab5a promotes its sequestration to the PV, thereby keeping them as early endosomes and preventing its replacement with Rab7 necessary for their maturation into late endosomes and phagolysosomes. Extensive data of excellent quality are presented in support of the interpretations based on the "Rab cascade model" and the novel discovery of miR494 with regulatory role specific to THP-1- and HPBM-derived human macrophages.

The foregoing paragraph provides a glimpse of the current conceptual basis and technical approaches to dissect early *Leishmania*-macrophage membrane interactions in vitro. New discoveries as described warrant further investigation in greater details to bridge the gap of their discordance with the previous findings and to advance the field. Some recommendations are given below for consideration:

Foremost is perhaps to examine the PV in the infected macrophages ex vivo derived from lesion aspirates of patients' spleen, bone marrow, or skin and, if known, reservoir animals. Examination of such samples for the vacuolar membrane marker proteins and the vacuolar contents will shed light on the properties of well-established PV in clinical infection with direct relevance to the diseases. Clinical correlation of laboratory discoveries has become increasingly mandatory for acceptance by examining archived disease tissues for verification. Such clinical materials are readily available from kala-azar patients for investigation in the endemic countries, such as India. It would be highly desirable to directly examine, in the natural setting, the very

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Box 3 (continued)

early infection of human macrophages by sand fly-delivered promastigotes. This is difficult, if not impossible, to accomplish. The closest simulation of such natural infection is to develop an in vitro organ system, which mimics human skin, e.g., 3D printed skin with draining vasculatures for examining macrophages and other phagocytes in vitro for endocytosis of *Leishmania* delivered by infected vectors. Given that such an experimental model is not available, the next best to consider is perhaps to obtain in vivo infected macrophages for ex vivo study of their PV, e.g., inoculate mammalian peritoneal cavity or artificially produced skin blister/pouch with infective promastigotes plus sand fly saliva. In vivo infected macrophages are then withdrawn from these sites periodically for ex vivo examinations of their PV in a time course. While still artificial, this experimental approach is perhaps closer to reality than the methodology in use, i.e., exposure of glass- or plastic-adhered macrophages to in vitro grown promastigotes alone in culture medium. The merit of this in vitro system is its simplicity for use to study endocytosis of inert particles, from which "Rab cascade model" is derived as a plausible explanation for phagosome maturation and its regulation as discussed. In that sense, by using the similar in vitro system, the work under discussion contributes significantly to this model by the discovery of miR494 for its novel role in regulating Rab5a. Intervention of this and other regulatory molecules by gp63 and LPG is a very acceptable scenario, considering that both are released, as they are downregulated during promastigote-to-amastigote differentiation after *Leishmania* infection of macrophages. *Leishmania* differentiation, akin to cellular development, is expected to follow an orderly program of molecular reorganization. There are known changes of the surface architectures and secretory molecules, in addition to gp63 and LPG, released by *Leishmania* from early to late stages of this differentiation. All these programed events are expected to work in tandem, contributing to the remodeling the PV for its maturation, i.e., creation of a microenvironment conducive to the replication of amastigotes. At least in one in vitro model, intracellular *Leishmania* differentiation appears to take a week or longer to complete based on the switch in tubulin biosynthesis as the molecular marker (9). Thus, a large gap appears to emerge in the experimental approaches to assess the molecular events and in the time frame of the observations between previous and more current studies, i.e., the week-long maturation of the PV for parasite replication versus a couple of days or less for phagosome maturation. In addition, information collected after short-term infection, e.g., 48 h does not foretell events beyond this time frame, including phagosome-lysosome fusion, as reported previously. Further investigation to bridge the gaps entails the consideration of all experimental approaches using

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in vitro models, which enable *Leishmania* not only to complete their differentiation but also to replicate as amastigotes, i.e., long-term infection of human macrophages (1) and macrophage cell lines (10, Fig. 4), and those from experimentally well-infected animals for PV in a steady-state infection.

Recent work, including the latest paper under discussion, has significant bearing on our quest for understanding the *Leishmania* mechanisms of intracellular parasitism. Our renewed attention in that direction is necessitated by the state-of-the-art approach, as it represents progresses in the science of cell biology research. Whether or not the discussion provided is viewed as pertinent, it brings up a significant issue. That is, a close and proactive collaboration among leishmaniacs in different fields will be necessary for advances toward the resolution of the issue at hand in the context of leishmaniasis.

References

1. Berman JD, Dwyer DM, Wyler DJ. Multiplication of *Leishmania* in human macrophages in vitro. Infect Immun. 1979;26:375–9.
2. Chang KP. Endocytosis of *Leishmania*-infected macrophages. Fluorometry of pinocytic rate, lysosome-phagosome fusion and intralysosomal pH, p. 231-234. In: Van den Bossche H, editor. The host invader interplay. Amsterdam: Elsevier/NorthHolland, Biomedical Press; 1980.
3. Chang KP. Leishmanicidal mechanisms of human polymorphonuclear phagocytes. Am J Trop Med Hyg. 1981;30:322–33.
4. Trouet A. Isolation of modified liver lysosomes. Methods Enzymol. 1974;31:323–9. PMID: 4370710; Tulkens P, Trouet A. The concept of drug-carriers in the treatment of parasitic diseases. In: Müller-Ruchholtz W, Müller-Hermelink HK, editors. Function and structure of the immune system. Advances in experimental medicine and biology, Vol. 114. Boston, MA: Springer; 1979.
5. Moradin N, Descoteaux A. *Leishmania* promastigotes: building a safe niche within macrophages. Front Cell Infect Microbiol. 2012;2:121. doi: https://doi.org/10.3389/fcimb.2012.00121. PMID: 23050244.
6. Matte C, Descoteaux A. Exploitation of the host cell membrane fusion machinery by *Leishmania* is part of the infection process. PLoS Pathog. 2016;12:e1005962. doi:https://doi.org/10.1371/journal.ppat.1005962.
7. Ndjamen B, Kang BH, Hatsuzawa K, Kima PE. *Leishmania* parasitophorous vacuoles interact continuously with the host cell's endoplasmic reticulum; parasitophorous vacuoles are hybrid compartments. Cell Microbiol. 2010;12:1480–94. doi:https://doi.org/10.1111/j.1462-5822.2010.01483.x.

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8. Canton J, Kima PE. Interactions of pathogen-containing compartments with the secretory pathway. Cell Microbiol. 2012;14:1676–86. doi: https://doi.org/10.1111/cmi.12000.
9. Fong D, Chang KP. Tubulin biosynthesis in the developmental cycle of a parasitic protozoan, *Leishmania mexicana*: changes during differentiation of motile and nonmotile stages. Proc Natl Acad Sci U S A. 1981;78:7624–8.
10. Chang KP, Reed SG, McGwire BS, Soong L. *Leishmania* model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. Acta Trop. 2003;85:375–90.

Box 4: Programmed Cell Death in the *Leishmania* Parasite

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Programmed cell death (PCD), commonly manifested as apoptosis, plays crucial roles in a multitude of physiological processes starting from embryogenesis to maintenance of the immune system. Initially believed to be the prerogative of multicellular organisms to use PCD for maintaining cellular homeostasis, it was later found to be prevalent in unicellular organisms as well (1). The term PCD and apoptosis have been used interchangeably and describe cell death with typical features of apoptosis. PCD was described in *Trypanosoma cruzi* and *Leishmania amazonensis* during the 1990s (2, 3).

Subsequently, with the demonstration of cell death in different *Leishmania* species, showing a phenotype similar to apoptosis generated a great interest in the field of *Leishmania* biology. The digenetic life cycle of this parasite provides possibilities of PCD at several points during their life cycle for maintenance of fitness of the colony. The fittest promastigotes residing in the midgut of the female sand fly have to pass onto the pharynx of the fly by removing unfit cells, likely discarded through PCD as necrotic removal would endanger the health of the sand fly. Although the type of death in the gut of the sand fly has not been examined, free-swimming forms of the parasite in culture were shown to undergo PCD under various stress conditions (4–6). Within the vertebrate host cells, the mammalian macrophages, the parasites are removed through the process of PCD to maintain the optimum number, thus creating a niche for favorable growth of the remaining amastigotes, the

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nonmotile intracellular forms (7). Several features of mammalian apoptosis like chromatin condensation, DNA fragmentation, loss of mitochondrial membrane potential, cell shrinkage, caspase-like activities, phosphatidylserine exposure, and cytochrome c release were demonstrated in the *Leishmania* parasite in vitro (4, 7). Cell lysates from *Leishmania* undergoing apoptosis were shown to cleave substrates for caspase-3, although no caspase has been identified in the *Leishmania* except for a metacaspase (6–8). Interestingly, pretreatment of cells with specific caspase inhibitors reduced the number of cells showing apoptosis-like features, e.g., DNA breakage and cleavage of a PARP-like protein, suggesting existence of proteins with caspase-like activity (4–6).

It was not only during developmental stages of the life cycle that PCD features were shown, exposure to agents that the parasites are normally exposed to, like the reactive oxygen species or drugs, also induced PCD features. Anti-leishmanial drugs like antimony, miltefosine, and amphotericin B were reported to precipitate PCD (9–11). Exposure to reactive oxygen species, heat shock, and staurosporine treatment also precipitates apoptosis of the parasites (4, 5, 12, 13). Like the higher eukaryotic system, the single mitochondrion of *Leishmania* spp. plays a pivotal role in PCD where imbalances in mitochondrial membrane potential like a fall or increase lead to cell death by apoptosis (5). Calcium appears to be heavily involved in *Leishmania* PCD. It is increased by exposure to several PCD-inducing agents. Reducing cytosolic calcium by chelating extracellular or intracellular calcium during oxidative stress prevents apoptosis that is preceded by abrogation of a loss of mitochondrial membrane potential (5, 7, 9, 14). Inhibitors of respiratory chain complexes I, II, and III provoke PCD in *Leishmania donovani* promastigotes. Mitochondrial hyperpolarization resulting from Complex I inhibition is preceded by increased superoxide production. Thenoyltrifluoroacetone and antimycin A, inhibitors of complexes II and III, respectively, dissipate the membrane potential causing PCD (15). Therefore, respiratory chain inhibition is an interesting prospect for drug targeting (16). Exposure of these protozoa to a mixture of reactive oxygen and nitrogen species can cause PCD that is reversible by antioxidants, like glutathione and calcium channel blockers (17). *Leishmania* spp. react to two related metalloids, arsenic and antimony, leading to cell death accompanied by typical apoptotic features that is preceded by an increase in reactive oxygen species. Mitochondrial dysfunction and a drop in ATP level are observed with a loss of membrane potential. During arsenic treatment, prevention of calcium influx reduces cell death, whereas supplementation of glutathione during antimony treatment saved cell loss (9). Therefore, multiple agents with

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different mechanisms of action could precipitate apoptosis-like death. Recently, apoptotic death in the *Leishmania* has been shown after exposure to amphotericin B, and zinc flux causes mitochondrial disruption, resulting from the accumulation of reactive oxygen species (18). Caspase-like activity was detected in *Leishmania* raising the possibility for the existence of this enzyme, although genome sequence did not reveal any ORF homologous to typical caspases. Caspase-independent death was described in the trypanosomatid parasites where endonuclease G, a mitochondrial enzyme, appears to be responsible for DNA fragmentation during apoptosis (19).

Interestingly, PCD may function beyond the provision of unwanted cell elimination to maintain fitness of the colony; it can be used to drive other functions like the ability to infect. These parasites have been shown to mimic an apoptotic cell phenotype by phosphatidylserine exposure. As a result, a given infective inoculum may consist of both live and apoptotic cells to facilitate a successful infection (20). In the case of *Leishmania* spp. infection in mice, such apoptotic mimicry in amastigotes has been described (21). *Leishmania* expresses a variety of defense mechanisms against exogenous stress, preventing them from undergoing apoptosis. For example, ergosterol upsurge during antimony treatment prevents cell death (22). Upregulation of defensive enzymes like tryparedoxin peroxidases of both the mitochondrial and cytosolic origin also prevents cell death induced by reactive oxygen species (17). Therefore, it is evident that PCD of *Leishmania* parasites may play a significant role in infection (23).

Although many aspects of the PCD have come to light, the molecular mechanism remains to be defined. Elucidation of the molecular events linked to apoptotic death of *Leishmania* spp. is of great importance because this information has the potential to help define a more comprehensive view of the cell death machinery in terms of evolutionary origin and identify new target molecules for chemotherapeutic drug development and therapeutic intervention.

References

1. Arnoult D, Akarid K, Grodet A, Petit PX, Estaquier J, Ameisen JC. On the evolution of programmed cell death: apoptosis of the unicellular eukaryote *Leishmania major* involves cysteine proteinase activation and mitochondrion permeabilization. Cell Death Differ. 2002;9:65–81.
2. Murphy NB, Welburn SC. Programmed cell death in procyclic *Trypanosoma brucei* rhodesiense is associated with differential expression of mRNAs. Cell Death Differ. 1997;4:365–70.

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3. Moreira ME, Del Portillo HA, Milder RV, Balanco JM, Barcinski MA. Heat shock induction of apoptosis in promastigotes of the unicellular organism *Leishmania (Leishmania) amazonensis*. J Cell Physiol. 1996;167:305–13.
4. Das M, Mukherjee SB, Shaha C. Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. J Cell Sci. 2001;114:2461–9.
5. Mukherjee SB, Das M, Sudhandiran G, Shaha C. Increase in cytosolic Ca $^{2+}$ levels through the activation of non-selective cation channels induced by oxidative stress causes mitochondrial depolarization leading to apoptosis-like death in *Leishmania donovani* promastigotes. J Biol Chem. 2002;277:24717–27.
6. Lee N, Bertholet S, Debrabant A, Muller J, Duncan R, Nakhasi HL. Programmed cell death in the unicellular protozoan parasite *Leishmania*. Cell Death Differ. 2002;9:53–64.
7. Sudhandiran G, Shaha C. Antimonial-induced increase in intracellular Ca^{2+} through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. J Biol Chem. 2003;278:25120–32.
8. Casanova M, Gonzalez IJ, Sprissler C, Zalila H, Dacher M, Basmaciyan L, Späth GF, Azas N, Fasel N. Implication of different domains of the *Leishmania* major metacaspase in cell death and autophagy. Cell Death Dis. 2015;6:e1933.
9. Mehta A, Shaha C. Mechanism of metalloid-induced death in *Leishmania* spp.: role of iron, reactive oxygen species, Ca^{2+}, and glutathione. Free Radic Biol Med. 2006;40:1857–68.
10. Paris C, Loiseau PM, Bories C, Bréard J. Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother. 2004;48:852–9.
11. Shadab M, Jha B, Asad M, Deepthi M, Kamran M, Ali N. Apoptosis-like cell death in *Leishmania donovani* treated with KalsomeTM10, a new liposomal amphotericin B. PLoS One. 2017;12:e0171306.
12. Sen N, Banerjee B, Das BB, Ganguly A, Sen T, Pramanik S, Mukhopadhyay S, Majumder HK. Apoptosis is induced in Leishmanial cells by a novel protein kinase inhibitor withaferin A and is facilitated by apoptotic topoisomerase I-DNA complex. Cell Death Differ. 2007;14:358–67.
13. Kumari A, Singh KP, Mandal A, Paswan RK, Sinha P, Das P, Ali V, Bimal S, Lal CS. Intracellular zinc flux causes reactive oxygen species

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mediated mitochondrial dysfunction leading to cell death in Leishmania donovani. PLoS One. 2017;12:e0178800.
14. Dolai S, Pal S, Yadav RK, Adak S. Endoplasmic reticulum stress-induced apoptosis in *Leishmania* through Ca^{2+}-dependent and caspase-independent mechanism. J Biol Chem. 2011;286:13638–46.
15. Mehta A, Shaha C. Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. J Biol Chem. 2004;279:11798–813.
16. Fidalgo LM, Gille L. Mitochondria and trypanosomatids: targets and drugs. Pharm Res. 2011;28:2758–70.
17. Iyer JP, Kaprakkaden A, Choudhary ML, Shaha C. Crucial role of cytosolic tryparedoxin peroxidase in *Leishmania donovani* survival, drug response and virulence. Mol Microbiol. 2008;68:372–91.
18. Kumari A, Singh KP, Mandal A, Paswan RK, Sinha P, Das P, Ali V, Bimal S, Lal CS. Intracellular zinc flux causes reactive oxygen species mediated mitochondrial dysfunction leading to cell death in *Leishmania donovani*. PLoS One. 2017;12:e0178800.
19. Gannavaram S, Vedvyas C, Debrabant A. Conservation of the pro-apoptotic nuclease activity of endonuclease G in unicellular trypanosomatid parasites. J Cell Sci. 2008;121:99–109.
20. Wanderley JL, Pinto da Silva LH, Deolindo P, Soong L, Borges VM, Prates DB, de Souza AP, Barral A, Balanco JM, do Nascimento MT, Saraiva EM, Barcinski MA. Cooperation between apoptotic and viable metacyclics enhances the pathogenesis of Leishmaniasis. PLoS One. 2009;4:e5733.
21. El-Hani CN, Borges VM, Wanderley JL, Barcinski MA. Apoptosis and apoptotic mimicry in *Leishmania*: an evolutionary perspective. Front Cell Infect Microbiol. 2012;2:96.
22. Mathur R, Das RP, Ranjan A, Shaha C. Elevated ergosterol protects *Leishmania* parasites against antimony-generated stress. FASEB J. 2015;29:4201–13.
23. Shaha C. Apoptosis in *Leishmania* species and its relevance to disease pathogenesis. Indian J Med Res. 2006;123:233–44.

Box 5: One Health for Leishmaniasis

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One of the major necessities, for more effective *Leishmania* elimination program in South Asia and for even the rest of the world, can be an integrative approach to introduce One Health programs and education with welfare as well as development programs. Hence a comprehensive policy framework is required for incorporation of One Health program for control and elimination of leishmaniasis. One Health programs are an amalgamation of multidisciplinary-integrated approach that brings about multiple benefits. One Health encompasses unification of animal, human, and environmental health into an interdisciplinary field of health sciences. The synergy between interdisciplinary fields helps in achieving the goals of biomedical research, education, and more effective public health programs as well as environmental protection. The One Health programs are all encompassing, which further aids toward better effective implementation of welfare programs for achieving sustainable development goals and the overall well-being of the community.

One Health program ensures a creation of a platform for information gathering, training of health workers, and educating the masses of an integrated approach for disease control and elimination both in human and animals (1). By utilization of modern information and communication technologies, along with an effective training of health workers, a robust surveillance system can be designed for areas that are endemic for anthroponotic VL (AVL) or zoonotic VL.

Comprehensive One Health approach explores and strengthens the existing programs using a multidimensional road map of all possible scientific streams. First parameter to analyze the effectiveness of control program should be to ascertain the mode of transmission of disease-causing VL

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parasite. The best method to prevent or curb VL disease is vector control especially in the endemic regions for AVL. Xenomonitoring of vector infection through a real-time dynamic surveillance design is needed to monitor the transmission of the *Leishmania* parasite vector species in endemic areas. The best example is the infection caused by *L. donovani* parasite through vector *Phlebotomus argentipes* in South Asia, predominantly in India, Nepal, and Bangladesh. The information of percentage of vector infected will be essential to ascertain the degree of spread of *Leishmania* parasite in the VL endemic regions.

Also in the context for vector control program, now there is a definite shift toward using synthetic pyrethroids that are pesticides derived from naturally occurring pyrethrins. The use of dichlorodiphenyltrichloroethane (DDT) is gradually being discontinued in many VL endemic regions of the world including South Asia due to environmental concerns. There has been introduction of pressure pumps for insecticide spraying; effectiveness of the same has to be also ascertained. Here a policy is also needed for proper use of pesticide as well as continuous monitoring for identifying the development of resistance against the pesticides among the vector population. Another important parameter is the reporting for occurrence of any adverse reaction to human population and the environment. The vector, i.e., the sand fly's ecological role, cannot be ignored and have to be researched thoroughly. The vector control envisages stopping the overpopulation of the vector and preventing transmission of VL infection but definitely not the total eradication of the vector population.

Further the second step is to monitor human reservoirs of VL parasite. In the AVL areas, the asymptomatic human populations, which harbor *Leishmania* parasites, including the cases of Post-kala-azar dermal leishmaniasis, assume significance. Even an active surveillance at short regular intervals will be helpful in evaluation of the load of parasite circulating in the environment at any given time. The use of dynamic surveillance becomes more important in the areas of zoonotic VL. In addition to the vector and the patients, the animal reservoirs have to be monitored for circulating parasites.

Third important step toward *Leishmania* control is to monitor the zoonotic reservoirs for VL, including the environmental changes it affects. The environmental changes are a continuous process and it affects inevitably the life cycle of the organisms that occupy its habitat. Thus it is important that monitoring of the leishmanial parasitic spread if any, also among the domesticated cattle, be undertaken (2). Domestication of cattle is a major source of livelihood among the farmers and the rural community in the VL endemic regions like the Indian subcontinent. The cattle shelters in most of the times

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harbor conducive habitats for several insects like mosquitoes and sand fly that are health hazard. In the Indian subcontinent, the VL disease is found to be anthroponotic in nature till date with *L. donovani* as the main causative agent of the VL infection and sand fly as vector, but constant monitoring of the VL parasite among the animal population is important still.

In other zoonotic VL endemic areas, for example, *L. infantum*, animal reservoirs are found in the canid population along with human population. These are mainly found in the Mediterranean regions, the Middle East, Central Asia, China, and the Americas. A thorough surveillance of the disease in both canine and human populations will help prevent disease outbreaks.

In several studies (3–5) to control zoonotic VL, researchers have emphasized One Health programs as required for effective management of transmission of disease. This can be achieved through a combined approach on one hand by obtaining information regularly from human, vectors, and animal reservoirs for the parasite in the endemic area and on the other hand as an integrated approach, by analysis of environmental factors necessary for disease spread. The environmental factors as we know lead to random genetic mutations; this can increase or decrease parasite infectivity and can also give rise to phenotypic changes in the parasite which also needs to be monitored periodically. The advent of the omics technology has opened new tools to monitor genetic and epigenetic changes among the organisms. It is imperative that the genomic and protein profiling of the parasite circulating in the environment have to be carried out periodically. Another important aspect is reporting of adverse reactions for the chemotherapeutic treatment agents. The policy thus would design mandatory protocols for health systems in reporting adverse events in a full proof and robust manner as part of surveillance system. Another addition to the surveillance policy is to have a comprehensive monitoring for the development of resistance against the chemotherapeutic agents. The grassroots public health clinics have involved in the policy framework.

The important step now is how to implement the concept with the given resources. The surveillance system requires adequate tools for diagnosis that has to be rapid, sensitive, easy to conduct, and cost-effective. The diagnosis with rK39 rapid diagnostic dip test is a sensitive proposition in detecting the presence of anti-*Leishmania* antibodies in the serum of the patients at a field level. Also now new variant of novel rapid rKE16 antigen-based test is being evaluated to be introduced in the VL elimination programs (6). The rapid dip test for VL mentioned here is routinely carried out in blood samples in place of serum due to lack of resources. In rKE39 test carried out with whole blood, the sensitivity is lesser in cases where the antibody produced is below the

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normal range, mostly among immunocompromised patients (7). The policy in such cases is such that patients will be screened and identified for suspected VL based on symptoms, even if rK39 test or another dip test comes out negative. Patients with symptoms can be referred to public health clinics (PHC). The PHC have to be equipped to carry out definitive test and provide treatment. India and other South Asian countries are slowly progressing toward equipping their PHC in VL endemic areas to be self-sufficient to provide treatment. VL as we know is the disease prevalent among the impoverished and immunocompromised. Thus the other problem is that of coinfection with diseases like pneumonia and TB that can occur in VL patients and that have to be properly diagnosed. PHC can also screen for HIV which is found to be prevalent in VL-endemic areas too. The PHC should be nodal points of training centers for ground-level health workers, so that they can identify symptoms in patients in the community, carry out surveillance and diagnosis in the field, and learn data gathering. The use of mobile net and telephony application tools can be a viable and speedy option for data collation and distribution to the block-, district-, state-, and national-level program managers as required. The One Health program also envisages as stated before that the environment is protected and its degradation is minimized and the community gets access to both proper sanitation and nutrition in a sustainable manner. As a holistic approach in addition to welfare programs, sustainable development goals have to be achieved. The PHC through health workers will also ascertain whether benefits of the other welfare program reach the target community. Further the One Health program will ensure educating the community about VL and other infections and about how to protect and prevent the infection. Policy should envisage that primary school teachers at rural level along with health workers have to be given proper training and incentives to hire them over a long period of time to educate and generate awareness among the masses about VL and other diseases along the need for environmental protection and conservation.

Thus the One Health approach when adopted in full measure will ensure that data are gathered properly, stored securely, and analyzed, which will aid to ascertain the NTD elimination program gaps and drawbacks. This will ensure proper course correction carried out to keep the elimination program for VL on track.

References

1. Barrett MA, Bouley TA, Stoertz AH, Stoertz RW. Integrating a One Health approach in education to address global health and sustainability challenges. Front Ecol Environ 2011;9(4):239–45. Published by: Wiley on

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Box 5 (continued)

behalf of the Ecological Society of America. Stable URL: http://www.jstor.org/stable/41149772. Accessed 21 May 2017 23:16 UTC.
2. Bhattarai NR, Van der Auwera G, Rijal S, Picado A, Speybroeck N, Khanal B, De Doncker S, Das ML, Ostyn B, Davies C, Coosemans M, Berkvens D, Boelaert M, Dujardin J-C. Domestic animals and epidemiology of visceral leishmaniasis, Nepal. Emerg Infect Dis. 2010;16(2). www.cdc.gov/eid.
3. Lorusso V, et al. Paediatric visceral leishmaniasis in Italy: a 'One Health' approach is needed. Parasit Vectors. 2013;6:123.
4. Hailu T. One Health approach prospect for integrated control and elimination of visceral leishmaniasis in Ethiopia: a narrative review article. Iran J Parasitol 2016;11(1):1–9.
5. Palatnik-de-Sousa CB, Day MJ. One Health: the global challenge of epidemic and endemic leishmaniasis. Parasit Vectors. 2011;4:197. http://www.parasitesandvectors.com/content/4/1/197.
6. Vaish M, Sharma S, Chakravarty J, Sundar S. Evaluation of two novel rapid rKE16 antigen-based tests for diagnosis of visceral leishmaniasis in India. Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India. J Clin Microbiol. 2012;50(9):3091–2. jcm.asm.org 3091.
7. Matlashewski G, Das VNR, Pandey K, Singh D, Das S, Ghosh AK, et al. Diagnosis of visceral leishmaniasis in Bihar, India: comparison of the rK39 rapid diagnostic test on whole blood versus serum. PLoS Negl Trop Dis. 2013;7(5):e2233. https://doi.org/10.1371/journal.pntd.0002233.

References

1. WHO. http://www.who.int/leishmaniasis/en/. Accessed Sept 2016.
2. CDC. https://www.cdc.gov/parasites/leishmaniasis/. Accessed Jan 2013.
3. Chang KP. Leishmaniases. In: Encyclopedia of life sciences. Wiley; 2012. doi:https://doi.org/10.1002/9780470015902.a0001954.pub3. http://www.els.net/WileyCDA/ElsArticle/refId-a0001954.html.
4. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7:e35671. https://doi.org/10.1371/journal.pone.0035671.
5. GBD 2013 DALYs and HALE Collaborators, Murray CJ, Barber RM, Foreman KJ, Abbasoglu Ozgoren A, Abd-Allah F, Abera SF, Aboyans V, et al. Global, regional, and national disability-adjusted life years (DALYs) for 306 diseases and injuries and healthy life expectancy (HALE) for 188 countries, 1990–2013: quantifying the epidemiological transition. Lancet. 2015;386:2145–91. https://doi.org/10.1016/S0140-6736(15)61340-X.
6. Chang KP, Bray RS, editors. Leishmaniasis. Part II: Specific. In: Ruitenburg EJ, MacInnis AJ, series editors. Human parasitic diseases, Vol. 1. Amsterdam: Elsevier Biomedical Press; 1985. Chapters 14–25, pp. 247–479.

7. Killick-Kendrick R. The race to discover the insect vector of kala-azar: a great saga of tropical medicine 1903–1942. Bull Soc Pathol Exot. 2013;106:131–7. https://doi.org/10.1007/s13149-013-0285-x.
8. PATH. Diagnostic gaps and recommendations for visceral leishmaniasis: assessment of user needs, use cases, and the diagnostic landscape. Seattle: PATH; 2016. http://sites.path.org/dx/files/2012/04/2016-04-08__Report_VL_gap_analysis_final.pdf
9. Shamsuzzaman AK, Hossain MA, Musa AK, Hasan MU, Dhar DK. A preliminary report on culture of Leishmania donovani in Mymensingh Medical College and evaluation of new immuno-chromatography test (ICT). Mymensingh Med J. 2003;12:51–4. 12715645
10. Khan MG, Alam MS, Bhuiyan AT, Jamil MA, Saha B, Islam M, et al. Short communication: evaluation of a new rapid diagnostic test for quality assurance by kala azar elimination programme in Bangladesh. J Parasitol Res. 2011;2011:862475. https://doi.org/10.1155/2011/862475.
11. Sivakumar R, Sharma P, Chang KP, Singh S. Cloning, expression and purification of a novel recombinant antigen from Leishmania donovani. Protein Expr Purif. 2006;46:156–65.
12. Burns JM Jr, Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG. Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. Proc Natl Acad Sci USA. 1993;90:775–9.
13. Islam MZ, Itoh M, Ul Islam MA, Saifuddin-Ekram AR, Rahman MA, Takagi H, et al. ELISA with recombinant rKRP42 antigen using urine samples: a tool for predicting clinical visceral leishmaniasis cases and its outbreak. Am J Trop Med Hyg. 2012;87:658–62. https://doi.org/10.4269/ajtmh.2012.12-0168.
14. Bublitz DC, Poché RM, Garlapati R. Measures to control phlebotomus argentipes and visceral leishmaniasis in India. J Arthropod Borne Dis. 2016;10:113–26.
15. Picado A, Dash AP, Bhattacharya S, Boelaert M. Vector control interventions for visceral leishmaniasis elimination initiative in South Asia, 2005–2010. Indian J Med Res. 2012;136:22–31.
16. Yaghoobi-Ershadi MR. Control of phlebotomine sand flies in Iran. J Arthropod-Born Dis. 2016;10:429–44.
17. Sevá AP, Ovallos FG, Amaku M, Carrillo E, Moreno J, Galati EA, et al. Canine-based strategies for prevention and control of visceral leishmaniasis in Brazil. PLoS One. 2016;11:e0160058. doi:10.1371/journal.pone.0160058. Erratum in: PLoS One. 2016;11:e0162854.
18. Hurwitz I, Fieck A, Read A, Hillesland H, Klein N, Kang A, et al. Paratransgenic control of vector borne diseases. Int J Biol Sci. 2011;7:1334–44. PMID: 22110385.
19. Chang KP, Kolli BK, New Light Group. New "light" for one-world approach toward safe and effective control of animal diseases and insect vectors from leishmaniac perspectives. Parasit Vectors. 2016;9:396. https://doi.org/10.1186/s13071-016-1674-3.
20. Roque AL, Jansen AM. Wild and synanthropic reservoirs of Leishmania species in the Americas. Int J Parasitol Parasites Wildl. 2014;3:251–62. https://doi.org/10.1016/j.ijppaw.2014.08.004.
21. Safyanova VM. Leishmaniases in USSR. In: Chang KP, Bray RS, editors. Leishmaniasis. Amsterdam: Elsevier Biomedical Press, 1985; Part II, Chapter 22, pp. 427–42.
22. Lun ZR, Wu MS, Chen YF, Wang JY, Zhou XN, Liao LF, et al. Visceral leishmaniasis in China: an endemic disease under control. Clin Microbiol Rev. 2015;28:987–1004. https://doi.org/10.1128/CMR.00080-14.
23. Singh S, Kumari V, Singh N. Predicting kala-azar disease manifestations in asymptomatic patients with latent Leishmania donovani infection by detection of antibody against recombinant K39 antigen. Clin Diagn Lab Immunol. 2002;9:568–72. Erratum in: Clin Diagn Lab Immunol. 2004;11:1199.
24. Das VN, Siddiqui NA, Verma RB, Topno RK, Singh D, Das S, et al. Asymptomatic infection of visceral leishmaniasis in hyperendemic areas of Vaishali district, Bihar, India: a challenge

to kala-azar elimination programmes. Trans R Soc Trop Med Hyg. 2011;105:661–6. https://doi.org/10.1016/j.trstmh.2011.08.005.
25. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S. Asymptomatic Leishmania infection: a new challenge for Leishmania control. Clin Infect Dis. 2014;58:1424–9. https://doi.org/10.1093/cid/ciu102.
26. Ganguly S, Das NK, Barbhuiya JN, Chatterjee M. Post-kala-azar dermal leishmaniasis–an overview. Int J Dermatol. 2010;49:921–31. https://doi.org/10.1111/j.1365-4632.2010.04558.x.
27. Gillespie PM, Beaumier CM, Strych U, Hayward T, Hotez PJ, Bottazzi ME. Status of vaccine research and development of vaccines for leishmaniasis. Vaccine 2016;3(34):2992–5. doi:10.1016/j.vaccine.2015.12.071.
28. Hofmeyer KA, Duthie MS, Laurance JD, Favila MA, Van Hoeven N, Coler RN, et al. Optimizing immunization strategies for the induction of antigen-specific CD4 and CD8 T cell responses for protection against intracellular parasites. Clin Vaccine Immunol. 2016;23(9):785–94.
29. Reed SG, Coler RN, Mondal D, Kamhawi S, Valenzuela JG. Leishmania vaccine development: exploiting the host-vector-parasite interface. Expert Rev Vaccines. 2016;15:81–90. https://doi.org/10.1586/14760584.2016.1105135.
30. Mo AX, Pesce J, Hall BF. Visceral leishmaniasis control and elimination: is there a role for vaccines in achieving regional and global goals? Am J Trop Med Hyg. 2016;16-0184.
31. Seyed N, Taheri T, Rafati S. Post-genomics and vaccine improvement for leishmania. Front Microbiol. 2016;7:467. 27092123. https://doi.org/10.3389/fmicb.2016.00467.
32. Kumar R, Engwerda C. Vaccines to prevent leishmaniasis. Clin Transl Immunol. 2014;3:e13. 25505961. https://doi.org/10.1038/cti.2014.4.
33. Srivastava S, Shankar P, Mishra J, Singh S. Possibilities and challenges for developing a successful vaccine for leishmaniasis. Parasit Vectors. 2016;9:277. https://doi.org/10.1186/s13071-016-1553-y.
34. Jha TK. Epidemiology of drug-resistant kala-azar in India and neighboring countries. In: Jha TK, Noiri E, editors. Kala Azar in South Asia: current status and challenges ahead. Dordrecht: Springer Science; 2011. p. 21–34.
35. Singh OP, Singh B, Chakravarty J, Sundar S. Current challenges in treatment options for visceral leishmaniasis in India: a public health perspective. Infect Dis Poverty. 2016;5:19. https://doi.org/10.1186/s40249-016-0112-2.
36. Mishra J, Saxena A, Singh S. Chemotherapy of leishmaniasis: past, present and future. Curr Med Chem. 2007;14:1153–69.
37. Field MC, Horn D, Fairlamb AH, Ferguson MA, Gray DW, Read KD, et al. Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need. Nat Rev Microbiol. 2017. https://doi.org/10.1038/nrmicro.2016.193.
38. Sundar S, Jha TK, Thakur CP, Sinha PK, Bhattacharya SK. Injectable paromomycin for Visceral leishmaniasis in India. N Engl J Med. 2007;356:2571–81.
39. Jamil KM, Haque R, Rahman R, Faiz MA, Bhuiyan AT, Kumar A, et al. Effectiveness study of paromomycin im injection (pmim) for the treatment of visceral leishmaniasis (VL) in Bangladesh. PLoS Negl Trop Dis. 2015;9:e0004118. https://doi.org/10.1371/journal.pntd.0004118.
40. Mukhopadhyay R, Mukherjee S, Mukherjee B, Naskar K, Mondal D, Decuypere S, et al. Characterisation of antimony-resistant Leishmania donovani isolates: biochemical and biophysical studies and interaction with host cells. Int J Parasitol. 2011;41:1311–21. https://doi.org/10.1016/j.ijpara.2011.07.013.
41. Patterson S, Wyllie S, Norval S, Stojanovski L, Simeons FR, Auer JL, et al. The anti-tubercular drug delamanid as a potential oral treatment for visceral leishmaniasis. Elife. 2016;5. pii: e09744. doi:10.7554/eLife.09744.
42. Rijal S, Ostyn B, Uranw S, Rai K, Bhattarai NR, Dorlo TP, Beijnen JH, et al. Increasing failure of miltefosine in the treatment of kala-azar in Nepal and the potential role of parasite

drug resistance, reinfection, or noncompliance. Clin Infect Dis. 2013;56:1530–8. https://doi.org/10.1093/cid/cit102.
43. Mondal D, Hasnain MG, Hossain MS, Ghosh D, Ghosh P, Hossain H, et al. Study on the safety and efficacy of miltefosine for the treatment of children and adolescents with post-kala-azar dermal leishmaniasis in Bangladesh, and an association of serum vitamin E and exposure to arsenic with post-kala-azar dermal leishmaniasis: an open clinical trial and case-control study protocol. BMJ Open. 2016;6:e010050. https://doi.org/10.1136/bmjopen-2015-010050.
44. Khare S, Nagle AS, Biggart A, Lai YH, Liang F, Davis LC, et al. Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. Nature. 2016. https://doi.org/10.1038/nature1933.
45. Refai W, Madarasingha N, Sumanasena B, Weerasingha S, De Silva A, Fernandopulle R, et al. Efficacy, safety and cost-effectiveness of thermotherapy in the treatment of Leishmania donovani-induced cutaneous leishmaniasis: a randomized controlled clinical trial. Am J Trop Med Hyg. (in press).
46. Dhimal M, Ahrens B, Kuch U. Climate change and spatiotemporal distributions of vector-borne diseases in Nepal–a systematic synthesis of literature. PLoS One. 2015;10(6): e0129869. https://doi.org/10.1371/journal.pone.0129869. eCollection 2015
47. Rock KS, Quinnell RJ, Medley GF, Courtenay O. Progress in the mathematical modelling of visceral leishmaniasis. Adv Parasitol. 2016;94:49–131. https://doi.org/10.1016/bs.apar.2016.08.001.
48. Le Rutte EA, Coffeng LE, Bontje DM, Hasker EC, Postigo JA, Argaw D, et al. Feasibility of eliminating visceral leishmaniasis from the Indian subcontinent: explorations with a set of deterministic age-structured transmission models. Parasit Vectors. 2016;9:24. https://doi.org/10.1186/s13071-016-1292-0.
49. Chowdhury R, Mondal D, Chowdhury V, Faria S, Alvar J, Nabi SG, et al. How far are we from visceral leishmaniasis elimination in Bangladesh? An assessment of epidemiological surveillance data. PLoS Negl Trop Dis. 2014;8:e3020. doi:https://doi.org/10.1371/journal.pntd.0003020. eCollection 2014 Aug. PMID: 25144317.
50. Olliaro PL, Shamsuzzaman TA, Marasini B, Dhariwal AC, Be-Nazir A, Mondal D, et al. Investments in research and surveillance are needed to go beyond elimination and stop transmission of Leishmania in the Indian subcontinent. PLoS Negl Trop Dis. 2017;11(1): e0005190. https://doi.org/10.1371/journal.pntd.0005190.
51. Cameron MM, Acosta-Serrano A, Bern C, Boelaert M, den Boer M, Burza S, et al. Understanding the transmission dynamics of Leishmania donovani to provide robust evidence for interventions to eliminate visceral leishmaniasis in Bihar, India. Parasit Vectors. 2016;9:25. doi:https://doi.org/10.1186/s13071-016-1309-8.
52. WHO. Regional strategic plan for integrated neglected tropical diseases control in south-east Asia region. 2012. http://apps.searo.who.int/pds_docs/B4867.pdf?ua=1.
53. Hasker E, Singh SP, Malaviya P, Picado A, Gidwani K, Singh RP, et al. Visceral leishmaniasis in rural Bihar, India. Emerg Infect Dis. 2012;18:1662–4. https://doi.org/10.3201/eid1810.111083.
54. Hirve S, Boelaert M, Matlashewski G, Mondal D, Arana B, Kroeger A, et al. Transmission dynamics of visceral leishmaniasis in the Indian subcontinent – a systematic literature review. PLoS Negl Trop Dis. 2016;10:e0004896. https://doi.org/10.1371/journal.pntd.0004896.
55. Meheus F, Boelaert M. The burden of visceral leishmaniasis in South Asia. Trop Med Int Health. 2010;15(Suppl 2):1–3. https://doi.org/10.1111/j.1365-3156.2010.02564.x.
56. Yangzom T, Cruz I, Bern C, Argaw D, den Boer M, Vélez ID, et al. Endemic transmission of visceral leishmaniasis in Bhutan. Am J Trop Med Hyg. 2012;87:1028–37. https://doi.org/10.4269/ajtmh.2012.12-0211.
57. Thakur CP. History of kala azar. 2007. http://www.who.int/global_health_histories/seminars/presentation35a.pdf.

58. Steverding D. The history of leishmaniasis. Parasit Vectors. 2017;10:82. https://doi.org/10.1186/s13071-017-2028-5.
59. Nadeem S, Shamroz S, Iqbal T, ul Manan M, Salaam A. Frequency of visceral leishmaniasis in Abbottabad from 2005 to 2009. J Ayub Med Coll Abbottabad. 2010;22:58–60.
60. Siriwardena HV, Udagedara CU, Karunaweera ND. Clinical features, risk factors and efficacy of cryotherapy in cutaneous leishmaniasis in Sri Lanka. Ceylon Med J. 2003;48:10–2.
61. Siriwardana HV, Noyes HA, Beeching NJ, Chance ML, Karunaweera ND, Bates PA. Leishmania donovani and cutaneous leishmaniasis, Sri Lanka. Emerg Infect Dis. 2007;13:476–8.
62. Karunaweera ND, Rajapaksa US. Is leishmaniasis in Sri Lanka benign and be ignored? J Vector Borne Dis. 2009;46:13–7.
63. Karunaweera ND. Leishmaniasis: path toward elimination from the Indian subcontinent. Trop Parasitol. 2016;6:2–4. http://www.tropicalparasitology.org/text.asp?2016/6/1/2/175023
64. Manamperi NH, Oghumu S, Pathirana N, de Silva VC, Abeyewickreme W, Satoskar AR, et al. In situ immunopathological changes in cutaneous leishmaniasis due to Leishmania donovani. Parasite Immunol. 2017;39. https://doi.org/10.1111/pim.12413.
65. Kumar NP, Srinivasan R, Anish TS, Nandakumar G, Jambulingam P. Cutaneous leishmaniasis caused by Leishmania donovani in the tribal population of the Agasthyamala Biosphere Reserve forest, Western Ghats, Kerala, India. J Med Microbiol. 2015;64:157–63. https://doi.org/10.1099/jmm.0.076695-0.
66. Ranasinghe S, Zhang WW, Wickremasinghe R, Abeygunasekera P, Chandrasekharan V, Athauda S, et al. Leishmania donovani zymodeme MON-37 isolated from an autochthonous visceral leishmaniasis patient in Sri Lanka. Pathog Glob Health. 2012;106:421–4. 23265615. https://doi.org/10.1179/2047773212Y.0000000054.
67. Zhang WW, Ramasamy G, McCall LI, Haydock A, Ranasinghe S, Abeygunasekara P, et al. Genetic analysis of Leishmania donovani tropism using a naturally attenuated cutaneous strain. PLoS Pathog. 2014;10:e1004244. doi:https://doi.org/10.1371/journal.ppat.1004244. PMID: 24992200.
68. McCall LI, Zhang WW, Matlashewski G. Determinants for the development of visceral leishmaniasis disease. PLoS Pathog. 2013;9:e1003053. https://doi.org/10.1371/journal.ppat.1003053.
69. Srivastava D, Vyas MCR, Joshi CK. Clinico epidemiological study of cutaneous leishmaniasis in Bikaner (Rajasthan). J Commun Dis. 1987;19:326–31.
70. Agrawal S, Khandelwal K, Bumb RA, Oghumu S, Salotra P, Satoskar AR. Pediatric cutaneous leishmaniasis in an endemic region in India. Am J Trop Med Hyg. 2014;91:901–4. https://doi.org/10.4269/ajtmh.13-0737.
71. Khan NH, Bari AU, Hashim R, Khan I, Muneer A, Shah A, et al. Cutaneous leishmaniasis in Khyber Pakhtunkhwa province of Pakistan: clinical diversity and species-level diagnosis. Am J Trop Med Hyg. 2016;95:1106–14.
72. Adegboye OA, Adegboye M. Spatially correlated time series and ecological niche analysis of cutaneous leishmaniasis in Afghanistan. Int J Environ Res Public Health. 2017;14. pii: E309. doi:https://doi.org/10.3390/ijerph14030309. PMID: 28304356.
73. Myint CK, Asato Y, Yamamoto Y, Kato H, Bhutto AM, Soomro FR, et al. Polymorphisms of cytochrome b gene in Leishmania parasites and their relation to types of cutaneous leishmaniasis lesions in Pakistan. J Dermatol. 2008;35:76–85. https://doi.org/10.1111/j.1346-8138.2008.00419.x.
74. Sharma NL, Mahajan VK, Ranjan N, Verma GK, Negi AK, Mehta KI. The sandflies of the Satluj river valley, Himachal Pradesh (India): some possible vectors of the parasite causing human cutaneous and visceral leishmaniases in this endemic focus. J Vector Borne Dis. 2009;46:136–40.
75. Sharma NL, Mahajan VK, Kanga A, Sood A, Katoch VM, Mauricio I, et al. Localized cutaneous leishmaniasis due to Leishmania donovani and Leishmania tropica: preliminary

findings of the study of 161 new cases from a new endemic focus in Himachal Pradesh. India. Am J Trop Med Hyg. 2005;72:819–24.
76. Chang KP, Sharma NL (Unpublished data, including RFLP and sequence analysis of the ~1.4 kb *nagt* that was PCR-amplified from DNAs of the lesion materials, which were incubated in 3N medium for differentiation of amastigotes into promastigotes with very little, if any growth of the latter).
77. Sukmee T, Siripattanapipong S, Mungthin M, Worapong J, Rangsin R, Samung Y, et al. A suspected new species of Leishmania, the causative agent of visceral leishmaniasis in a Thai patient. Int J Parasitol. 2008;38:617–22. https://doi.org/10.1016/j.ijpara.2007.12.003.
78. Bualert L, Charungkiattikul W, Thongsuksai P, Mungthin M, Siripattanapipong S, Khositnithikul R, Naaglor T, et al. Autochthonous disseminated dermal and visceral leishmaniasis in an AIDS patient, southern Thailand, caused by Leishmania siamensis. Am J Trop Med Hyg. 2012;86:821–4. https://doi.org/10.4269/ajtmh.2012.11-0707.
79. Noppakun N, Kraivichian K, Siriyasatien P. Disseminated dermal leishmaniasis caused by Leishmania siamensis in a systemic steroid therapy patient. Am J Trop Med Hyg. 2014;91:869–70. https://doi.org/10.4269/ajtmh.13-0711.
80. Siriyasatien P. Leishmaniasis and sand fly vectors in Thailand. Bangkok: D-One Books; 2016.
81. Das VN, Pandey RN, Siddiqui NA, Chapman LA, Kumar V, Pandey K, et al. Longitudinal study of transmission in households with visceral leishmaniasis, asymptomatic infections and PKDL in highly endemic villages in Bihar, India. PLoS Negl Trop Dis. 2016;10:e0005196. https://doi.org/10.1371/journal.pntd.0005196.
82. Anonymous. National road map for kala-azar elimination. 2014. http://www.nvbdcp.gov.in/Doc/Road-map-KA_2014.pdf.
83. Anonymous. https://www.dndi.org/diseases-projects/leishmaniasis/. Accessed 23 Apr 2017.
84. Anonymous. Acting against kala azar in Bihar. MSF Activity Report – India. 2014;9:25. http://www.msfindia.in/country-region/acting-against-kala-azar-bihar. doi:https://doi.org/10.1186/s13071-016-1309-8.
85. Anonymous. Southeast Asian health ministers collaborate in the elimination of visceral leishmaniasis. 2014. http://outbreaknewstoday.com/southeast-asian-health-ministers-collaborate-in-the-elimination-of-visceral-leishmaniasis-92085/. Accessed 14 Sept 2014.
86. Medley GF, Hollingsworth TD, Olliaro PL, Adams ER. Health-seeking behaviour, diagnostics and transmission dynamics in the control of visceral leishmaniasis in the Indian subcontinent. Nature. 2015;528:S102–8. https://doi.org/10.1038/nature16042.
87. Bauer S, Morris MT. Glycosome biogenesis in trypanosomes and the de novo dilemma. PLoS Negl Trop Dis. 2017;11:e0005333. https://doi.org/10.1371/journal.pntd.0005333. eCollection 2017
88. Docampo R, Huang G. Acidocalcisomes of eukaryotes. Curr Opin Cell Biol. 2016;41:66–72. https://doi.org/10.1016/j.ceb.2016.04.007.
89. Rodriguez-Contreras D, Aslan H, Feng X, Tran K, Yates PA, Kamhawi S, Landfear SM. Regulation and biological function of a flagellar glucose transporter in Leishmania mexicana: a potential glucose sensor. FASEB J. 2015;29:11–24. 25300620. https://doi.org/10.1096/fj.14-251991.
90. Landfear SM, Tran KD, Sanchez MA. Flagellar membrane proteins in kinetoplastid parasites. IUBMB Life. 2015;67:668–76. 26599841. https://doi.org/10.1002/iub.1411.
91. Rougeron V, De Meeûs T, Kako Ouraga S, Hide M, Bañuls AL. "Everything you always wanted to know about sex (but were afraid to ask)" in Leishmania after two decades of laboratory and field analyses. PLoS Pathog. 2010;6:e1001004. https://doi.org/10.1371/journal.ppat.1001004.
92. TriTrypDB. Structural and functional genomic database for parasites of the family Trypanosomatidae, including Leishmania. http://tritrypdb.org/tritrypdb/.

93. de Jesus JB, Mesquita-Rodrigues C, Cuervo P. Proteomics advances in the study of Leishmania parasites and leishmaniasis. Subcell Biochem. 2014;74:323–49. https://doi.org/10.1007/978-94-007-7305-9_14.
94. Singh AK, Roberts S, Ullman B, Madhubala R. A quantitative proteomic screen to identify potential drug resistance mechanism in α-difluoromethylornithine (DFMO) resistant Leishmania donovani. J Proteomics. 2014;102:44–59. https://doi.org/10.1016/j.jprot.2014.02.030.
95. Bag AK, Saha S, Sundar S, Saha B, Chakrabarti A, Mandal C. Comparative proteomics and glycoproteomics of plasma proteins in Indian visceral leishmaniasis. Proteome Sci. 2014;12:48. https://doi.org/10.1186/s12953-014-0048-z.
96. Jamdhade MD, Pawar H, Chavan S, Sathe G, Umasankar PK, Mahale KN, et al. Comprehensive proteomics analysis of glycosomes from Leishmania donovani. OMICS. 2015;19:157–70. doi:https://doi.org/10.1089/omi.2014.0163; 99.
97. Pawar H, Kulkarni A, Dixit T, Chaphekar D, Patole MS. A bioinformatics approach to reanalyze the genome annotation of kinetoplastid protozoan parasite Leishmania donovani. Genomics. 2014;104:554–61. https://doi.org/10.1016/j.ygeno.2014.09.008.
98. Pawar H, Sahasrabuddhe NA, Renuse S, Keerthikumar S, Sharma J, Kumar GS, et al. A proteogenomic approach to map the proteome of an unsequenced pathogen – Leishmania donovani. Proteomics. 2012;12:832–44. https://doi.org/10.1002/pmic.201100505.
99. Nirujogi RS, Pawar H, Renuse S, Kumar P, Chavan S, Sathe G, et al. Moving from unsequenced to sequenced genome: reanalysis of the proteome of Leishmania donovani. J Proteomics. 2014;97:48–61. https://doi.org/10.1016/j.jprot.2013.04.021.
100. Pawar H, Renuse S, Khobragade SN, Chavan S, Sathe G, Kumar P, et al. Neglected tropical diseases and omics science: proteogenomics analysis of the promastigote stage of Leishmania major parasite. OMICS. 2014;18:499–512. https://doi.org/10.1089/omi.2013.0159.
101. Singh N, Sundar S. Integrating genomics and proteomics permits identification of immunodominant antigens associated with drug resistance in human visceral leishmaniasis in India. Exp Parasitol. 2017;176:30–45. https://doi.org/10.1016/j.exppara.2017.02.019.
102. Cantacessi C, Dantas-Torres F, Nolan MJ, Otranto D. The past, present, and future of Leishmania genomics and transcriptomics. Trends Parasitol. 2015;31:100–8. https://doi.org/10.1016/j.pt.2014.12.012.
103. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A historical overview of the classification, evolution, and dispersion of leishmania parasites and sandflies. PLoS Negl Trop Dis. 2016;10:e0004349. https://doi.org/10.1371/journal.pntd.0004349. Erratum in: PLoS Negl Trop Dis. 2016;10:e0004770.
104. Waki K, Dutta S, Ray D, Kolli BK, Akman L, Kawazu S, et al. Transmembrane molecules for phylogenetic analyses of pathogenic protists: leishmania-specific informative sites in hydrophilic loops of trans-endoplasmic reticulum N-acetylglucosamine-1-phosphate transferase. Eukaryot Cell. 2007;6:198–210. PMID: 17142569.
105. Schönian G, Kuhls K, Mauricio IL. Molecular approaches for a better understanding of the epidemiology and population genetics of Leishmania. Parasitology. 2011;138:405–25. https://doi.org/10.1017/S0031182010001538.
106. Imamura H, Downing T, Van den Broeck F, Sanders MJ, Rijal S, Sundar S, et al. Evolutionary genomics of epidemic visceral leishmaniasis in the Indian subcontinent. Elife. 2016;5. pii: e12613. https://doi.org/10.7554/eLife.12613.
107. Toz SO, Culha G, Zeyrek FY, Ertabaklar H, Alkan MZ, Vardarlı AT, et al. Real-time ITS1-PCR based method in the diagnosis and species identification of Leishmania parasite from human and dog clinical samples in Turkey. PLoS Negl Trop Dis. 2013;7:e2205. https://doi.org/10.1371/journal.pntd.0002205. PMID: 23675543.
108. Singh N, Chikara S, Sundar S. SOLiD™ sequencing of genomes of clinical isolates of Leishmania donovani from India confirm leptomonas co-infection and raise some key questions. PLoS One. 2013;8:e55738. https://doi.org/10.1371/journal.pone.0055738.

109. Loría-Cervera EN, Andrade-Narváez FJ. Animal models for the study of leishmaniasis immunology. Rev Inst Med Trop Sao Paulo. 2014;56:1–11. https://doi.org/10.1590/S0036-46652014000100001. PMID: 24553602.
110. Scott P, Novais FO. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. Nat Rev Immunol. 2016;16:581–92. https://doi.org/10.1038/nri.2016.72.
111. Liu D, Uzonna JE. The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. Front Cell Infect Microbiol. 2012;2:83. https://doi.org/10.3389/fcimb.2012.00083.
112. Dostálová A, Volf P. Leishmania development in sand flies: parasite-vector interactions overview. Parasit Vectors. 2012;5:276. https://doi.org/10.1186/1756-3305-5-276.
113. Titus RG, Ribeiro JM. Salivary gland lysates from the sand fly Lutzomyia longipalpis enhance Leishmania infectivity. Science. 1988;239:1306–8.
114. Belkaid Y, Kamhawi S, Modi G, Valenzuela J, Noben-Trauth N, Rowton E, et al. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis. J Exp Med. 1998;188:1941–53.
115. Abdeladhim M, Kamhawi S, Valenzuela JG. What's behind a sand fly bite? The profound effect of sand fly saliva on host hemostasis, inflammation and immunity. Infect Genet Evol. 2014;28:691–703. https://doi.org/10.1016/j.meegid.2014.07.028.
116. Oliveira F, Rowton E, Aslan H, Gomes R, Castrovinci PA, Alvarenga PH, et al. A sand fly salivary protein vaccine shows efficacy against vector-transmitted cutaneous leishmaniasis in nonhuman primates. Sci Transl Med. 2015;7:290ra90. https://doi.org/10.1126/scitranslmed.aaa3043.
117. Chang KP, Chaudhuri G, Fong D. Molecular determinants of Leishmania virulence. Annu Rev Microbiol. 1990;44:499–529.
118. Ueno N, Wilson ME. Receptor-mediated phagocytosis of Leishmania: implications for intracellular survival. Trends Parasitol. 2012:335–44. https://doi.org/10.1016/j.pt.2012.05.002.
119. Roy S, Mandal C. Leishmania donovani utilize sialic acids for binding and phagocytosis in the macrophages through selective utilization of siglecs and impair the innate immune arm. PLoS Negl Trop Dis. 2016;10:e0004904. https://doi.org/10.1371/journal.pntd.0004904.
120. Rana T, Misra S, Mittal MK, Farrow AL, Wilson KT, Linton MF, et al. Mechanism of down-regulation of RNA polymerase III-transcribed non-coding RNA genes in macrophages by Leishmania. J Biol Chem. 2011; 286:6614-6626. PubMed Central PMCID: PMC3057815.
121. Farrow AL, Rana T, Mittal MK, Misra S, Chaudhuri G. Leishmania-induced repression of selected non-coding RNA genes containing B-box element at their promoters in alternatively polarized M2 macrophages. Mol Cell Biochem. 2011;350:47–57. PMID: 21165676.
122. Courret N, Fréhel C, Gouhier N, Pouchelet M, Prina E, Roux P, Antoine J. Biogenesis of Leishmania-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites. J Cell Sci. 2002;115:2303–16.
123. Real F, Mortara RA. The diverse and dynamic nature of Leishmania parasitophorous vacuoles studied by multidimensional imaging. PLoS Negl Trop Dis. 2012;6:e1518. https://doi.org/10.1371/journal.pntd.0001518.
124. Séguin O, Descoteaux A. Leishmania, the phagosome, and host responses: the journey of a parasite. Cell Immunol. 2016. pii: S0008-8749(16)30076-4. https://doi.org/10.1016/j.cellimm.2016.08.004.
125. Mittra B, Andrews NW. IRONy OF FATE: role of iron-mediated ROS in Leishmania differentiation. Trends Parasitol. 2013:489–96. https://doi.org/10.1016/j.pt.2013.07.007.
126. Mukherjee B, Mukhopadhyay R, Bannerjee B, Chowdhury S, Mukherjee S, Naskar K, et al. Antimony-resistant but not antimony-sensitive Leishmania donovani up-regulates host IL-10 to overexpress multidrug-resistant protein 1. Proc Natl Acad Sci U S A. 2013;110:E575–82. 23341611. https://doi.org/10.1073/pnas.1213839110.

127. Gurung P, Kanneganti TD. Innate immunity against Leishmania infections. Cell Microbiol. 2015;17:1286–94. https://doi.org/10.1111/cmi.12484.
128. de Menezes JP, Saraiva EM, da Rocha-Azevedo B. The site of the bite: Leishmania interaction with macrophages, neutrophils and the extracellular matrix in the dermis. Parasit Vectors. 2016;9:264. https://doi.org/10.1186/s13071-016-1540-3.
129. Atayde VD, Hassani K, da Silva Lira Filho A, Borges AR, Adhikari A, et al. Leishmania exosomes and other virulence factors: Impact on innate immune response and macrophage functions. Cell Immunol. 2016;309:7–18. https://doi.org/10.1016/j.cellimm.2016.07.013.
130. Wege AK, Florian C, Ernst W, Zimara N, Schleicher U, Hanses F, Schmid M, Ritter U. Leishmania major infection in humanized mice induces systemic infection and provokes a nonprotective human immune response. PLoS Negl Trop Dis. 2012;6:e1741. https://doi.org/10.1371/journal.pntd.0001741. PMID: 22848771.
131. Chang KP, McGwire BS. Molecular determinants and regulation of Leishmania virulence. Kinetoplastid Biol Dis. 2002;1:1. http://www.kinetoplastids.com/content/1/1/1. PMID: 12234388.
132. Chang KP, Reed SG, McGwire BS, Soong L. Leishmania model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. Acta Trop. 2003;85:375–90.
133. Ready PD. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 2014;6:147–54. https://doi.org/10.2147/CLEP.S44267.
134. Hasnain G, Shomik MS, Ghosh P, Rashid MO, Hossain S, Hamano S, et al. Post-Kala-azar dermal leishmaniasis without previous history of visceral leishmaniasis. Am J Trop Med Hyg. 2016;95:1383–5.
135. Coleman M, Foster GM, Deb R, Pratap Singh R, Ismail HM, Shivam P, et al. DDT-based indoor residual spraying suboptimal for visceral leishmaniasis elimination in India. Proc Natl Acad Sci U S A. 2015;112:8573–8. https://doi.org/10.1073/pnas.
136. Singh S. Personal communication in support of previous serological and molecular evidence in "Singh N, Mishra J, Singh R, Singh S. Animal reservoirs of visceral leishmaniasis in India. J Parasitol. 2013;99:64–7. https://doi.org/10.1645/GE-3085".
137. Singh S. Changing trends in the epidemiology, clinical presentation, and diagnosis of Leishmania-HIV co-infection in India. Int J Infect Dis. 2014;29:103–12. https://doi.org/10.1016/j.ijid.2014.07.011.
138. Ejazi SA, Bhattacharya P, Bakhteyar MA, Mumtaz AA, Pandey K, Das VN, et al. Noninvasive diagnosis of visceral leishmaniasis: development and evaluation of two urine-based immunoassays for detection of leishmania donovani infection in India. PLoS Negl Trop Dis. 2016;10:e0005035. 27741241. https://doi.org/10.1371/journal.pntd.0005035.
139. Bhattacharya P, Ghosh S, Ejazi SA, Rahaman M, Pandey K, Ravi Das VN, et al. Induction of IL-10 and TGFβ from CD4+CD25+FoxP3+ T cells correlates with parasite load in Indian kala-azar patients infected with Leishmania donovani. PLoS Negl Trop Dis. 2016;10:e0004422. https://doi.org/10.1371/journal.pntd.0004422.
140. Singh N, Kumar R, Engwerda C, Sacks D, Nylen S, Sundar S. Tumor necrosis factor alpha neutralization has no direct effect on parasite burden, but causes impaired IFN-γ production by spleen cells from human visceral leishmaniasis patients. Cytokine. 2016;85:184–90. https://doi.org/10.1016/j.cyto.2016.06.013. PMID: 27372917.
141. Kumar R, Singh N, Gautam S, Singh OP, Gidwani K, Rai M, et al. Leishmania specific CD4 T cells release IFNγ that limits parasite replication in patients with visceral leishmaniasis. PLoS Negl Trop Dis. 2014;8:e3198. https://doi.org/10.1371/journal.pntd.0003198.
142. Faleiro RJ, Kumar R, Bunn PT, Singh N, Chauhan SB, Sheel M, et al. Combined immune therapy for the treatment of visceral leishmaniasis. PLoS Negl Trop Dis. 2016;10:e0004415. https://doi.org/10.1371/journal.pntd.0004415.
143. Chandel HS, Pandey SP, Roy S, Doyen N, Saha B. TLR-CD40 cross-talk in anti-leishmanial immune response. Front Immunol. 2014;5:220. https://doi.org/10.3389/fimmu.2014.00220.

144. Anand S, Madhubala R. Twin attributes of tyrosyl tRNA-synthetase of Leishmania donovani: a housekeeping protein translation enzyme and a mimic of host chemokine. J Biol Chem. 2016. pii: jbc.M116.727107.
145. Roy K, Mandloi S, Chakrabarti S, Roy S. Cholesterol corrects altered conformation of MHC-II protein in Leishmania donovani infected macrophages: implication in therapy. PLoS Negl Trop Dis. 2016;10:e0004710. https://doi.org/10.1371/journal.pntd.0004710.
146. Rashid U, Sultana R, Shaheen N, Hassan SF, Yaqoob F, Ahmad MJ, et al. Structure based medicinal chemistry-driven strategy to design substituted dihydropyrimidines as potential antileishmanial agents. Eur J Med Chem. 2016;115:230–44. https://doi.org/10.1016/j.ejmech.2016.03.022.
147. Kaur J, Kumar P, Tyagi S, Pathak R, Batra S, Singh P, et al. In silico screening, structure-activity relationship, and biologic evaluation of selective pteridine reductase inhibitors targeting visceral leishmaniasis. Antimicrob Agents Chemother. 2011;55:659–66. https://doi.org/10.1128/AAC.00436-10.
148. Maji M, Mazumder S, Bhattacharya S, Choudhury ST, Sabur A, Shadab M, et al. A lipid based antigen delivery system efficiently facilitates MHC Class-I antigen presentation in dendritic cells to stimulate CD8(+) T cells. Sci Rep. 2016;6:27206. https://doi.org/10.1038/srep27206.
149. Chhajer R, Bhattacharyya A, Didwania N, Shadab M, Das N, Palit P, et al. Leishmania donovani Aurora kinase: a promising therapeutic target against visceral leishmaniasis. Biochim Biophys Acta. 1860;2016:1973–88. https://doi.org/10.1016/j.bbagen.2016.06.005.
150. Mishra AK, Singh N, Agnihotri P, Mishra S, Singh SP, Kolli BK, et al. Discovery of novel inhibitors for Leishmania Nucleoside Diphosphate Kinase (NDK) based on its structural and functional characterization. J Comput Aid Mol Des. 2017. https://doi.org/10.1007/s10822-017-0022-9. [Epub ahead of print].
151. Asthana S, Gupta PK, Jaiswal AK, Dube A, Chourasia MK. Targeted chemotherapy of visceral leishmaniasis by lactoferrin-appended amphotericin B-loaded nanoreservoir: in vitro and in vivo studies. Nanomedicine (Lond). 2015;10:1093–109. https://doi.org/10.2217/nnm.14.182.
152. Nadhman A, Khan MI, Nazir S, Khan M, Shahnaz G, Raza A, et al. Annihilation of Leishmania by daylight responsive ZnO nanoparticles: a temporal relationship of reactive oxygen species-induced lipid and protein oxidation. Int J Nanomed. 2016;11:2451–61. https://doi.org/10.2147/IJN.S105195.
153. Banerjee A, Bhattacharya P, Joshi AB, Ismail N, Dey R, Nakhasi HL. Role of pro-inflammatory cytokine IL-17 in Leishmania pathogenesis and in protective immunity by Leishmania vaccines. Cell Immunol. 2016. pii: S0008-8749(16)30053-3. https://doi.org/10.1016/j.cellimm.2016.07.004.
154. Gannavaram S, Dey R, Avishek K, Selvapandiyan A, Salotra P, Nakhasi HL. Biomarkers of safety and immune protection for genetically modified live attenuated leishmania vaccines against visceral leishmaniasis – discovery and implications. Front Immunol. 2014;5:241. https://doi.org/10.3389/fimmu.2014.00241.
155. Basu R, Bhaumik S, Basu JM, Naskar K, De T, Roy S. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of Leishmania donovani that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. J Immunol. 2005;174:7160–71.
156. Dey A, Sharma P, Redhu NS, Singh S. Kinesin motor domain of Leishmania donovani as future vaccine candidate. Clin Vaccine Immunol. 2008;15:836–42. https://doi.org/10.1128/CVI.00433-07.
157. Sharma A, Madhubala R. Ubiquitin conjugation of open reading frame F DNA vaccine leads to enhanced cell-mediated immune response and induces protection against both antimony-susceptible and -resistant strains of Leishmania donovani. J Immunol. 2009;183:7719–31. https://doi.org/10.4049/jimmunol.0900132.

158. Patel N, Singh SB, Basu SK, Mukhopadhyay A. Leishmania requires Rab7-mediated degradation of endocytosed hemoglobin for their growth. Proc Natl Acad Sci U S A. 2008;105:3980–5. https://doi.org/10.1073/pnas.0800404105.
159. Guha R, Gupta D, Rastogi R, Vikram R, Krishnamurthy G, Bimal S, et al. Vaccination with leishmania hemoglobin receptor-encoding DNA protects against visceral leishmaniasis. Sci Transl Med. 2013;5:202ra121. https://doi.org/10.1126/scitranslmed.3006406.
160. Baharia RK, Tandon R, Sharma T, Suthar MK, Das S, Siddiqi MI, et al. Recombinant NAD-dependent SIR-2 protein of Leishmania donovani: immunobiochemical characterization as a potential vaccine against visceral leishmaniasis. PLoS Negl Trop Dis. 2015;9: e0003557. https://doi.org/10.1371/journal.pntd.0003557.
161. Pawar H, Chavan S, Mahale K, Khobragade S, Kulkarni A, Patil A, et al. A proteomic map of the unsequenced kala-azar vector Phlebotomus papatasi using cell line. Acta Trop. 2015;152:80–9. https://doi.org/10.1016/j.actatropica.2015.08.012.
162. Gupta AK, Srivastava S, Singh A, Singh S. De novo whole-genome sequence and annotation of a Leishmania strain isolated from a case of post-kala-azar dermal leishmaniasis. Genome Announc. 2015;3. pii: e00809-15. https://doi.org/10.1128/genomeA.00809-15.
163. Barry E, Raj S. Wary of outside influence, India limits foreign involvement in health system. 2016. http://www.nytimes.com/2016/04/06/world/asia/india-foreign-health-consultants-narendra-modi.html?ref=asia&_r=0.
164. Palatnik-de-Sousa CB, Day MJ. One Health: the global challenge of epidemic and endemic leishmaniasis. Parasit Vectors. 2011;4:197. https://doi.org/10.1186/1756-3305-4-197.

Human Amebiasis: Insight into the Biology and Immunopathogenesis

Preeti Shahi and Kris Chadee

Contents

1 Introduction

Several members of the genus *Entamoeba* infect humans including *E. histolytica*, *E. moshkovskii*, and *E. dispar*. Among these, only *E. histolytica* is considered pathogenic and is the causal agent of amebiasis. Amebiasis is the second most common cause of death from a parasite worldwide after malaria with considerable

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morbidity and mortality [1, 2]. Based on the site of infection, the clinical features of amebiasis range from asymptomatic colonization to amebic colitis (dysentery or diarrhea) and invasive extraintestinal amebiasis, which appears most commonly in the form of liver abscesses [2]. According to the World Health Organization, amebiasis occurs worldwide, with approximately 50 million people infected annually, causing close to 100,000 deaths per year. Therefore, it is important to understand the epidemiology, infection, and control of the disease. The aim of this chapter is to provide a current understanding of human amebiasis, which illustrates the parasite biology, mechanism of pathogenesis, diagnosis, and prevention.

Amebiasis is more common in areas of poor sanitation and nutrition particularly in the tropical and developing countries [3]. In the United States and Europe, amebiasis is found primarily in immigrants mostly from endemic areas. People who live in the United States that border Mexico have the most cases of amebic infection [4]. The dominance of human amebiasis varies geographically. For instance, birth cohort studies in Bangladesh indicate that approximately 50% of infants showed amebic diarrhea every year, with repeated infections that further led to malnourishment and stunting [4–6]. Comparative studies in Egypt and South Africa found that Egypt was dominated with amebic colitis, whereas amebic liver abscess was common in South Africa [7]. The prevalence of invasive infection was also reported in Nigeria and other areas in Central and South America, Asia, and Africa [8, 9].

The main mode of transmission of amebiasis occurs via ingestion of food and/or water that is contaminated with feces and *E. histolytica* cysts [10, 11], but in some cases amebic infection has been also reported in urban areas in the United States among men who had sex with men [12]. Following ingestion, the parasites remain nonpathogen in 90% of infected individuals (asymptomatic amebiasis), while in 10% of the cases, trophozoites can invade the intestinal mucosa, cause dysentery, and probably via portal circulation migrate to the liver where they produce abscesses (extraintestinal amebiasis) [13, 14]. In extreme cases, trophozoites can also invade other parts of the body like the brain and the lungs [15]. The conditions which trigger symptomatic infection are still unknown; however, this might be partly due to differences in the pathogenicity of the infecting strains [16] and/or the parasitic genotype [17] or variation in the host immune response against the infection [18].

2 *Entamoeba histolytica* Biology

E. histolytica is distributed globally and is a substantial health risk mostly in tropical and developing countries, which have poor sanitary and hygienic practices. The parasite's primary hosts are humans [4]. *E. histolytica* is a dimorphic organism whose life cycle consists of two stages: trophozoites, a cell-invasive form which can be found in the human intestine, and cysts, an infective form which is found in the external environment. The conversion between the two stages is usually

reversible [19]. The parasite enters the body through the ingestion of food or water contaminated with human feces [4]. Following ingestion, cysts survive the acidic pH of the stomach and proceed toward the intestine. In the small intestine, excystation occurs and trophozoites migrate to the colon. Generally, in the colon, trophozoites start multiplying via binary fission, remain as a nonpathogen, and fulfill their energy requirements by ingesting gut microbiota and nutrients from mucus and starch secretions of intestinal epithelial cells (IECs) of the host. In the colon, trophozoites produce quadrinucleate cysts, and both trophozoites and cysts are excreted along with feces that can infect new hosts [20]. Cysts can survive for prolonged periods outside the host, while the trophozoites survive only for a few hours. *E. histolytica* cysts are the parasite dormant infective form which is often found in the external environment. This form is a quadrinucleate round shaped with a size range of 10–15 μm. Covered with chitin-containing cell wall, cysts are highly stable to stomach acids, allowing their efficient infectivity [4]. Unlike inert cyst, trophozoites have a pleomorphic shape that ranges in size between 10 and 50 μm and are highly motile. Since the parasite lack features of aerobic eukaryotic metabolism including the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, energy for the motility comes from anaerobic metabolism of glucose and pyruvate to ethanol [21]. This unicellular parasite is a type I amitochondriate protist, lacking both mitochondria and hydrogenosomes. Instead, parasite poses a mitochondrial relic termed mitosome, which is involved in the assembly of Fe–S clusters, an ancient metabolic pathway present in all mitochondria [22]. Generally, the more close the relationship between parasites and hosts, the stronger the parasite depends on its host's physiology for survival and reproduction [23]. Indeed, residing in the human colon, *E. histolytica* has access to many bacterial and host-metabolites [24].

3 Pathogenesis of *E. histolytica*

Recent advances in the development of in vitro, in vivo, and ex vivo models of disease, new genetic approaches, the identification of key *E. histolytica* virulence factors, and the recognition of crucial elements of the host response to infection have led to significant insights into the pathogenesis of amebic infection (Fig. 1). These important steps are responsible for amebic dysentery/amebiasis and are the result of a tightly regulated and coordinated action of diverse factors during invasion and damage of target cells. Some important molecules have been studied for their role in tissue invasion: adherence, cytotoxicity, cell killing, and phagocytosis as well as the onset of host immune responses [25]. A few factors will be discussed here.

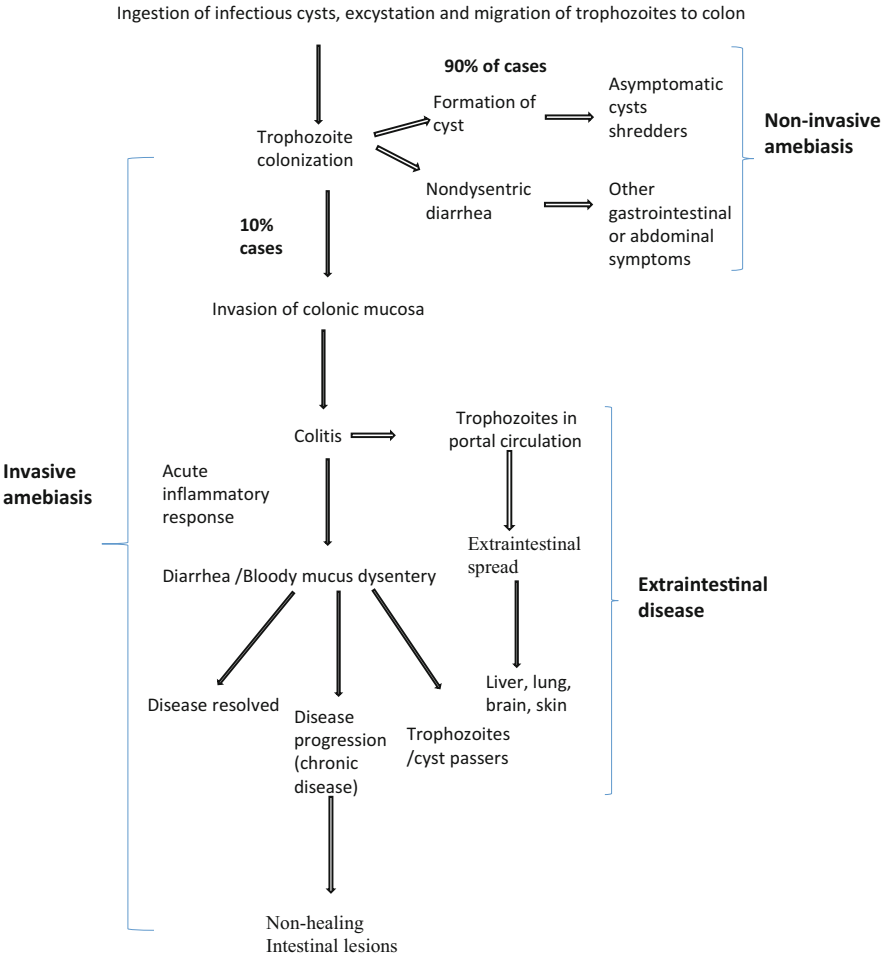

Fig. 1 Schematic representation on pathogenesis and disease manifestation of amebiasis. Infection of *E. histolytica* is acquired through ingestion of contaminated food or water. In 90% of cases, amebic infections are asymptomatic, while in 10% of cases, trophozoites start invading the mucosal barrier and penetrate the underlying tissues, which lead to diarrhea or dysentery. After invading the tissue, trophozoite may enter the portal circulation and spread to the liver, lung, and other soft organs to establish extraintestinal disease. Of the intestinal forms, mostly dysentery is resolved by host immune system. However, if dysentery/diarrhea is not cleared by host immune system, disease progression occurs, and this leads to nonhealing intestinal lesions

3.1 E. histolytica *Colonization and Exposure to Intestinal Environment*

In the large intestine, *E. histolytica* excysts into trophozoite and enters the host by invasion of the intestinal mucosal epithelium. During the course of infection, trophozoites colonize the mucus layer by binding of the parasite's Gal-lectin to galactose and GalNAc residues on colonic MUC2, a major structural component of mucus that covers the intestinal tract. For successful colonization and survival, the trophozoites must respond to environmental elements in the large intestine to start the invasion process [26]. Among the various environmental challenges encountered by the parasite are the surrounding biome, biofilm substrate, various assaults of host's immune system including oxidative stress, complement activation and phagocytosis, and drastic change in pH and glucose concentration [19, 27–30]. In the gut, the parasites are constantly interacting with intestinal microbiota, and it is one of the signals that start the infection process. The interaction between *E. histolytica* and microbiota was the subject of many studies which concluded that intestinal gut microbiota has an important role in the virulence of the ameba and it protects the parasite against oxidative stress [30–33].

3.2 E. histolytica *Invasion*

3.2.1 Gal/GalNAc Lectin

Host cells destruction begins with the binding of trophozoites to target cells. An important molecule involved in this process is the galactose/*N*-acetylgalactosamine (Gal/GalNAc)-inhibitable lectin [34]. This lectin is a multifunctional heterodimeric protein composed of a disulfide-linked heavy (170 kDa, HgL) and light (35/31 kDa, LgL) subunit, which is non-covalently associated with an intermediate subunit of 150 kDa. This protein plays a vital role in interaction with the brush border intestinal epithelial cells [35]. All of these subunits are encoded by multigene families [34]. Interestingly, apart from adhesion to cells, lectin was also found to participate in *E. histolytica* resistance to human complement system attacks [36]. HgL subunit of Gal/GalNAc contains a cysteine-rich domain (CRD) that participates in evasion from the complement system via a remarkable similarity to human inhibitor CD59, complement attack complex. The CRD of HgL subunit also activates macrophages to produce TNF-α and NO, and this leads to amebicidal activity [37]. A study showed the contribution of Gal/GalNAc to the recruitment of inflammatory cells and cytokine production through the analysis of HgL-dependent signaling pathways [34] that further leads to apoptosis- and ameba-induced cell death [38–40]. Thus, the Gal/GalNAc lectin is one of the essential factors in the pathogenesis of *E. histolytica*.

3.3 Cysteine Proteases

The function of *E. histolytica* cysteine proteases (EhCPs) in pathogenesis is marked by the relative absence of these CPs in the morphologically identical but noninvasive sp. *E. dispar* [41]. The contact of the parasite to target cells via Gal/GalNAc is followed by invasion of host tissue. In this regard, *E. histolytica* cysteine proteinases play essential role in degradation of the mucus layer that ultimately leads to invasion [42]. The genome of *E. histolytica* comprises 80 genes encoding proteases, including 50 CPs of the papain superfamily [43, 44]. Among the *E. histolytica* CPs participating in the pathogenic process, CP-A5 is the prime candidate. This CP-A5 is unique to *E. histolytica*, localizes at the amebic surface [45], and is involved in human colon invasion through their ability to degrade extracellular matrix (ECM) as well as MUC2, the major component of colon mucus [46, 47]. A study showed the direct involvement of CPs in immune evasion by degrading host antibodies and complement in the fluid phase [44]. In vivo studies in SCID mice and hamster demonstrated that inhibition of cysteine proteinase activity significantly decreases liver abscess formation [48, 49]. Generally, the mechanism of immune evasion mediated by EhCPs is involved in cleavage of IgA and IgG, processing of complement C3, inactivation of complement C3a and C5a, generation of mature IL-1β from pro-IL-1β, and inactivation of pro-IL-18, all of which reduce host defenses [50]. Taken together, these data suggest very important roles for EhCPs for causing intestinal inflammation, tissue damage in colitis, and extraintestinal infections.

3.4 Amebapores

Parasite invasion in the host is followed by the release of amebapore that results in the swelling, surface blebbing, and lysis of target cells. Amebapores are proteins of 77 amino acids, stored in vesicles, and are responsible for cytolytic activity of ameba. Three types of amebapores were characterized so far, A–C, when type C was reported to be the most active [51]. In the gut, amebapore seems to have a role in damaging the surface membrane of gram-positive bacteria; however, high concentration of amebapore or removal of the wall with lysozyme was required to kill outer membrane-shielded gram-negative bacteria [51]. Incubation of eukaryotic target cells with sublytic concentrations of amebapores showed that these peptides were also able to induce cell necrosis in addition to their known function in cell lysis [52]. Antisense inhibition of amebapore led to a decrease of trophozoites' cytolytic and bactericidal activity and also to impaired ability to create amebic liver abscesses [53, 54]. Taken together, these studies indicate that amebapore has potential cytolytic and antibacterial activities [51, 55]. In addition to the abovementioned amebic virulence factors, other amebic factors such as

proteins enriched in lysine and glutamic acid (KERP1), lipophosphoglycan (LPPG), peroxiredoxin, and arginase are also implicated in pathogenicity [25, 56].

3.5 *Phagocytosis*

Stool samples from patients with invasive parasite have shown the presence of trophozoites that contain ingested erythrocytes and have higher rate of phagocytosis than healthy human carrier [57]. Several studies have also shown that phagocytosis constitutes one of the key virulence determinants of *E. histolytica* [58–61]. Phagocytic-deficient mutant of *E. histolytica* exhibited low virulence as compared to wild type in hamster liver model [58, 59]; thus, phagocytosis-mediated host apoptotic cell killing could enable the parasite to evade host immune responses [62].

4 Host Immune Responses

Following host invasion, trophozoites induce host immune responses (cell-mediated and humoral immunity), and host tissue destruction leads to disease. In order to survive against the various assaults of the host immune system, trophozoites must have the ability to repress the host immune system and to control the parasitism's environment [63].

4.1 *Innate Immune Responses*

Prior to the development of adaptive immunity, parasite initial host resistance is based on innate immunity. Ameba confronts natural barriers in the intestine and in extraintestinal sites following tissue invasion. Therefore, the mucus barrier of the intestine serves as a primary physical barrier against pathogens in the gut that prevent trophozoites attachment to the underlying mucosal epithelial cells [63, 64]. MUC2 mucin secreted by goblet cells binds to *E. histolytica* Gal-lectin, responsible for colonization in the gut, and acts as a physical barrier to impair the adherence of parasite to the underlying epithelium [63–65]. Another innate resistance mechanism that may reduce trophozoite infection is activation of complement pathways by the parasitic [66]. During the initial stage of invasion, parasites bind to pathogen recognition receptors (PRRs) on intestinal epithelial cells (IECs) via the amebic Gal/GalNAc, and these IECs are considered to be the first line of defense against the parasitic infections [67]. *E. histolytica* in contact with IECs activates the

NF-κB signaling pathway to induce the secretion of pro-inflammatory mediators including IL-1β, IL-6, IL-8, IL-12, IFN-γ, and TNF-α, which later recruit immune cells including neutrophils and macrophage to the site of infection [46, 68, 69]. The main amebicidal activity of neutrophils activated by IFN-γ, TNF-α, or lipopolysaccharides is carried out by the release of reactive oxygen species [70]. A study in mice found that amebic lesion was predominated by neutrophils where macrophages were rarely seen, suggesting an essential role for neutrophils in clearance of ameba [15]. In amebic liver abscesses (ALA) in mice, macrophages were found to be responsible for tissue damage and abscesses formation [71]. Amebicidal activity of activated macrophages is mediated by the production of nitric oxide from L-arginine [70]. Several parasitic antigens are known to activate macrophages through pattern recognition receptors. Macrophages exposed to Gal/GalNAc have higher expression of Toll-like receptors, which triggers pro-inflammatory cytokine production via the NF-κB pathway [63, 72]. Macrophages deficient in TLR2 and TLR4 show a weakened immune response to parasitic antigens suggesting an important role for pattern recognition to the immune response [73]. A study in Bangladesh found an association between higher TNF-α production from stimulated peripheral blood mononuclear cells and *E. histolytica* diarrhea. In this study, the authors demonstrated that high potential destructive response from TNF-α may lead to increased inflammation and therefore disease [74].

4.1.1 Inflammasome

Inflammasomes are cytosolic multiprotein complexes considered as pathogenicity sensor consists of NOD-like receptor, caspase-1, and the adaptor protein ASC [75]. This intracellular multiprotein complex has been identified as activated pathways on contact with live *E. histolytica* that link specific pro-inflammatory stimuli to the activation of caspase-1. Active caspase-1 initiates highly potent inflammatory response by inducing secretion and release of pro-inflammatory cytokines IL-1β, IL-18, IL-1α, and IP-10 into the tissues [75]. Activation of inflammasomes seems to be an advantage for *E. histolytica* to limit immune clearance in the host.

4.2 Adaptive Immunity (Humoral and Cell-Mediated Response)

Several studies indicate that 81–100% of invasive amebiasis patients developed circulatory antibodies against the trophozoites [76, 77]. Mucosal IgA against the Gal/GalNAc heavy chain was shown to be one of the most abundant Ig in the human intestine that could reduce trophozoite colonization in the gut [62, 65, 78–80]. A study of school children in Bangladesh showed that mucosal secretory antibody IgA provided protection against reinfection against *E. histolytica* [81–83]. Apart from mucosal IgA, IgG was also found to be dominant against *E. histolytica* infection

and provided protection from parasite reinfection as well [83]. A study showed higher level of IgG in patients with intestinal amebiasis and ALA [77]. These findings suggest that humoral immunity plays an important role against amebic infection.

Cell-mediated immune responses are the essential factors for host defense against the parasite. T-cell involvement has been reported in protection against *E. histolytica* mediated by IFN-γ and IL-17 [84]. IFN-γ produced by peripheral mononuclear cells were responsible for parasite clearance and protection against repeated infection [85, 86]. These findings are in agreement with murine vaccination studies, where vaccine-induced protection against trophozoite infection could be passively transferred to naive animals mediated by interferon-gamma-producing T cells [84]. Collectively, these studies indicate that both innate and adaptive immunities are directly involved in protection against amebiasis.

5 Pathology and Clinical Signs of Amebiasis

Pathology of human amebiasis is characterized by different and destructive forms of lesions in the intestinal or extraintestinal sites [87]. Different forms of intestinal or extraintestinal amebiasis are characterized by unique features such as (a) amebic dysentery or amebic colitis, (b) fulminating amebic colitis, (c) amebic appendicitis, (d) ameboma or amebic granulomas, (e) amebic liver abscess, (f) extensive necrosis, and (g) liquefaction of all affected tissues [87, 88]. Abdominal pain and frequent stool containing both blood and mucus followed by intestinal inflammation is a characteristic feature of amebic dysentery, and symptoms range from mild diarrhea to classic dysentery [15, 89]. Other features of intestinal lesions are severe and need immediate medical care. A necrotic ulcerous lesion may lead to peritonitis which is a feature of fulminating amebic colitis, while amebomas are white nodular mass that extend in the lumen and are related to inflammation, edema, and necrosis of both mucosa and submucosa [90]. Trophozoites may later spread to extraintestinal sites such as the liver, lungs, brain, skin, and rarely urogenital structures where abscesses are formed consisting of circumscribed regions of dead and liquefied cells, cellular debris with few inflammatory cells, and trophozoites [88, 89]. Infection at these extraintestinal targets can develop months to years following colon infection and often rise as symptomatic infection. Patients develop symptoms over 2–4 week period and consist of low pain in the upper right quadrant, the lower right chest, and the right shoulder tip [4, 91].

6 Diagnosis

Diagnosis of amebiasis is usually based on microscopic, serological, antigen detection, and PCR-based method. Since the last decade, molecular biology-based diagnosis is becoming more important to detect various diseases including amebiasis. Accurate diagnosis of *E. histolytica* is very important to prevent furthered transmission of the parasite [92].

6.1 Microscopic Examinations

In the past, *E. histolytica* has been diagnosed based on microscopic examination of the parasite morphology. Microscopic examination is mostly based on the presence or absence of hematophagous trophozoites in the stool samples. The disadvantage of this method is the lack of sensitivity and specificity and inability to differentiate *E. histolytica* from morphologically similar but noninvasive sp. *E. dispar* [93, 94].

6.2 Biochemical Method

A biochemical method used for the diagnosis of the disease involves the culture of stool samples followed by isoenzyme analysis which can consistently distinguish *Entamoeba* sp. [95]. However, due to several limitations including difficulty in performing isoenzyme analysis, cultivation of *E. histolytica* from stool or liver abscess and time-consuming procedures make this method unsuitable for use in developing countries [96–98].

6.3 Serological Test

6.3.1 Antibody Detection

Patients with symptomatic *E. histolytica* infection develop detectable anti-amebic antibodies, and also persons recovering from amebiasis have detectable serum antibodies; therefore, serological test can be helpful. *E. dispa*r-infected patients do not develop serum anti-amebic antibody titers [81, 99]. Since levels of anti-amebic antibodies remain elevated in the serum for years, serodiagnosis in endemic regions is of limited use [99, 100].

6.3.2 Antigen Detection Method

Antigen detection methods in stool sample has several advantages over previously described methods including better sensitivity, specificity, and large-scale screening tool. Gal/GalNAc lectin is one of the most studied species-specific antigens which is replacing other techniques for both clinical and research purposes [101, 102]. Limitation of this technique is the requirement for fresh, unfixed stool specimens and incapability of the test to differentiate *E. histolytica* and *E. dispar*.

6.4 Molecular Diagnosis

Several PCR-based methods have been developed that are highly sensitive and helpful that can detect and identify *E. histolytica* DNA in stool samples [103–107] in the diagnosis of amebiasis [108–112]. However, in developing countries, this method may not be well suited because it requires specialized skills, equipment, and cost [109]. Recently, a new method was developed for the diagnosis of *E. histolytica* known as loop-mediated isothermal amplification (LAMP). This diagnosis method is cost-effective and easy to perform and could be a valuable and useful diagnostic tool particularly in the developing world where amebiasis is endemic [113].

7 Treatment and Control

Treatment for *E. histolytica* varies depending on whether it is symptomatic or asymptomatic infection. For invasive infection, nitroimidazole derivative (metronidazole, tinidazole, ornidazole) is the drug of choice particularly metronidazole [4]. Approximately, 90% of patients with amebic dysentery have positive response to metronidazole. In the case of amebic colitis or fulminating amebic colitis with perforation, patients can be treated with metronidazole followed by a luminal agent such as iodoquinol with the addition of antibiotic to deal with intestinal bacteria [114–117]. Several studies have reported the importance of metronidazole treatment with the addition of a luminal agent to remove parasites that are colonized and for the treatment of amebic liver abscess except in some extreme severe cases, which may require surgery [118–120]. Treatment with luminal agents such as iodoquinol or diloxanide furoate should be given for asymptomatic infections to remove the infection and to inhibit the potential for invasive disease and reduce the risk to the public considering that individuals will also shed cysts [114, 115].

8 Prevention

In order to inhibit *E. histolytica* infection, contamination of food and water with human feces must be prevented [121]. Therefore, water filtrations or boiling water and the use of appropriate water and sewage system are effective methods to prevent contamination. To promote better understanding on the negative effect of amebic infection, public awareness programs should be encouraged that will help in reducing the infection rate of the parasite. Despite the fact that provision of adequate sanitation worldwide could help to limit the number of patients that get amebiasis, this situation is doubtful in the future. Effective vaccine development that prevents amebiasis will be an important task to completely eradicate the disease considering the fact that antigen-based vaccine has been developed that protects animals against intestinal amebiasis and amebic liver abscesses [4, 122].

9 Conclusions

E. histolytica is a tissue-lysing protozoan parasite that causes amebiasis worldwide, particularly in endemic areas of developing countries. This parasite is an interesting biological system that lacks most eukaryotic organelles and has the ability to colonize the large intestine. For unknown reasons, the parasite becomes aggressive and invades the intestinal mucosa, which can cause severe disease. In recent years, our understanding on the molecular mechanisms involved in pathogenesis has greatly advanced. However, it is not clear how or what factors change the parasite pathophysiology, as it changes its role from a nonpathogen to an agonist pathogen. Therefore, a deeper understanding on the pathogenesis of amebiasis would require insights into parasite cell biology, genetics, and comparative genomics of strains compared to nonpathogenic strains. Ultimately, better characterization of protein receptor/ligand interaction involved in pathogenesis will provide candidates for future vaccine development. Additionally, as discussed before, various diagnosis methods for the detection of disease in patients with amebic dysentery and abscess, these methods appear encouraging, but most of them are still just research tools. Finally, much work needs to be done for the development of sensitive, rapid, and appropriate techniques for the detection of amebiasis in developing countries.

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References

1. World Health Organization. Amoebiasis. Wkly Epidemiol Rec. 1997;72(14):97–100. 9100475
2. Fotedar R, Stark D, Beebe N, Marriott D, Ellis J, Harkness J. Laboratory diagnostic techniques for *Entamoeba* species. Clin Microbiol Rev. 2007;20(3):511–32. https://doi.org/10.1128/CMR.00004-07.
3. Bennett JE, Dollin R, Blaser MJ. Mandell, Douglas, and Bennett's principles and practice of infectious diseases, vol. 2. Amsterdam: Elsevier; 2014. p. 3463–80.
4. Stanley SL. Amoebiasis. Lancet. 2003;361(9362):1025–34. https://doi.org/10.1016/S0140-6736(03)12830-9.
5. Korpe PS, Liu Y, Siddique A, Kabir M, Ralston K, Ma JZ, et al. Breast milk parasite-specific antibodies and protection from amebiasis and cryptosporidiosis in Bangladeshi infants: a prospective cohort study. Clin Infect Dis. 2013;56(7):988–92.
6. Mondal D, Petri WA, Sack RB, Kirkpatrick BD, Haque R. *Entamoeba histolytica*-associated diarrheal illness is negatively associated with the growth of preschool children: evidence from a prospective study. Trans R Soc Trop Med Hyg. 2006;100(11):1032–8.
7. Stauffer W, Abd-Alla M, Ravdin JI. Prevalence and incidence of *Entamoeba histolytica* infection in South Africa and Egypt. Arch Med Res. 2006;37(2):266–9.
8. Tengku SA, Norhayati M. Public health and clinical importance of amoebiasis in Malaysia: a review. Trop Biomed. 2011;28(2):194–222.
9. Ogunlesi TA, Okeniyi JAO, Oyelami OA. Pyogenic meningitis in Ilesa, Nigeria. Indian Pediatr 2005;42(10):1019–1023. PubMed PMID:16269839.
10. Ximénez C, Morán P, Rojas L, Valadez A, Gómez A. Reassessment of the epidemiology of amebiasis: state of the art. Infect Genet Evol. 2009;9(6):1023–32.
11. Ali IKM, Clark CG, Petri WA. Molecular epidemiology of amebiasis. Infect Genet Evol. 2008;8(5):698–707.
12. Allason-Jones E, Mindel A, Sargeaunt P, Williams P. *Entamoeba histolytica* as a commensal intestinal parasite in homosexual men. N Engl J Med. 1986;15(6):353–6.
13. Haque R, Huston CD, Hughes M. Amebiasis. Engl J. 2003;348(16):1565–73.
14. Haque R, Mondal D, Karim A, Molla IH, Rahim A, Faruque ASG, et al. Prospective case-control study of the association between common enteric protozoal parasites and diarrhea in Bangladesh. Clin Infect Dis. 2009;48(9):1191–7.
15. Espinosa-Cantellano M, Martínez-Palomo A. Pathogenesis of intestinal amebiasis: from molecules to disease. Clin Microbiol Rev. 2000;13(2):318–33.
16. Burch DJ, Li E, Reed S, Jackson TFHG, Stanley SL. Isolation of a strain-specific *Entamoeba histolytica* cDNA clone. J Clin Microbiol. 1991;29(4):696–701.
17. Ali IKM, Haque R, Alam F, Kabir M, Siddique A, Petri WA. Evidence for a link between locus R-R sequence type and outcome of infection with *Entamoeba histolytica*. Clin Microbiol Infect. 2012;18(7)
18. Mortimer L, Chadee K. The immunopathogenesis of *Entamoeba histolytica*. Exp Parasitol. 2010;126(3):366–80.
19. Lohia A. The cell cycle of *Entamoeba histolytica*. Mol Cell Biochem. 2003;253 (1–2):217–22.
20. Singh U, Ehrenkaufer GM. Recent insights into *Entamoeba* development: identification of transcriptional networks associated with stage conversion. Int J Parasitol. 2009;39(1):41–7.
21. Reeves RE. Metabolism of *Entamoeba histolytica* Schaudinn, 1903. Adv Parasitol. 1984;23: 105–42. 6085216
22. Martin W, Müller M. The hydrogen hypothesis for the first eukaryote. Nature. 1998; 392(6671):37–41.
23. Ginger ML. Niche metabolism in parasitic protozoa. Philos Trans R Soc Lond Ser B Biol Sci. 2006;361(1465):101–18.

24. Loftus B, Anderson I, Davies R, Alsmark UCM, Samuelson J, Amedeo P, et al. The genome of the protist parasite *Entamoeba histolytica*. Nature. 2005;433(7028):865–8.
25. Faust DM, Guillen N. Virulence and virulence factors in *Entamoeba histolytica*, the agent of human amoebiasis. Microbes Infect. 2012;14(15):1428–41.
26. Beck DL, Boettner DR, Dragulev B, Ready K, Nozaki T, Petri WA. Identification and gene expression analysis of a large family of transmembrane kinases related to the Gal/GalNAc lectin in *Entamoeba histolytica*. Eukaryot Cell. 2005;4(4):722–32.
27. Tovy A, Hertz R, Siman-Tov R, Syan S, Faust D, Guillen N, et al. Glucose starvation boosts *Entamoeba histolytica* virulence. PLoS Negl Trop Dis. 2011;5(8):e1247.
28. Wolfe AJ. The acetate switch. Microbiol Mol Biol Rev. 2005;69(1):12–50.
29. Shahi P, Trebicz-Geffen M, Nagaraja S, Alterzon-Baumel S, Hertz R, Methling K, et al. Proteomic identification of oxidized proteins in *Entamoeba histolytica* by resin-assisted capture: insights into the role of arginase in resistance to oxidative stress. PLoS Negl Trop Dis. 2016;10(1):e0004340.
30. Padilla-Vaca F, Anaya-Velazquez F. Insights into *Entamoeba histolytica* virulence modulation. Infect Disord Drug Targets. 2010;10(4):242–50.
31. Mirelman D. Ameba-bacterium relationship in amebiasis. Microbiol Rev. 1987;51 (2):272–84.
32. Bhattacharya A, Anand MT, Paul J, Yadav N, Bhattacharya S. Molecular changes in *Entamoeba histolytica* in response to bacteria. J Eukaryot Microbiol. 45(2):28S–33S.
33. Bhattacharya A, Ghildyal R, Prasad J, Bhattacharya S, Diamond LS. Modulation of a surface antigen of *Entamoeba histolytica* in response to bacteria. Infect Immun. 1992;60(4):1711–3.
34. Petri WA, Haque R, Mann BJ. The bittersweet interface of parasite and host: lectin-carbohydrate interactions during human invasion by the parasite *Entamoeba histolytica*. Annu Rev Microbiol. 2002;56:39–64.
35. Seigneur M, Mounier J, Prevost MC, Guillen N. A lysine- and glutamic acid-rich protein, KERP1, from *Entamoeba histolytica* binds to human enterocytes. Cell Microbiol. 2005;7(4): 569–79.
36. Braga LL, Ninomiya H, McCoy JJ, Eacker S, Wiedmer T, Pham C, et al. Inhibition of the complement membrane attack complex by the galactose-specific adhesion of *Entamoeba histolytica*. J Clin Invest. 1992;90(3):1131–7.
37. Séguin R, Mann BJ, Keller K, Chadee K. The tumor necrosis factor alpha-stimulating region of galactose-inhibitable lectin of *Entamoeba histolytica* activates gamma interferon-primed macrophages for amebicidal activity mediated by nitric oxide. Infect Immun. 1997;65 (7):2522–7.
38. Saffer LD, Petri WA. Role of the galactose lectin of *Entamoeba histolytica* in adherence-dependent killing of mammalian cells. Infect Immun. 1991;59(12):4681–3.
39. Vines RR, Ramakrishnan G, Rogers JB, L a L, Mann BJ, Petri WA. Regulation of adherence and virulence by the *Entamoeba histolytica* lectin cytoplasmic domain, which contains a beta2 integrin motif. Mol Biol Cell. 1998;9(8):2069–79.
40. Tavares P, Rigothier MC, Khun H, Roux P, Huerre M, Guillén N. Roles of cell adhesion and cytoskeleton activity in *Entamoeba histolytica* pathogenesis: a delicate balance. Infect Immun. 2005;73(3):1771–8.
41. Bruchhaus I, Jacobs T, Leippe M, Tannich E. *Entamoeba histolytica* and *Entamoeba dispar*: differences in numbers and expression of cysteine proteinase genes. Mol Microbiol. 1996;22(2):255–63.
42. Moncada D, Keller K, Chadee K. *Entamoeba histolytica* cysteine proteinases disrupt the polymeric structure of colonic mucin and alter its protective function. Infect Immun. 2003;71 (2):838–44.

43. Tillack M, Biller L, Irmer H, Freitas M, Gomes MA, Tannich E, et al. The *Entamoeba histolytica* genome: primary structure and expression of proteolytic enzymes. BMC Genomics. 2007;8:170.
44. Que X, Reed SL. Cysteine proteinases and the pathogenesis of amebiasis. Clin Microbiol Rev. 2000;13(2):196–206.
45. Jacobs T, Bruchhaus I, Dandekar T, Tannich E, Leippe M. Isolation and molecular characterization of a surface-bound proteinase of *Entamoeba histolytica*. Mol Microbiol. 1998;27 (2):269–76.
46. Bansal D, Ave P, Kerneis S, Frileux P, Boche O, Baglin AC, et al. An ex-vivo human intestinal model to study *Entamoeba histolytica* pathogenesis. PLoS Negl Trop Dis. 2009;3 (11):e551.
47. Lidell ME, Moncada DM, Chadee K, Hansson GC. *Entamoeba histolytica* cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. Proc Natl Acad Sci USA. 2006;103(24):9298–303.
48. Stanley SL, Zhang T, Rubin D, Li E. Role of the *Entamoeba histolytica* cysteine proteinase in amebic liver abscess formation in severe combined immunodeficient mice. Infect Immun. 1995;63(4):1587–90.
49. Ankri S, Stolarsky T, Bracha R, Padilla-Vaca F, Mirelman D. Antisense inhibition of expression of cysteine proteinases affects *Entamoeba histolytica*-induced formation of liver abscess in hamsters. Infect Immun. 1999;67(1):421–2.
50. He C, Nora GP, Schneider EL, Kerr ID, Hansell E, Hirata K, et al. A novel *Entamoeba histolytica* cysteine proteinase, EhCP4, is key for invasive amebiasis and a therapeutic target. J Biol Chem. 2010;285(24):18516–27.
51. Leippe M, Andre J, Nickel R, Tannich E, Muller-Eberhard HJ. Amoebapores, a family of membranolytic peptides from cytoplasmic granules of *Entamoeba histolytica*: isolation, primary structure, and pore formation in bacterial cytoplasmic membranes. Mol Microbiol. 1994;14(5):895–904.
52. Berninghausen O, Leippe M. Necrosis versus apoptosis as the mechanism of target cell death induced by *Entamoeba histolytica*. Infect Immun. 1997;65(9):3615–21.
53. Bracha R, Nuchamowitz Y, Leippe M, Mirelman D. Antisense inhibition of amoebapore expression in *Entamoeba histolytica* causes a decrease in amoebic virulence. Mol Microbiol. 1999;34(3):463–72.
54. Zhang X, Zhang Z, Alexander D, Bracha R, Mirelman D, Stanley SL. Expression of amoebapores is required for full expression of *Entamoeba histolytica* virulence in amebic liver abscess but is not necessary for the induction of inflammation or tissue damage in amebic colitis. Infect Immun. 2004;72(2):678–83.
55. Leippe M, Andrä J, Müller-Eberhard HJ. Cytolytic and antibacterial activity of synthetic peptides derived from amoebapore, the pore-forming peptide of *Entamoeba histolytica*. Proc Natl Acad Sci USA. 1994;91:2602–6.
56. Lejeune M, Rybicka JM, Chadee K. Recent discoveries in the pathogenesis and immune response toward *Entamoeba histolytica*. Future Microbiol. 2009;4(1):105–18.
57. McCaul TF. Transmission electron microscopy observations of phagocytosis in trophozoites of *Entamoeba histolytica* in contact with tissue culture cells. Zeitschrift für Parasitenkd. 1977;52(3):203–11.
58. Orozco E, Suarez ME, Sanchez T. Differences in adhesion, phagocytosis and virulence of clones from *Entamoeba histolytica*, strain HM1: IMSS. Int J Parasitol. 1985;15(6):655–60.
59. Orozco E, Guarneros G, Martinez-Palomo A, Sánchez T. *Entamoeba histolytica*. Phagocytosis as a virulence factor. J Exp Med. 1983;158(5):1511–21.
60. Sateriale A, Vaithilingam A, Donnelly L, Miller P, Huston CD. Feed-forward regulation of phagocytosis by *Entamoeba histolytica*. Infect Immun. 2012;80(12):4456–62.
61. Bracha R, Mirelman D. Virulence of *Entamoeba histolytica* trophozoites. Effects of bacteria, microaerobic conditions, and metronidazole. J Exp Med. 1984;160(2):353–68.

62. Huston CD, Boettner DR, Miller-Sims V, Petri WA. Apoptotic killing and phagocytosis of host cells by the parasite *Entamoeba histolytica*. Infect Immun. 2003;71(2):964–72.
63. Moonah SN, Jiang NM, Petri WA. Host immune response to intestinal amebiasis. PLoS Pathog. 2013;9(8):e1003489.
64. Begum S, Quach J, Chadee K. Immune evasion mechanisms of *Entamoeba histolytica*: progression to disease. Front Microbiol. 2015;6:1394.
65. Chadee K, Petri WA, Innes DJ, Ravdin JI. Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. J Clin Invest. 1987;80(5):1245–54.
66. Calderon J, Schreiber RD. Activation of the alternative and classical complement pathways by *Entamoeba histolytica*. Infect Immun. 1985;50(2):560–5.
67. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. Int Rev Immunol. 2011;30(1):16–34.
68. Yu Y, Chadee K. *Entamoeba histolytica* stimulates interleukin 8 from human colonic epithelial cells without parasite-enterocyte contact. Gastroenterology. 1997;112:1536–47.
69. Galvan-Moroyoqui JM, del Carmen Dominguez-Robles M, Meza I. Pathogenic bacteria prime the induction of Toll-like receptor signalling in human colonic cells by the Gal/GalNAc lectin Carbohydrate Recognition Domain of *Entamoeba histolytica*. Int J Parasitol. 2011;41(10):1101–12.
70. Denis M, Chadee K. Human neutrophils activated by interferon-gamma and tumour necrosis factor-alpha kill *Entamoeba histolytica* trophozoites in vitro. J Leukoc Biol. 1989;46(3): 270–4.
71. Helk E, Bernin H, Ernst T, Ittrich H, Jacobs T, Heeren J, et al. TNFα-mediated liver destruction by kupffer cells and Ly6Chi monocytes during *Entamoeba histolytica* infection. PLoS Pathog. 2013;9(1):e1003096.
72. Kammanadiminti SJ, Mann BJ, Dutil L, Chadee K. Regulation of toll-like receptor-2 expression by the Gal-lectin of *Entamoeba histolytica*. FASEB J. 2004;18(1):155–7.
73. Maldonado-Bernal C, Kirschning CJ, Rosenstein Y, Rocha LM, Rios-Sarabia N, Espinosa-Cantellano M, et al. The innate immune response to *Entamoeba histolytica* lipopeptido-phosphoglycan is mediated by toll-like receptors 2 and 4. Parasite Immunol. 2005;27(4): 127–37.
74. Peterson KM, Shu J, Duggal P, Haque R, Mondal D, Petri WA, et al. Association between TNF-alpha and *Entamoeba histolytica* diarrhea. Am J Trop Med Hyg. 2010;82(4):620–5.
75. Mortimer L, Moreau F, Cornick S, Chadee K. Gal-lectin-dependent contact activates the inflammasome by invasive *Entamoeba histolytica*. Mucosal Immunol. 2013;7(4):1–13.
76. Ravdin JI, Jackson TFHG, Petri WA, Murphy CF, Ungar BLP, Gathiram V, et al. Association of serum antibodies to adherence lectin with invasive amebiasis and asymptomatic infection with pathogenic *Entamoeba histolytica*. J Infect Dis. 1990;162(3):768–72.
77. Kaur U, Sharma AK, Sharma M, Vohra H. Distribution of *Entamoeba histolytica* Gal/GalNAc lectin-specific antibody response in an endemic area. Scand J Immunol. 2004;60(5):524–8.
78. Lamm ME. Current concepts in mucosal immunity IV. How epithelial transport of IgA antibodies relates to host defense. Am J Phys. 1998;274(4 Pt 1):G614–7.
79. Ravdin JI, Moreau F, Sullivan JA, Petri WA, Mandell GL. Relationship of free intracellular calcium to the cytolytic activity of *Entamoeba histolytica*. Infect Immun. 1988;56 (6):1505–12.
80. Lotter H, Zhang T, Seydel KB, Stanley SL, Tannich E. Identification of an epitope on the *Entamoeba histolytica* 170-kD lectin conferring antibody-mediated protection against invasive amebiasis. J Exp Med. 1997;185(10):1793–801.
81. Haque R, Mondal D, Duggal P, Kabir M, Roy S, Farr BM, et al. *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. Infect Immun. 2006;74 (2):904–9.

82. Haque R, Duggal P, Ali IM, Hossain MB, Mondal D, Sack RB, et al. Innate and acquired resistance to amebiasis in bangladeshi children. J Infect Dis. 2002;186(4):547–52.
83. Haque R, Ali IM, Sack RB, Farr BM, Ramakrishnan G, Petri WA. Amebiasis and mucosal IgA antibody against the *Entamoeba histolytica* adherence lectin in Bangladeshi children. J Infect Dis. 2001;183(12):1787–93.
84. Guo X, Barroso L, Becker SM, Lyerly DM, Vedvick TS, Reed SG, et al. Protection against intestinal amebiasis by a recombinant vaccine is transferable by T cells and mediated by gamma interferon. Infect Immun. 2009;77(9):3909–18.
85. Haque R, Mondal D, Shu J, Roy S, Kabir M, Davis AN, et al. Correlation of interferon-gamma production by peripheral blood mononuclear cells with childhood malnutrition and susceptibility to amebiasis. Am J Trop Med Hyg. 2007;76(2):340–4.
86. Lotter H, Gonzalez-Roldan N, Lindner B, Winau F, Isibasi A, Moreno-Lafont M, et al. Natural killer T cells activated by a lipopeptidophosphoglycan from *Entamoeba histolytica* are critically important to control amebic liver abscess. PLoS Pathog. 2009;5(5):e1000434.
87. Brandt H, Tamayo RP. Pathology of human amebiasis. Hum Pathol. 1970;1(3):351–85.
88. Seeto RK, Rockey DC. Amebic liver abscess: epidemiology, clinical features, and outcome. West J Med. 1999;170(2):104–9.
89. Li E, Stanley J. Protozoa. Amebiasis. Gastroenterol Clin N Am. 1996;25:471–92.
90. Lucas R, Upcroft JA. Clinical significance of the redefinition of the agent of amoebiasis. Revista Latinoamericana de Microbiologia. 2001;43(4):183–7.
91. Johnson RL, Rogers JW, Zinneman HH. Amebic liver abscess. Am J Dig Dis. 1968;13(9): 822–9.
92. Parija SC, Mandal J, Ponnambath DK. Laboratory methods of identification of *Entamoeba histolytica* and its differentiation from look-alike *Entamoeba spp*. Trop Parasitol. 2014;4(2): 90–5.
93. Walsh JA, Walsh JA. Problems in recognition and diagnosis of amebiasis: Estimation of the global magnitude of morbidity and mortality. Rev Infect Dis. 1986;8(2):228–38.
94. Williams JE. Diagnostic medical parasitology. Parasitol Today. 1998;14(3):125–6.
95. Tanyuksel M, Petri WA. Laboratory diagnosis of amebiasis. Clin Microbiol Rev. 2003;16(4): 713–29.
96. Clark CG, Diamond LS. Methods for cultivation of luminal parasitic protists of clinical importance. Clin Microbiol Rev. 2002;15(3):329–41.
97. Haque R, Neville LM, Hahn P, Petri WA. Rapid diagnosis of *Entamoeba* infection by using *Entamoeba* and *Entamoeba histolytica* stool antigen detection kits. J Clin Microbiol. 1995; 33(10):2558–61.
98. Haque R, Faruque AS, Hahn P, Lyerly DM, Petri WA. *Entamoeba histolytica* and *Entamoeba dispar* infection in children in Bangladesh. J Infect Dis. 1997;175(3):734–6.
99. Haghighi A, Rezaeian M. Detection of serum antibody to *Entamoeba histolytica* in various population samples of amebic infection using an enzyme-linked immunosorbent assay. Parasitol Res. 2005;97(3):209–12.
100. Haque R, Ali IM, Petri WA. Prevalence and immune response to *Entamoeba histolytica* infection in preschool children in Bangladesh. Am J Trop Med Hyg. 1999;60(6):1031–4.
101. Mirelman D, Nuchamowitz Y, Stolarsky T. Comparison of use of enzyme-linked immunosorbent assay-based kits and PCR amplification of rRNA genes for simultaneous detection of *Entamoeba histolytica* and *E. dispar*. J Clin Microbiol. 1997;35(9):2405–7.
102. Haque R, Mollah NU, Ali IKM, Alam K, Eubanks A, Lyerly D, et al. Diagnosis of amebic liver abscess and intestinal infection with the TechLab *Entamoeba histolytica* II antigen detection and antibody tests. J Clin Microbiol. 2000;38(9):3235–9.
103. Troll H, Marti H, Weiss N. Simple differential detection of *Entamoeba histolytica* and *Entamoeba dispar* in fresh stool specimens by sodium acetate-acetic acid-formalin concentration and PCR. J Clin Microbiol. 1997;35(7):1701–5.
104. Roy S, Kabir M, Mondal D, Ali IKM, Petri WA, Haque R. Real-time-PCR assay for diagnosis of *Entamoeba histolytica* infection. J Clin Microbiol. 2005;43(5):2168–72.

105. Tannich E, Horstmann RD, Knobloch J, Arnold HH. Genomic DNA differences between pathogenic and nonpathogenic *Entamoeba histolytica*. Proc Natl Acad Sci USA. 1989; 86(13):5118–22.
106. Tachibana H, Kobayashi S, Okuzawa E, Masuda G. Detection of pathogenic *Entamoeba histolytica* DNA in liver abscess fluid by polymerase chain reaction. Int J Parasitol. 1992; 22(8):1193–6.
107. Clark CG, Diamond LS. *Entamoeba histolytica*: a method for isolate identification. Exp Parasitol. 1993;77(4):450–5.
108. Rivera WL, Tachibana H, Kanbara H. Application of the polymerase chain reaction (PCR) in the epidemiology of *Entamoeba histolytica* and *Entamoeba dispar* infections. Tokai J Exp Clin Med. 1998;23(6):413–5.
109. Haque R, Ali IKM, Akther S, Petri WA. Comparison of PCR, isoenzyme analysis, and antigen detection for diagnosis of *Entamoeba histolytica* infection. J Clin Microbiol. 1998;36(2):449–52.
110. Khairnar K, Parija SC. A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of *Entamoeba histolytica*, *E. moshkovskii* and *E. dispar* DNA in stool samples. BMC Microbiol. 2007;7:47.
111. Gonin P, Trudel L. Detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* isolates in clinical samples by PCR and enzyme-linked immunosorbent assay. J Clin Microbiol. 2003;41(1):237–41.
112. Paglia MG, Visca P. An improved PCR-based method for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in formalin-fixed stools. Acta Trop. 2004; 92(3):273–7.
113. Samie A, ElBakri A, AbuOdeh R. Amoebiasis in the tropics: epidemiology and pathogenesis. In: Rodriguez-Morales AJ, editor. Current topics in tropical medicine: InTech; 2012.
114. Pritt BS, Clark CG. Amebiasis. Mayo Clin Proc. 2008;83(10):1154–60.
115. Blessmann J, Ali IKM, Ton Nu PA, Dinh BT, Ngo Viet TQ, Le Van A, et al. Longitudinal study of intestinal *Entamoeba histolytica* infections in asymptomatic adult carriers. J Clin Microbiol. 2003;41(10):4745–50.
116. Monga NK, Wig JD, Sood KC, Dutta DV, Kaushik SP. Amebic peritonitis. Int Surg. 1977;62 (8):431–2.
117. Pehrson PO, Bengtsson E. A long-term follow up study of amoebiasis treated with metronidazole. Scand J Infect Dis. 1984;16(2):195–8.
118. Akgun Y, Tacyildiz IH, Celik Y. Amebic liver abscess: changing trends over 20 years. World J Surg. 1999;23(1):102–6.
119. Lasserre R, Jaroonvesama N, Kurathong S, Soh CT. Single-day drug treatment of amebic liver abscess. Am J Trop Med Hyg. 1983;32(4):723–6.
120. Weinke T, Grobusch MP, Güthoff W. Amebic liver abscess—rare need for percutaneous treatment modalities. Eur J Med Res. 2002;7(1):25–9.
121. Escobedo LG, Homedes N, von Alt K, Escobedo MA. Intestinal parasites in children from three West Texas border communities. J Sch Health. 2004;74(10):411–3. 1
122. Quach J, St-Pierre J, Chadee K. The future for vaccine development against *Entamoeba histolytica*. Hum Vaccin Immunother. 2014;10(6):1514–21.

Overview on Ascariasis in Humans in South Asia

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1 Introduction

Ascaris lumbricoides, a member of the soil-transmitted helminths, infects a staggering 800 million people yearly [1, 2]. It is hard to fully comprehend the worldwide impact of this nematode, but Dr. Peter Hotez, an acknowledged expert on neglected tropical diseases (NTDs), described it best as "the most important disease you have never heard of" [3]. Despite these high numbers, it is still classified as a neglected tropical disease [1, 2]. Neglected tropical diseases affect poor communities disproportionately but are often forgotten when it comes to resources and research funding [4]. Many research questions therefore remain unanswered, such as, why are children disproportionately infected? What is the role of host genetics in predisposition? What is the true degree of cognitive impairment suffered by children because of *A. lumbricoides*? What is the immune response against *Ascaris*?

Although acute symptomatology includes intestinal obstruction and morbidity, it is the chronic aspect of the disease such as growth retardation and cognitive impairment that produces the most impact at the population level [5–7]. Children in particular are most adversely affected by *Ascaris* infection and can consequently experience chronic symptoms [6, 7]. As the severity of symptoms correlates to worm burden, it is essential to investigate why some people are more heavily infected than others [8, 9]. As eradication of the parasite itself might prove to be very difficult without a vaccine, prevention is the best way to reduce the effects of acute and chronic symptoms on the population [10]. Prevention of *Ascaris* is often through mass drug administration [10]. The frequency of chemotherapy distribution depends on the local prevalence of the parasite, with frequencies ranging from twice a year to a case-to-case basis [10]. School-aged children especially are targeted in these campaigns, as this cohort is disproportionally infected with *Ascaris* and may excrete a disproportionate amount of eggs [11].

Few epidemiological studies are being conducted on *Ascaris* in the South Asian region. This apparent gap in knowledge is worrying, because *Ascaris* has a region-specific prevalence, abundance and egg production [12–14]. Without accurate epidemiological data per region, it will be hard to establish effective preventative measures, such as the above-mentioned mass drug administration programmes, but also sanitary improvements to reduce acute and chronic morbidity.

1.1 Epidemiology

In 2010, *Ascaris lumbricoides* infected 819 million people worldwide, of which 297.8 million cases occurred in the South Asian region [15]. Ascariasis additionally is responsible for 499,599 years lived with disability in South Asia and 1,110,600 globally [15]. Despite these high numbers, ascariasis is still considered a neglected tropical disease [1, 2]. The World Health Organization estimated that in the year

2010, around 2224 deaths could be attributed to the parasite [16], making *Ascaris* one of the most common foodborne parasitic infections [16].

A. lumbricoides is transmitted through the faecal-oral route, making it most prevalent in poverty-stricken settings with insufficient sanitary conditions [17–19]. A study performed in government-owned tea plantations in Sri Lanka found that *Ascaris* infection in children between 3 and 12 years was linked to poor sanitary settings; however, this difference was only found in some parts of the country [20]. Traub et al. [21] further identified a range of risk factors linked to *Ascaris* infection in an Indian population: Hindu religion, lower education level (or maternal education in the case of children), higher density of people in one household, owning pigs and lack of anthelminthic treatment. The authors also found that, although gender was not a risk factor for *Ascaris* infection, females between the ages of 16 and 20 did show an "overproportional" parasite intensity. This observed sex variation was attributed to a difference in work environments, with men mostly working in factories or other indoor environments and the women mainly being outdoor tea-pickers [21]. Gunawardena et al. [22] found similar risk factors for *Ascaris* infection in Sri Lankan children: lower altitude of below 500 meters, maternal education, household sanitation and gender. The influence of household conditions on *Ascaris* infection was confirmed through the identification of a positive correlation in an Indian fishing village near Madras between average worm burden and increasing number of family members [23]. Additionally, in low-income households in Bangladesh, it was identified that finished flooring was associated with a 44% decrease in *Ascaris* infection [24]. The authors concluded that a combination of deworming, increased hygienic sanitation and flooring may have resulted in a stronger reduction of *Ascaris* prevalence than deworming on its own [24].

In short, factors such as gender-related behaviour, environment and the socioeconomic situation including housing conditions, cultural practices, defecation habits and available infrastructure all have an influence on *Ascaris* infection [20–26].

1.2 Predisposition

Ascaris affects certain demographics disproportionally, with children between 5 and 15 years most heavily infected and children over the age of 15, together with adults, having lower infection intensities [27]. Regardless of these age-related differences, it is clear that certain people are more heavily infected than others [9, 28–30]. This means that most of the worms in an endemic area can be found in a small set of people [27]. These people, carrying the majority of the worm burden, are often described as "wormy people" [9], with heavily infected individuals often clustering in one family [31]. Subsequent to anthelminthic treatment, patients reacquire a similar worm burden to what they possessed before treatment, a phenomenon known as predisposition [32]. The overdispersed frequency distribution was first described for parasites in general by Crofton [33] and for *Ascaris*

specifically by Croll and Ghadirian [9]. Predisposition has subsequently been demonstrated for a variety of geographical locations in humans, ranging from India to Nigeria [28, 29, 32, 34, 35], and in naturally and experimentally infected pigs [36]. The mechanisms that determine predisposition are not yet fully elucidated and are likely to be multifactorial [8]. Using probability theory, McCallum [37] found that both long- (i.e. host genetics and socio-economic status) and short-term (i.e. host-acquired immune system) variables were involved.

It is this population, of heavily infected individuals, that is most at risk of having severe symptoms, morbidity and mortality due to heavy worm burdens [8]. Additionally, because of their high parasite burden, such individuals can potentially excrete more eggs and thus enhance the parasite spread [8]. Understanding the underlying mechanisms for this diversity in parasite load is therefore important with respect to enhancing parasite control and diminishing morbidity and mortality.

One of the unanswered questions regarding heterogeneity is at which point in the life cycle of *Ascaris* does predisposition occur? Taking an experimental approach, Lewis et al. [38] used a mouse model to assess a range of inbred strains of mice for resistance and susceptibility to *Ascaris* infection. The authors identified two mouse strains as model organisms for heterogeneity in *Ascaris* infection with one strain susceptible (C57BL/6J) and one relatively resistant (CBA/Ca) to *A. suum* [38].

Two main factors have been identified as contributors to the observed predisposition at the individual level of infection [39]: host genetics and host immunity. As for the role of the immune system, studies showed that putatively immune children demonstrated higher levels of *Ascaris*-specific anti-ABA IgE antibodies and pro-inflammatory proteins [28]. Deslyper et al. [40] also used C57BL/6J and CBA/Ca mice and identified an intrinsic difference in susceptibility between the two strains. These authors investigated the protein abundances in the liver of the mice at day 4 postinfection and found that the relatively resistant mouse strain, CBA/Ca, had an intrinsically higher abundance of oxidative phosphorylation cycle (OXPHOS) proteins. These proteins are part of the cellular respiration process and produce reactive oxygen species (ROS) as a by-product [41, 42]. Under infection, both mouse strains had an increased relative abundance of OXPHOS proteins but with the resistant strain still having a higher relative abundance than the susceptible strain [40]. These results indicate a potential role of ROS in early elimination of *Ascaris*. Additionally, CBA/Ca mice had an intrinsically higher relative abundance of ribosomal proteins than C57BL/6J mice. This difference became more pronounced under infection [40].

At the genetic level, the MHC genes were among the first to be implicated to play a role in predisposition. Holland et al. [43] found that the presence of the A30–31 antigen was more prevalent in consistently infected children. Subsequently, Williams-Blangero et al. [44] identified, in the Jirel population of East Nepal, that 30–50% of difference in worm burden between individuals could be attributed to host genetics. Using variance component linkage analysis, the authors measured the covariance between infection and genotype, which resulted in the identification of three loci on chromosomes 8, 11 and 13 as having an influence on susceptibility [45, 46]. An additional three suggestive loci were recognized in a

later study, using a bigger sample size and thus enhancing the power. This analysis indicated a total of four chromosomes of interest: 13, 11, 8 and 1 [45, 46]. The authors propose the TNFSF13B gene as a candidate for further investigation. Other studies confirmed these findings, suggesting TNFSF13B, together with LIG4, as important factors that might explain heterogeneity in *Ascaris* infection [47], with a possible role of these two genes in the modulation of an IgE and IgG response against the parasite in favour of a protection against *Ascaris* infection. Additionally, Peisong et al. [48] identified a STAT6 gene variant in a Chinese population that was linked with lower *Ascaris* burden.

In order to understand the role of the household level, the influence of exposure on predisposition was examined by Walker et al. [49], using data obtained from Hall et al. [50] who investigated a population in Bangladesh, using new Bayesian statistical model methods. Walker et al. [49] identified that predisposition is strongly linked within households. Additionally, an increased risk was observed for households with earth floors, without latrines and who use a common tap for their water necessities. The authors further identified a sex difference, with a higher prevalence of infection observed in adult women than adult men [49]. This sex difference was not observed in children and is likely to be ascribed to the sociological aspects of the society, where women tend to stay at home (exactly the area that has been identified as the place where most infections occur by the authors) and teenage boys and men tend to go to work during the day. As for the age-related differences in worm burden, the authors [49] point to a rapid behavioural change, as they start exploring their environment, in early childhood as likely cause for the observed decrease in baseline mean worm burden with increased age. This effect seems to be particularly strong in the first 3 life years. In sum, the authors found that individual differences play a minimal role in predisposition, whereas the household was found to be the main risk factor.

1.3 Animal Models

Although pigs are a natural host for *A. suum* infection, which in itself is closely related to the human *A. lumbricoides*, their use as model system is challenging because of cost, husbandry, size of the pigs and the lack of inbred strains [51]. A large range of animal models has been explored for *Ascaris* infection: mice, guinea pigs, rabbits, labs, goats, gerbils, rats and cows [51]. Because only pigs are natural hosts, all other model organisms have incomplete life cycles, with only the early stages of infection occurring [51], making them abnormal hosts. Additionally, Holland et al. [51] found "that in the vast majority of these models, susceptibility and resistance to *Ascaris* infection in either the liver or the lungs has not been clearly established".

Experiments on rats were conducted by Davaine as early as 1863 [52]. Stewart continued Davaine's experiments, using rats and mice [53], where he identified the presence of *Ascaris* larvae in the gut, the liver and the lungs. Slotved et al. [54]

subsequently recognized that the migratory path of *A. suum* in both pig and mouse was the same, making mice an ideal model organism. Furthermore, the migratory path of the *A. suum* larvae in mice is similar to *A. lumbricoides* in humans [54, 55]. Mice also have a higher larval recovery rate than other animals such as rats, guinea pigs and rabbits [56] and even pigs [57]. This is due to their relative host size and parasite size [38].

Lewis et al. [38] built on the early mouse work of Mitchell et al. [58] by testing a range of different mouse strains in order to mimic the susceptibility and resistance to *Ascaris* found in humans [38]. A range of different mouse strains were tested to mimic the susceptibility and resistance found in humans [38]. C57BL/6J and CBA/Ca mice were identified as a susceptible and resistant, respectively, against *Ascaris* infection [38], with C57BL/6J mice demonstrating a peak in larval numbers at 7 days postinfection and CBA/Ca mice remaining relatively resistant [51]. A change in dose of infective eggs did not modify the relative susceptibility, indicating an intrinsic difference in host factors between the two strains [38].

2 Pathogen

2.1 Types

There are two species of *Ascaris*, *Ascaris lumbricoides* Linnaeus, 1758, and *A. suum* Goeze, 1782, with the former infecting humans and the latter infecting pigs [59]. However, there has been considerable debate in the literature about whether these ascarids are truly separate species [60, 61]. Both species are morphologically very similar, with only small difference in denticle and lip morphology observed [59, 62, 63]. The use of molecular techniques was therefore an essential development, with mitochondrial DNA (mtDNA) and the first internal transcribed spacer (ITS-1) being the most frequently used molecular markers. mtDNA is often used for identifying cryptic species, with a 2% sequence variation between individuals of the same species and 10–20% between closely related species being observed [64–66]. ITS-1, conversely, has $\leq$1% sequence variation between individuals of the same species and between closely related nematode species [67, 68]. mtDNA has a higher mutation rate as ITS-1, making it preferable when analysing small sample sizes [65]. As for *Ascaris*, a difference was observed between the two in both ITS-1 and mtDNA [69, 70], with a 1.3% difference in the ITS-1 [69] and 3–4% for the mtDNA [70], which the authors interpret as evidence for two separate species. Liu et al., conversely, compared whole mtDNA extracted from *A. lumbricoides* and *A. suum* from both human and porcine sources, respectively, and found a 1.9% sequence difference, indicating a single species [71].

Another area of debate has been the possibility of cross transmission. In nonendemic areas, pig-to-human infections have been demonstrated; however, in endemic regions such cross infections are harder to verify [72–75]. Evidence for cross transmission in endemic settings was established through the identification of

Ascaris hybrids, as hybrids can only occur when the two different species were at one time present together in the same gut [72, 74, 76, 77]. The question as to whether *Ascaris* can cross infect is an important one for the development of appropriate control measures as it determines if *Ascaris* infection should be considered as a zoonosis [76]. Although rare, cross infections could have important implications regarding drug-resistant gene transfer, formation of hybrid genes, potentially increasing parasite virulence or host immune evasion [78, 79]. The presence of hybrids does not immediately imply a single species. It is possible that after mating the eggs die off straight away or that the offspring of the hybrids could be sterile or have lower fitness, all of which would be supporting the two species theory [80]. In the case of *Ascaris*, however, it is thought that the hybrids are fertile [76, 77, 80].

If the two species theory prevails, questions about the last common ancestor will arise. Anderson et al. first found that 17% of genetic variation between *Ascaris* obtained from pig samples was explained by differences in geographical location [81]. Criscione et al. [76] confirmed these results through the investigation of *Ascaris*, isolated from both human and porcine origins and which were gathered from different parts of the world. The authors found that *Ascaris* tends to cluster together depending on the region rather than based on their host organism, i.e. *A. suum* and *A. lumbricoides* cluster together per region. The authors thus concluded that *Ascaris* diverged into infecting humans and pigs separately in different locations and that this evolutionary event therefore must have happened several times. In contrast, Betson et al. [72] found evidence for a single host switch and subsequent geographical differentiation; however, they further suggest that an alternative model of multiple host switches is also a possibility [72, 76]. The above, taken together with evidence for restricted gene flow between the species, suggests the possibility of the presence of multiple species of *Ascaris* in humans and pigs [82].

Solving these species problems will prove to be essential for transmission and control purposes [82].

2.2 Life Cycle

The life cycle of *A. lumbricoides* in humans is very similar to the life cycle of *A. suum* in pigs [83]. The infective eggs, which contain L3 larvae covered with the L2 cuticle [84], are ingested and hatch in the small intestine. These larvae migrate to the caecum where they penetrate the mucosal barrier [55] and migrate to the liver via the portal blood. In the liver, the L2 cuticle is shed after which the larvae migrate to lungs on day 6–8 postinfection [57]. Here the larvae continue to penetrate the alveolar spaces and migrate to the pharynx. Subsequently they are swallowed and return to the small intestine at day 8–10 p.i. [57]. On day 10 p.i., L3 stage larvae moult into the L4 stage, after which they reach sexual maturity. Another moult occurs on day 24 p.i. resulting in L5 stage larvae [85], which are

expelled from the gut at week 23 [86]. Adult female worms measure between 20 and 35 cm, in contrast to smaller males that range from 15 to 20 cm [83]. Adult worms can survive for 1–2 years in the gut [29, 87].

Female worms produce 200,000 eggs per day; however, egg production is variable with changes in egg production occurring on a daily basis [88]. Optimal conditions for survival of infective eggs in the environment were found to be wet and dark, whereas a dry, sunny environment kills the eggs after a few weeks [89]. The eggs are sticky and can therefore get stuck to various objects such as utensils, furniture, money, fingers and door handles and food such as fruit and vegetables [90]. In the soil, the larvae within the eggs undergo two moults [84], developing into L1 larvae, which takes around 10 days at optimal conditions (28–32 °C) [91]. This delay means that it is not fresh faeces, but rather old faeces, that contain infectious material. The traces of old faeces could therefore already have disappeared, while the infectious material can still be present [92].

In endemic areas, eggs are ingested frequently. Wong et al. [93] monitored children of two households that received treatment at the start of the trial and subsequent confirmation, using Kato-Katz thick smear, that the children were parasite-free. The authors then determined the amount of soil the children consumed by measuring quantity of non-dietary silica present in the stool. After determining the density and distribution of the eggs in the play area of the children, the authors were able to estimate that the children on average ingest 9–20 *Ascaris* eggs per year. Three months later the children received another round of mebendazole, allowing for the measurement of their new worm burden. The authors were then able to compare estimated infection rate with the observed infection rate; the results then indicated that between 12 and 90% of ingested eggs developed into adult worms. Although ingestion is the most common way of infection, inhalation and swallowing of eggs from the air are also possible routes of entry in hyperendemic regions [94–96].

The exact reason why the parasite goes through such complex cycle, i.e. larval migration, is not fully known [51]. One theory [97] suggests it is an evolutionary remnant from when the parasite used to penetrate the skin. Other theories suggest that the complex life cycle provides fitness benefits to the parasite due to a reduced risk of immune-mediated damage and death occurring in the different tissues [98, 99]. Evidence for this theory was found through introducing infective larvae in the blood stream in the pigs, which resulted in a lack of larval migration and slower larval development [100].

3 Clinical Features

Most individuals infected with *A. lumbricoides* will only harbour a few worms [30, 32] and will not experience significant symptoms [101, 102]. Moderate infections can cause acute illness with symptoms such as nausea, diarrhoea and abdominal pain, which usually disappear shortly after onset [103]. Heavily infected

individuals, however, can have a wide range of symptoms which are divided into chronic and acute symptoms [104]. Chronic symptoms of *Ascaris* infection have a major public health impact [5]. Acute symptoms, conversely, are rare but more serious [104] with an increased risk of mortality. de Silva et al. [105] estimated that per 12 million acute cases, 1000 deaths occur.

The most prevalent symptoms of chronic disease are growth impairment and malnutrition [5, 92]. Malabsorption of nutrients such as fats, vitamin A and β-carotene is caused by the parasite damaging the intestinal villi and is, in combination with anorexia, the main cause of malnutrition [106]. Anorexia, together with damaged intestinal villi, can result in lactose intolerance [106]. Additionally, malnutrition in children can cause impairment of growth and physical ability, together with reduced work capacity and cognition [5, 107]. Awasthi and Pande [108] studied a paediatric population (ages between 6 and 12 months) in North India for a period of 18 months. The authors found, at the end of the study after administration of albendazole and vitamin A, a significant increase in weight for these children. Hall et al. [92] described problems involved when assessing the nutritional status of patients infected with geohelminths. The authors highlight the necessity of having an untreated control group to fully establish the extent of growth impairment in children with a gastrointestinal parasite, as non-malnourished children will experience growth and weight gain naturally.

Using four different cognitive tests, Ezeamama et al. [109] investigated the influence of soil-transmitted helminths on cognitive function in Filipino children. With respect to *Ascaris*, the investigators found an increase in test scores for the learning subscale of the Wide Range Assessment of Memory and Learning test, with decreased worm burden. The authors attributed these cognitive impairments to nutritional deficits, iron in particular, inflammatory symptoms and abdominal pain which can be distracting [6, 7]. Cognitive development is difficult to measure, as cognition is often influence by multiple causes [110]. Careful reporting of additional environmental risk factors is therefore essential for correct interpretation of these studies [110]. Furthermore, because a wide range of different functions could be impaired, a range of different cognitive tests needs to be conducted in order to map all of those affected functions [110]. The improvement of the cognitive impairment is often hard to measure after treatment, as both remedial education and psychosocial stimulation are considered contributing factors [110].

The effect geohelminths have on cognitive function has been debated, with some papers arguing there is no evidence [111] and others that there is a strong link [27, 112]. The human brain requires a high percentage of the body's metabolic energy, ranging from 87% in newborns to 27% in an adult female [113]. Parasites inhabiting the intestinal tract interfere with the body's metabolic energy balance through interference with the nutrient absorption [114]. The Cochrane Review by Taylor-Robinson et al. [111] could not find any link between deworming and cognitive development. However, Medley et al. [115] point out that "Critics of these reviews have argued that many of the included trials suffer from a number of methodological shortcomings that may bias the results, and that better-designed studies are required in this area". The authors, however, did identify three main

pitfalls for cognitive studies [4]: the lack of consensus on which cognitive domain needs to be investigated, the lack of standardization and the absence of test validation in developing countries.

The increased energetic need due to infection has been described in pigs only, where a net increase of 50–100% of wet weight of the small intestine was observed [116]. This was ascribed to a hypertrophy, mainly of the *tunica muscularis* in the bowel, in order to push food past the worms. The increased energy expenditure could result in energy loss in other parts of the body.

Both intestinal obstruction and biliary complications are the most common acute symptom of *Ascaris* infection [107]. Intestinal obstruction accounts for 57% of all complications; it is most often found in young children (5–10 years) because of their smaller intestinal lumen [104]. *Ascaris* can lead to partial blockages [117, 118], resulting in intussusception, volvulus and eventually complete obstruction [118]. All the aforementioned symptoms can cause bowel infarction and intestinal perforation with potential peritonitis. After intestinal perforation, the dead worms can induce granulomatous peritonitis [27]. Intestinal obstruction occurs mostly in children, whereas hepatobiliary and pancreatic ascariasis are more common in adults, because the larger biliary tree in adults can house an adult worm [27]. Blockage not only occurs in the intestines; the adult worms can move around the body causing blockages in different locations resulting in biliary colic, cholecystitis, cholangitis, pancreatitis and hepatic abscesses [118]. Adult worms can sometimes enter the appendix and induce symptoms very much like appendicitis [27]. Children with a high fever may suffer from moving *Ascaris* worms, which causes the worms to emerge from the nasopharynx or anus [27].

Migrating larvae can induce a range of symptoms, including asthma, coughing, skin rashes, fever, eosinophilia and substernal pain, with dying larvae causing more harm than the living ones [119–121]. While the larvae are migrating through the lung as part of their life cycle, they cause a range of symptoms including blood eosinophilia, respiratory symptoms and pulmonary infiltrates termed “Loeffler’s syndrome” [122, 123]. Dead worms can release their eggs and cause a granulomatous reaction, which can induce liver abscesses [17]. Additionally, McSharry et al. [124] found that acute phase proteins, produced in the liver, were increased in putatively immune children from Nigeria. This may indicate the presence of an immune mechanism which targets the hepatic stage of the parasite [124].

4 Molecular and Biological Aspects

4.1 Excretome/Secretome

The excretome/secretome (ES) from a parasite consists of both proteins that are actively secreted and passively released. ES proteins are known to have immunomodulatory functions in several parasites; one such parasite is *Fasciola hepatica*, which is known to secrete thioredoxin peroxidase [125] in addition to a range of

antioxidant enzymes [126]. Additionally, Spolski et al. [127] showed that the ES from *Taenia crassiceps* supressed a Th1 response.

The ES of *A. suum* has been studied sparsely and little is known about its influence on the host. Chehayeb et al. [128] found that in vitro, *A. suum* secreted a range of lipid-binding proteins. Among those lipid-binding proteins, which parasites possibly use to obtain lipids from their host, the authors discovered the presence of vitellogenins which are known to play a major role in oogenesis and development of the larvae. Another one of these lipid-binding proteins, ABA-1, is the most abundantly secreted protein and a major allergen; its function, however, remains unclear [128, 129]. Additionally, Chehayeb et al. [128] found proteases and protease inhibitors being excreted/secreted, both of which are thought to play a role in immunomodulation, with proteases having an extra function in tissue migration. Furthermore, Antunes et al. [130] found that PAS-1, an ES protein from both larval and adult stages of *Ascaris*, had an immunomodulating effect through the suppression of leucocyte infiltration in addition to the production of the pro-inflammatory cytokines IL-1β and IL-6.

As parasites excrete/secrete different proteins during their life cycle, Wang et al. [131] investigated which proteins are found during the different stages of development of *Ascaris*. The authors found that just two proteins were excreted/secreted during each of the different larval stages: serpins and 14–3-3 protein. Serine protease inhibitors are believed to have a dual function of, first, protecting the larvae from being digested and, second, coating the larvae in these serpins, hereby potentially masking them from the host's immune system [131]. The 14–3-3 protein was found to be excreted in other parasites such as *S. mansoni*. Its function remains elusive to date, but the protein is believed to be involved in the host-parasite relationship [131, 132].

Further identification and characterization of these proteins is not only necessary for our understanding of the disease progression, but it may also offer novel vaccine candidates and potential new therapies.

4.2 Genome

A draft genome of *A. suum* has been sequenced by Jex et al. [133]. The draft genome predicted the presence of 775 excretory/secretory proteins, 301 immunogen proteins and 26 immunomodulatory proteins including proteases which have been identified as Th2 immunomodulating proteins [133, 134]. Additionally, several immunomodulating homologues were predicted, including B-cell inhibitors, neutrophil inhibitors, etc. In addition, some immune evasion proteins were predicted through the mimicking of host proteins [133].

A range of peptidases linked to feeding and migration were also identified, together with homologues to olfactory chemosensory genes of volatile compounds [133]. The latter are thought to play an important part in migration of the parasite

[133]. Moreover, the authors identified sex-specific genes, for the males associated with spermatogenesis and for the females associated with oogenesis and egg laying.

This draft genome can play a significant role in the search for new drug targets [133]. These can be found by targeting either essential genes or by interfering with essential enzymatic chokepoints, which are defined by the authors as "enzymatic reactions that uniquely produce and/or consume a molecular compound" [133].

5 *Ascaris* and the Immune System

5.1 Immune Response

Upon oral infection, *Ascaris* larvae migrate through the digestive tract to the caecum, the site where the larvae penetrate the gut [55]. Gut-dwelling helminths trigger the innate immune system through damaged epithelial cells, which will secrete cytokines [135, 136]. These cytokines are called "alarmins"; they are IL-25 and IL-33 [135, 136]. These alarmins activate nuocytes, which are non-B non-T cells [135–137]. Nuocytes then have the ability to secrete IL-4 and IL-13 and alternatively activate macrophages [137]. Neill et al. [135] demonstrated the importance of these nuocytes and their cytokine production in the expulsion of *Nippostrongylus brasiliensis*.

Parallel to this, the alarmins, together with parasite-derived excretory/secretory products, are able to activate antigen-presenting cells, which subsequently can activate Th2 cells, thus inducing the Th2 response [137]. Parasite-derived excretory/secretory products are detected by the host most likely through C-type lectin receptors [138]. As for *A. suum*, Yoshida et al. [139] identified a novel C-type lectin of *A. suum* called *A. suum* C-type lectin-1 (*As*-CTL-1) in the lung tissue of infected rabbits. The authors found that *As*-CTL-1 has 38% similarity to a known C-type lectin from *Toxocara canis*: *Tc*-CTL-4.

In the gut, IL-13 induces an increase in epithelial cell turnover and goblet cell differentiation [137, 140]. Goblet cells in their turn produce mucins [137, 140, 141]. Mucins produced by goblet cells play an important role in the clearing of *Trichuris muris*, where it was found that mice lacking mucin 5a have difficulty expelling the parasite compared to mucin 5a–secreting mice [141]. Relmβ is also induced by IL-13 and disrupts the chemotactic sensors of parasites through direct binding [142, 143].

Dendritic cells have been shown to be essential for a Th2 response activation [144, 145]. An experimental approach by Pythian-Adams et al. [146] showed that *Schistosoma mansoni* infections require the presence of dendritic cell to induce a Th2 response.

Masure et al. [147] found that pigs continuously exposed to *A. suum* eggs for 14 weeks manage to develop immunity against subsequent infections, resulting in a 99.7% reduction in larvae. The authors linked this observed immunity to immunological changes in the caecum including eosinophilia, mastocytosis and a

hyperplasia of goblet cells [147]. They additionally found an increase in the recruitment of eosinophils to the caecum in immune animals upon reinfection. This was paired with an increase in IL-5, IL-13, CCL11 and eosinophil peroxidase transcripts. IL-5 is involved in the development of eosinophils and their recruitment from the bone marrow into the blood [148], and eosinophil peroxidase is involved in ROS production [149]. In vitro experiments showed that *A. suum* larvae induce degranulation of eosinophils [147]. In all, their results seem to indicate an important role for eosinophils and ROS in the development of immunity against *Ascaris* and subsequent larval expulsion [147].

After ingestion, the larvae migrate to the liver. Little is known regarding an immune response against *Ascaris* in the liver. The liver shows macroscopic signs of an innate immune response, with the presence of white spots in the livers of pigs infected with *A. suum* [150] and humans infected with *A. lumbricoides* [151]. There are two types of these white spots: granulation tissue and lymphonodular [152]. The former mainly contains eosinophils, neutrophils and macrophages; the latter mainly contains lymphoid cells.

The liver is an immunomodulatory organ [153], which could explain why *Ascaris* incorporated this organ as part of its life cycle. This is a route which is also used by *Plasmodium*; Bertolino and Bowen [154] suggested that the malaria parasite goes through a hepatic, pre-erythrocytic stage, potentially to evade the immune system.

The surviving larvae subsequently move from the liver to the lungs [57]. During the lung stage of the life cycle, an intense inflammatory response was observed in the lungs of BALB/c mice, induced at first by an influx of neutrophils, followed by eosinophils and mononuclear cells [155]. Lewis et al. [156] showed, using hydrocortisone to suppress inflammation, that this inflammatory response is not responsible for the observed difference in heterogeneity of infection. In fact, the authors identified the hepatic or post-hepatic stage as the most likely time for resistance to manifest itself.

Using BALB/c mice infected with *Ascaris*, Gazzinelli-Guimarães et al. [157] found that the earliest change in cytokine profile in the lungs was an increase of IL-5 concentration at day 4 postinfection, coinciding with the migration of the larvae towards the lung. IL-5 has been shown to play a role in the eosinophilia observed in helminth infections [157]. Because this stage is too early for this cytokine to be produced in CD4+ cells, the authors postulated that the IL-5 is secreted by either resident lung cells or nuocytes [136].

A profound increase in both eosinophils and neutrophils was confirmed in a mouse model using *A. suum* infected BALB/c mice, with a peak at day 14 postinfection [158]. Recent work by Nogueira et al. [155] demonstrated that this rise in eosinophils and mononuclear cells is more pronounced in reinfected mice, when compared to single infections. The authors also observed that this increase in eosinophils correlated with a decrease in parasite burden, suggesting a potential role for eosinophils in the clearance of infection. However, the authors note, this intense inflammatory response could be associated with tissue repair rather than worm expulsion [156].

5.2 Immunoglobulins

As for the adaptive immune response, an increase in IgE concentration is associated with *Ascaris* infection. High levels of specific IgE antibodies against one *Ascaris* protein in particular (anti-ABA-1) have been associated with natural immunity against *Ascaris* infection [124, 159]. Paterson et al. [160], however, found that ABA1 is "not intrinsically allergenic". The authors argued that the IgE response observed in *Ascaris* infections is a combination of several factors, exposure route, antigen dose and other Th2-promoting molecules, which together make ABA-1 a bystander antigen [160]. IgE was found to bind the Fc epsilon RI (high-affinity IgE) receptor on eosinophils and induce eosinophilic degranulation [161].

IgE has been found to play an important role in eosinophil-mediated cytotoxicity in *S. mansoni* infection [161]. The role of IgE, however, is a source of debate, with some evidence suggesting that high levels of *Ascaris*-specific IgE antibodies have a protective effect [159], whereas a study performed in Bangladesh found that heavily infected children have higher anti-*Ascaris* IgE than lightly infected children [162].

Miguel et al. [163] found an increase in anti-*Ascaris* IgA antibody-secreting cells (ASC) at day 10 p.i. in the lamina propria of the proximal and distal jejunum of pigs, which the authors attribute to a delayed response to larval penetration after infection. These ASCs started decreasing at day 21 p.i. The authors additionally measured increased serum anti-*Ascaris* IgA, with IgA anti-ABF serum levels remaining high throughout infection and IgA anti-L3-ES serum levels dropping after day 21 p.i. Secreted anti-IgA antibodies play an important role in the gut as they form a protective barrier for pathogens and toxins to enter the gut epithelium, through trapping and eliminating potential pathogens [164].

5.3 Th2 Response

The presence of a strong Th2 response in *Ascaris* infection has been found to be important for worm clearance, with an increased Th2 response playing a significant role in age-dependent resistance to *Ascaris* infection [165]. A Th2 immune response is associated with IL-4 and IL-5 production, two cytokines that induce IgE production and eosinophilia [166], with IL-4 in particular mediating the class switch to IgE B cells [167]. However, Nogueira et al. [155] in their model of reinfected mice found evidence of a mixed Th2/Th17 response. Th17 cells produce IL-17, which in turn produces CXCR2 ligands, which are chemoattractant for neutrophils [168]. Additional evidence for their findings was found in C57BL/6 mice infected with *S. japonicum*, which were found to produce IL-17A [168]. Furthermore, Nogueira et al. [155] found that in reinfected mice, during the first days of the repeat infection, there was a significantly higher level of IL-4. The authors [155] suggest the mixed Th2/Th17 response is part of tissue remodelling, with the larval

migration and subsequent tissue damage causing an inflammatory response that needs to be controlled.

5.4 Regulations of the Immune System

Regulation of the immune response is an important part of the immune system. Several immune modulating cells were identified to play a role in helminth infections such as alternatively activated macrophages, Treg cells and regulatory B cells [137]. Matera et al. [169] found a statistically significant increase of innate CD4+ CD25+ Treg cells in *Ascaris*-infected individuals compared to controls. The authors suggest this regulation of the immune response could be beneficial for both the host and the parasite. The parasite would be able to sustain prolonged infection [169]. The host on the other hand, the authors point out, would benefit from a dampened immune response, which would otherwise lead to pathology [169].

However, Matera et al. [169] found that in *Ascaris*-infected patients, despite an increase in Treg cells, no expected rise in IL-10 was observed. Some evidence [170], however, points towards these Treg cells modulating the immune response using IL-10. Nascimento et al. [170] induced autoimmune hepatitis in BALB/c mice, using a lectin called concanavalin A. In an attempt to reduce hepatic inflammation and tissue damage, caused presumably by concanavalin A-induced polyclonal B-cell and T-cell activation, the researchers administered *A. suum* extract. The authors found that both prophylactic and therapeutic administration of this extract showed statistically significant improvements in serum markers, which the authors believe was the result of increased IL-10 concentrations. IL-10 would then inhibit the co-stimulatory molecules from antigen-presenting cells [170]. This research highlights the potential of immune modulation of *Ascaris* infections. Antunes et al. [130] identified the PAS-1 antigen from *A. suum* as an anti-inflammatory protein which can inhibit LPS-induced inflammation in an IL-10-mediated manner. IL-10 is an important regulator of inflammation; it is produced by a wide range of cells and acts as an anti-inflammatory cytokine [171].

5.5 Other Proteins Involved

McSharry et al. [124] found that Nigerian children, putatively immune to *Ascaris*, had a higher concentration of the inflammatory markers C-reactive protein, ferritin and eosinophil cationic protein, when compared to a group predisposed to infection. Eosinophil cationic protein was found to be upregulated in Ecuadorian children chronically infected with *Ascaris* [172].

5.6 Natural Immunity

Due to the immunomodulatory aspects of helminth infection, it is hard to develop natural immunity against it [173]. The immunomodulatory properties of *A. suum* work through the secretion of phosphorylcholine, which have pathogen-associated molecular pattern properties [27]. These interfere with the immune system by disturbing lymphocyte proliferation pathways. Additionally glycosphingolipids, produced by *Ascaris*, have been shown to inhibit LPS-induced Th1 cytokine production (IFN-γ). PAS-1, PAS-2 and PAS-3 all inhibit the antibody response of BALB/c mice [174].

6 Diagnosis and Treatment

6.1 Diagnosis

A wide range of diagnostic tools have been developed for the identification of helminths. Diagnostics can be divided up into two divisions: firstly, indirect diagnostics, which looks at the egg count and presumes a correlation between the amount of eggs secreted and the amount of worms present in the gut. Secondly, direct diagnostics, in which the worms themselves are counted as measure of worm burden.

Each test has its own sensitivity, and the need for different sensitivities might change [175]. At the start of control programmes, when there is a high prevalence of the parasite in the population, a quick method with low sensitivity is required to screen large parts of the population [175]. However, later on during the programme, it will become necessary to detect light infections, and a higher sensitivity will be required [175].

The easiest and most reliable method is microscopy, which only requires a microscope with magnification 10X objective [39, 176]. Microscopy is cheap and easy to use in a low socio-economic setting [27, 177, 178]. It is a quantitative method that provides a measure of the eggs per gram of faeces (epg) [27, 177, 178]. The eggs are brown or yellow and measure approximately 55–75 μm by 35–55 μm [179]. This technique, however, cannot be used to distinguish between *A. suum* and *A. lumbricoides* [60]. There are several ways of performing microscopy, the easiest being an examination of a thick smear of the sampled faeces [39]. In the Kato-Katz method, glycerol is added to a set volume of filtered faeces, in order to clear the faecal detritus, which takes about 30 min [39, 180]. This is then applied to a template on a slide and the eggs are counted. The number of counted eggs is subsequently multiplied by a factor, depending on the used template [181].

Based on epg, the World Health Organization divides people into three categories: light-intensity infections (1–4999 epg), moderate-intensity infections (5000–49,999 epg) and heavy-intensity infection (>50,000 epg) [10]. It is assumed

that egg counts are related to the number of sexually mature female parasites present in the gut [182]. Microscopical examination, however, can still be influenced by a number of potentially confounding factors: (1) Diagnosis can be missed due to low-intensity infections or the presence of male worms only; both scenarios would lead to a reduced or absent egg production [39]. (2) The females have to be fertilized by males; if not, unfertilized eggs are produced [179]. These are longer and thinner than fertilized eggs [179]. However, only few of these are released and can therefore be hard to find [179]. (3) Eggs are sometimes clumped in the faecal matter, mixing the sample before subsampling can help, preferably with 0.9% of saline and a surfactant solution to separate eggs further [39, 183]. (4) The number of adult worms in the gut can determine the amount of eggs released by the females, which is called density-dependent fecundity, meaning that with increased worm burden, the females produce fewer eggs [13, 184–186]. (5) Geographical differences in egg production have been observed [13]. Hall and Holland [13] identified a difference in egg counts when comparing a study performed in Nigeria [28] and another one in Bangladesh [14]. Children infected with *Ascaris* in Nigeria had a 6 to 13 times greater egg count for similar worm burdens [13]. The authors point out that when only egg counts are considered, none of the children from the Bangladeshi cohort would have been categorized as heavily infected. However, 13% of this cohort would have been identified in this category if worm counts were used. An earlier study, also based in Bangladesh, gave similar results [12–14]. Given these geographical differences, Hall and Holland [13] suggest setting local epg thresholds to identify heavily infected individuals, rather than using global categories.

Quantification of infection is thus best achieved through infection intensity (worm burden in particular) rather than prevalence [39]. Worm burden can be determined through expulsion of the parasites using anthelmintic drugs [39]. However, this is a challenging process with human subjects.

Flotation and sedimentation techniques are also used for the detection and diagnosis of *Ascaris* eggs [39]. For the sedimentation method, gravitation is used to extract the eggs, with the potential of adding water, ethyl acetate and formol-ether [187, 188]. The eggs can subsequently be found in the sediment which can be microscopically examined [39]. The formol-ether technique has the benefit that it can be used in settings where no microscope is available, as the samples are fixed and can thus be examined at a later time [175]. As for the flotation technique, either $ZnSO_4$ or sugar is added [187, 189], the eggs can subsequently be found in the top layer, which can be aspirated and investigated using microscopy [39]. Two new flotation-based techniques are available, FLOTAC and mini-FLOTAC [190, 191]. Nikolay et al. [175] found that the FLOTAC method was more sensitive than direct microscopy. However, FLOTAC requires a centrifuge [190] and is more time-consuming than microscopy [192]. The mini-FLOTAC was developed to overcome those obstacles, making it easier to use with the same high sensitivity as the FLOTAC [193].

Infections can also be diagnosed using imaging techniques such as X-ray photographs [194], tomographic images [194], sonographic images [195] or endoscopy [195].

Detecting the metabolites (2-methyl-butyramide and 2-methyl-valeramide) of the worm in urine is another available method; however, this is very impractical as it requires a solvent extraction followed by gas-liquid chromatography [39, 196].

Furthermore, it is possible to find *Ascaris* DNA in faecal samples using a polymerase chain reaction with specially developed *Ascaris* primers [197, 198].

Two methods in particular are considered unreliable for diagnosis and should be avoided [39]. The first is the use of clinical symptoms to diagnose *Ascaris*, as there are no specific symptoms or signs [39]. The second method is antibody detection in blood and saliva, as the presence of these antibodies does not imply a current infection [199, 200].

The true prevalence of *Ascaris* infection in a community is always higher than the apparent prevalence [115]. However, Medley et al. [115] found that it is only those communities with an intermediate true prevalence (30–70%) that actually benefit from improved sensitivity in diagnostic tests.

6.2 Treatment

The WHO has five anthelminthic drugs on its list of essential medicines: albendazole, levamisole, mebendazole, niclosamide and pyrantel [201]. These can be given to both adults and children [201, 202]. Benzimidazoles in particular are often used in mass treatment programs against soil-transmitted helminths [203]. Ivermectin, although not included on the list, is also effective [204].

Treatments are aimed at eliminating adult worms from the gut [27]. Benzimidazole-based drugs (albendazole, mebendazole) work through binding with the β-tubulin of the worm, which inhibits microtubule polymerization, causing the adult worm to die [205]. Both levamisole and pyrantel pamoate do not kill but rather paralyse the worms; they can therefore be used to collect adult worms to measure infection intensity [39]. Both are agonists of nicotinic acetylcholine receptors [206].

Anthelmintic resistance in *A. lumbricoides* has not been observed so far [207, 208]. Other parasitic nematodes, such as *Haemonchus contortus*, have shown anthelmintic resistance against benzimidazoles, which is often caused by a single-nucleotide polymorphism in β-tubulin. Three single-nucleotide polymorphisms are known at codon positions: 200 (T → A), 167 (T → A) or 198 (A → C) [209–212]. The result of these mutations is that benzimidazoles cannot bind β-tubulin anymore and therefore lose its anthelmintic resistance [213]. The homozygous mutation at position 167 was found to be present at a high incidence in *A. lumbricoides* eggs taken from Haiti, Kenya and Panama [214]. Treatment with albendazole did not increase the incidence of the SNP in both the Haiti and Panama cohort, possibly indicating that the polymorphism occurs

naturally [214]. The Kenyan samples did show an increased incidence of these SNPs in the post-treatment group; however, this could be due to the difference in sample size in the pre- and post-treatment groups [214]. Overall the treatment with albendazole was found to be successful, indicating that this particular polymorphism has no influence on drug efficacy in *A. lumbricoides* [214]. The SNP at location 200 was found to not be present at all in *A. lumbricoides* in Panama, Uganda and Zanzibar [215].

7 Coinfections and Allergies

7.1 Ascaris *Coinfection*

The Th2 response observed in helminth infections has various consequences, including reduced immunological reactions to Th1 response-inducing vaccines [216–219] and a decreased incidence of predominantly Th1 response diseases [220]. In general, this strong immunological reaction can have implications for other nonparasite antigens, leaving the host with an impaired Th1 response [166, 221]. This impaired immune state can potentially lead to impaired viral clearance [222]. Some infections and diseases are often associated with *Ascaris*, such as HIV, malaria and allergies; these will be discussed in more detail below.

7.1.1 *Ascaris* and HIV

It is estimated that worldwide there are 22 million people coinfected with HIV and *Ascaris* [223]. It is still unclear what the exact effect of *Ascaris* infection is on the HIV viral load, with some studies suggesting that the infections exert no influence on each other [224, 225] and other studies suggesting a positive [226] or negative [227, 228] correlation.

HIV progression is measured through the decline of CD4+ T cells [226]. It is thought that the Th2 shift induced during *Ascaris* infection causes a reduction in Th1 T cells, which are necessary to activate CD8+ T cells [223, 226]. These CD8+ T cells would normally eliminate HIV-infected CD4+ T cells [223, 226]. Based on this, it has been suggested that deworming would reduce the risk of HIV infection and the disease progression [226]. Abossie and Petros [226] investigated this theory and found an increase in CD4+ T cells both at 15 weeks and 6 months after anthelminthic treatment.

Other studies, however, suggest that there is a correlation between HIV progression and *Ascaris* infection [227, 228]. This theory suggests that HIV can replicate better in Th2 cells than Th1 cells and the shift towards Th2 cells, due to *Ascaris* infection, could therefore increase the virus' chances of replication [223]. Additionally, a low systemic immune activation, such as seen in helminth infections [229], is considered a risk factor for HIV transmission [230]. A generalized immune

suppression caused by *Ascaris* infection could be responsible for those studies which fail to detect any link between HIV and *Ascaris* infection [223].

7.1.2 *Ascaris* and Malaria

Malaria and *Ascaris* are geographically often found together. The effect the two have on each other could therefore have important implications. As with HIV coinfection, it remains unclear what, if any, the extent of the effect is, with several studies suggesting a protective effect of *A. lumbricoides* on malaria [231], some studies suggesting the opposite [231, 232] and still others suggesting no influence [231]. If there is in fact an association, the consequences of a mass *Ascaris* eradication programme would need to be considered, with potential requirement of adding several antiparasitic drugs [233].

Both possibilities are associated with a number of theories explaining these findings; however, the exact mechanism is not understood, as the immunological response to both parasites separately is not fully understood yet. One such theory is that the preferred Th2/Treg response during *Ascaris* infection reduces the Th1 response necessary to expel *Plasmodium* parasites [234, 235]. Alternatively, the rise of IgE in *Ascaris*-infected patients leads to activation of CD23, which in turn induces an increase in IL-10 [236]. IL-10 induces, through a rise in nitric oxide synthase, a rise an nitric oxide which would be detrimental for *Plasmodium* parasites [236].

Conversely, the beneficial effect of a coinfection can be explained through the balance between pro-inflammatory and immunoregulatory cytokines from both infections, which can result in asymptomatic malaria infections [223]. Making that the immunomodulatory cytokines produced during an *Ascaris* infection may thus have a beneficial effect on malaria infection.

Superficially it may thus appear that deworming people would have a detrimental effect on the population regarding malaria morbidity [223]. However, helminth infection is linked to an increase in gametocytes, increasing the chance of malaria transmission [237]. Additionally, *Ascaris* coinfection has been linked to an increase mixed malaria infection (*P. vivax* and *P. falciparum*) [238], making a coinfection both beneficial and detrimental.

7.2 *Allergies and* Ascaris

The role of *Ascaris* in allergies remains controversial. *Ascaris* has been attributed the quality of reducing allergic reactions, including asthma [239, 240]. Other studies, conversely, could not find a protective effect of parasite infection against allergies [241, 242].

Cooper [243] identified four factors which could potentially play a role in a potential positive effect of helminth infections on allergies:

1. Timing, where chronic infections are more likely to suppress allergies and periodic infections are more likely to enhance it.
2. Intensity of infection, with heavy infections inducing more immunomodulatory effects and reducing allergy.
3. Host genetics, individuals with a type of host genetics more susceptible to atopic diseases are more likely to have allergic reactions to parasitic infections but might be more resistant to infections.
4. Parasite, depending on the parasite species, a different allergic response might be elicited.

Ascaris in particular has been linked to potentially increasing the risk of asthma [244, 245]. A cross-sectional study performed in children from rural Bangladesh indicated that *Ascaris* infection was a risk factor for asthma and atopy [246]. Another study performed on Bangladeshi children found a link between anti-*Ascaris* IgE levels and an increase in bronchial hyperresponsiveness [247].

The influence of *Ascaris* on allergic reactions is possible either by (1) the enhancement or suppression of the immune system against the parasite or (2) cross-reactivity with helminth allergens and aeroallergens [243].

8 Prevention and Control

Ascaris prevention is done through regular administration of chemotherapeutic drugs to vulnerable populations: pre-school-aged children, school-aged children, women of reproductive age and particular at-risk groups [10]. In 2002, the WHO held a consultation on the participation of children between the age of 12 and 24 months in the mass treatment programmes [248]. The WHO confirmed it was safe to include this age group in the programmes. The need to include this group was highlighted by Kirwan et al. [249], who found that there was a high prevalence of *Ascaris* in the 12–25 month group of Nigerian children. The authors additionally found that *Ascaris* prevalence and intensity increased with age.

The WHO recommends chemotherapy should be given twice annually in areas where over 50% of the school-aged children are infected with soil-transmitted helminths and once annually for those areas where 20–50% of school-aged children are infected [10]. No mass treatment with chemotherapy of the population is advisable in areas where less than 20% of the children are infected with STH [10]. The preventative treatment of school-aged children is important for all age groups as this demographic contains most heavy infections and potentially excretes a disproportionate amount of infective eggs into the environment [11]. The administration of chemotherapy to this group will therefore benefit the entire community.

Interference with transmission is difficult, given that infective eggs of *Ascaris* are present in the environment. A vaccine would therefore be a great help to *Ascaris* elimination. Several vaccines have been trialled on pigs, such as ultraviolet-irradiated eggs, which gave 94% protection [250]. Other vaccines targeted specific

egg antigens of *A. suum* (As14&As16, As26, As37, As-Enol-1) [173, 251], antigen from the adult worm (As-GST-1) [251] and homologues from other parasites such as hookworm-derived Ac16 homologue and the RAL-1 homologue from filariae [173].

It is still more feasible to eliminate morbidity rather than the parasite itself [10]. This is for two reasons, the first being that anthelmintic treatments are very effective against heavy infections and can thus reduce morbidity efficiently [10]. Secondly, chemotherapy is easy to administer in countries with limited resources and is sometimes available for free [10]. Health education and improved sanitary facilities [252] should also be considered in order to reduce infection. Several studies showed that the use of latrines results in a significant improvement of infection rates, intensity and time between chemotherapy and reinfection [18, 253, 254]. Sewage disposal has also played an important role in Japan, South Korea and the United States of America, as part of successful *Ascaris* elimination campaigns [255]. However, economic development in general remains the best possible route to eliminating STH [107].

Using mathematical models, it is possible to predict if *Ascaris* transmission can be halted using conventional chemotherapy [255]. Hollingsworth et al. [255] found that a breakpoint in *Ascaris* transmission could be reached through several rounds of treatment, as each round reduces the average worm load [255]. However, the breakpoint at which the *Ascaris* population would go extinct is rather low, with an average of close to 0 worms per host is necessary [255]. This low number is mainly due to aggregation of parasites in few hosts and the bounce back with post-treatment levels [255].

9 Conclusion

Ascaris is a major burden on the world population, with the parasite particularly affecting poor communities in low-income countries [4]. Acute symptoms, though rare, are associated with morbidity [104]. However, it is the chronic symptoms that contribute to the significant public health impact of this neglected disease [5]. Some people, in particular, carry a disproportionate worm load, and it is these people that experience most severe symptoms [104]. Identifying the mechanisms behind *Ascaris* infection in general, and heterogeneity of infection in particular, is therefore a major research challenge which needs to be addressed in order to reduce the global burden of this parasite.

Mass control programmes are an effective way of reducing *Ascaris* infection [10]. A range of drugs are available for treatment, and these can be administered at different frequencies, ranging from mass drug administration twice a year in high-prevalence settings to a case-to-case basis in low-prevalence settings [10]. However, the main goal, to eradicate *Ascaris*, will prove difficult to accomplish with currently available methods [255]. Firstly, there is the matter of fecundity [255]. The excreted eggs are resistant to a range of environmental factors, making them resilient and

hard to eliminate [255]. Secondly, humans are unable to mount a sufficiently protective immune response against the penetrating larvae [255]. Given these difficulties, it is important to combine mass drug treatment with socio-economic improvements such as improved sanitary facilities.

The study of ascariasis through epidemiological means has its limitations as only one part of *Ascaris'* life cycle: the gut-dwelling worm can be investigated [51]. What happens during tissue migration by *Ascaris* larvae can only, due to ethical constraints, be studied in animal model systems at post mortem [51]. The introduction of a mouse model has been an important step in solving these mysteries. Douvres and Tromba [56] were among the first authors to use *A. suum* in a wide range of model systems (rabbits, guinea pigs, mice and swine). The authors concluded that mice, despite being non-natural hosts, were well suited as model organisms. Since then, the mouse model has been developed further with an increasing number of studies now employing mice to study *Ascaris* infection [38, 54, 155]. Using a mouse model, it was possible to identify the liver as most likely site for resistance to *Ascaris* to occur [156].

Future research will have to focus on answering elementary questions such as the exact immune response during *Ascaris* infection and the molecular mechanism behind heterogeneity of infection. Only when those questions are answered will it be possible to look further forward and develop new techniques and drugs to attempt full elimination of human ascariasis.

References

1. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Invest. 2008;118(4):1311–21. https://doi.org/10.1172/JCI34261. PubMed PMID: 18382743; PubMed Central PMCID: PMCPMC2276811
2. Ascaris. The neglected parasite. Amsterdam: Elsevier; 2013.
3. Hotez P. Foreword. *Ascaris*: the neglected parasite. Amsterdam: Elsevier; 2013. p. xiii–xiv.
4. Kvalsvig J, Albonico M. Effects of geohelminth infections on neurological development. In: Garcia HH, Tanowitz HB, Del Brutto OH, editors. Neuroparasitology and tropical neurology. Handbook of clinical neurology. Amsterdam: Elsevier; 2013.
5. Stephenson LS, Latham MC, Ottesen EA. Malnutrition and parasitic helminth infections. Parasitology. 2000;121(Suppl):S23–38. PubMed PMID: 11386688
6. Olsen A, Magnussen P, Ouma JH, Andreassen J, Friis H. The contribution of hookworm and other parasitic infections to haemoglobin and iron status among children and adults in western Kenya. Trans R Soc Trop Med Hyg. 1998;92(6):643–9. PubMed PMID: 10326110
7. Watkins WE, Pollitt E. "Stupidity or worms": do intestinal worms impair mental performance? Psychol Bull. 1997;121(2):171–91. PubMed PMID: 9100486
8. Holland CV. Predisposition to ascariasis: patterns, mechanisms and implications. Parasitology. 2009;136(12):1537–47. https://doi.org/10.1017/S0031182009005952. PubMed PMID: 19450374
9. Croll NA, Ghadirian E. Wormy persons: contributions to the nature and patterns of overdispersion with *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus* and *Trichuris trichiura*. Trop Geogr Med. 1981;33(3):241–8. PubMed PMID: 7314236

10. WHO. Soil-transmitted helminthiases: eliminating as public health problem soil-transmitted helminthiases in children: progress report 2001–2010 and strategic plan 2011–2020. Geneva: World Health Organization; 2012.
11. Anderson RM, Truscott JE, Pullan RL, Brooker SJ, Hollingsworth TD. How effective is school-based deworming for the community-wide control of soil-transmitted helminths? PLoS Negl Trop Dis. 2013;7(2):e2027. https://doi.org/10.1371/journal.pntd.0002027. PubMed PMID: 23469293; PubMed Central PMCID: PMCPMC3585037
12. Martin J, Keymer A, Isherwood RJ, Wainwright SM. The prevalence and intensity of *Ascaris lumbricoides* infections in Moslem children from northern Bangladesh. Trans R Soc Trop Med Hyg. 1983;77(5):5.
13. Hall A, Holland C. Geographical variation in *Ascaris lumbricoides* fecundity and its implications for helminth control. Parasitol Today. 2000;16(12):540–4. PubMed PMID: 11121853
14. Hall A, Anwar KS, Tomkins A, Rahman L. The distribution of *Ascaris lumbricoides* in human hosts: a study of 1765 people in Bangladesh. Trans R Soc Trop Med Hyg. 1999;93 (5):503–10. PubMed PMID: 10696405
15. Pullan RL, Smith JL, Jasrasaria R, Brooker SJ. Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. Parasit Vectors. 2014;7:37. https://doi.org/10.1186/1756-3305-7-37. PubMed PMID: 24447578; PubMed Central PMCID: PMCPMC3905661
16. Torgerson PR, Devleesschauwer B, Praet N, Speybroeck N, Willingham AL, Kasuga F, et al. World Health Organization estimates of the global and regional disease burden of 11 foodborne parasitic diseases, 2010: a data synthesis. PLoS Med. 2015;12(12):e1001920. https://doi.org/10.1371/journal.pmed.1001920. PubMed PMID: 26633705; PubMed Central PMCID: PMCPMC4668834
17. Brooker SJ, Pullan RL. Chapter 13—*Ascaris lumbricoides* and Ascariasis: estimating numbers infected and burden of disease. In: Holland C, editor. Ascaris: the neglected parasite. Amsterdam: Elsevier; 2013. p. 343–62.
18. Henry FJ. Reinfection with *Ascaris lumbricoides* after chemotherapy: a comparative study in three villages with varying sanitation. Trans R Soc Trop Med Hyg. 1988;82(3):460–4. PubMed PMID: 3232186
19. Mata L. Sociocultural factors in the control and prevention of parasitic diseases. Rev Infect Dis. 1982;4(4):871–9. PubMed PMID: 6214836
20. Sorensen E, Ismail M, Amarasinghe DK, Hettiarachchi I, Dassenaieke TS. The effect of the availability of latrines on soil-transmitted nematode infections in the plantation sector in Sri Lanka. Am J Trop Med Hyg. 1994;51(1):36–9. PubMed PMID: 8059913
21. Traub RJ, Robertson ID, Irwin P, Mencke N, Andrew Thompson RC. The prevalence, intensities and risk factors associated with geohelminth infection in tea-growing communities of Assam, India. Trop Med Int Health. 2004;9(6):688–701. https://doi.org/10.1111/j.1365-3156.2004.01252.x. PubMed PMID: 15189459
22. Gunawardena K, Kumarendran B, Ebenezer R, Gunasingha MS, Pathmeswaran A, de Silva N. Soil-transmitted helminth infections among plantation sector schoolchildren in Sri Lanka: prevalence after ten years of preventive chemotherapy. PLoS Negl Trop Dis. 2011;5(9): e1341. https://doi.org/10.1371/journal.pntd.0001341. PubMed PMID: 21980549; PubMed Central PMCID: PMCPMC3181244
23. Haswell-Elkins M, Elkins D, Anderson RM. The influence of individual, social group and household factors on the distribution of *Ascaris lumbricoides* within a community and implications for control strategies. Parasitology. 1989;98(Pt 1):125–34. PubMed PMID: 2717212
24. Benjamin-Chung J, Nazneen A, Halder AK, Haque R, Siddique A, Uddin MS, et al. The interaction of deworming, improved sanitation, and household flooring with soil-transmitted helminth infection in rural Bangladesh. PLoS Negl Trop Dis. 2015;9(12):e0004256. https://doi.org/10.1371/journal.pntd.0004256. PubMed PMID: 26624994; PubMed Central PMCID: PMCPMC4666415

25. Holland CV, Taren DL, Crompton DW, Nesheim MC, Sanjur D, Barbeau I, et al. Intestinal helminthiases in relation to the socioeconomic environment of Panamanian children. Soc Sci Med. 1988;26(2):209–13. PubMed PMID: 3347848
26. Kightlinger LK, Seed JR, Kightlinger MB. *Ascaris lumbricoides* intensity in relation to environmental, socioeconomic, and behavioral determinants of exposure to infection in children from southeast Madagascar. J Parasitol. 1998;84(3):480–4. PubMed PMID: 9645843
27. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, et al. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet. 2006;367 (9521):1521–32. https://doi.org/10.1016/S0140-6736(06)68653-4. PubMed PMID: 16679166
28. Holland CV, Asaolu SO, Crompton DW, Stoddart RC, Macdonald R, Torimiro SE. The epidemiology of *Ascaris lumbricoides* and other soil-transmitted helminths in primary school children from Ile-Ife, Nigeria. Parasitology. 1989;99(Pt 2):275–85. PubMed PMID: 2594419
29. Elkins DB, Haswell-Elkins M, Anderson RM. The epidemiology and control of intestinal helminths in the Pulicat Lake region of Southern India. I. Study design and pre- and post-treatment observations on *Ascaris lumbricoides* infection. Trans R Soc Trop Med Hyg. 1986;80(5):774–92. PubMed PMID: 3603617
30. Thein-Hlaing T-S, Htay-Htay-Aye M-L, Thein-Maung-Myint. Epidemiology and transmission dynamics of *Ascaris lumbricoides* in Okpo village, rural Burma. Trans R Soc Trop Med Hyg. 1984;78(4):497–504. PubMed PMID: 6237473
31. Forrester JE, Scott ME, Bundy DA, Golden MH. Clustering of *Ascaris lumbricoides* and *Trichuris trichiura* infections within households. Trans R Soc Trop Med Hyg. 1988;82 (2):282–8. PubMed PMID: 3188157
32. Croll NA, Anderson RM, Gyorkos TW, Ghadirian E. The population biology and control of *Ascaris lumbricoides* in a rural community in Iran. Trans R Soc Trop Med Hyg. 1982;76 (2):187–97. PubMed PMID: 7101403
33. Crofton HD. A quantitative approach to parasitism. Parasitology. 1971;63:343–64.
34. Seo BS, Cho SY, Chai JY. Frequency distribution of *Ascaris Lumbricoides* in rural Koreans with special reference on the effect of changing endemicity. Kisaengchunghak Chapchi. 1979;17(2):105–13. PubMed PMID: 12902750
35. Anderson RM, May RM. Population dynamics of human helminth infections: control by chemotherapy. Nature. 1982;297(5867):557–63. PubMed PMID: 7088139
36. Boes J, Medley GF, Eriksen L, Roepstorff A, Nansen P. Distribution of *Ascaris suum* in experimentally and naturally infected pigs and comparison with *Ascaris lumbricoides* infections in humans. Parasitology. 1998;117(Pt 6):589–96. PubMed PMID: 9881384
37. McCallum HI. Covariance in parasite burdens: the effect of predisposition to infection. Parasitology. 1990;100(Pt 1):153–9. PubMed PMID: 2179831
38. Lewis R, Behnke JM, Stafford P, Holland CV. The development of a mouse model to explore resistance and susceptibility to early *Ascaris suum* infection. Parasitology. 2006;132:289–300.
39. Walker M, Hall A. Basáñez. *Ascaris lumbricoides*: new epidemiological insights and mathematical approaches. In: Holland C, editor. *Ascaris*: the neglected parasite. Amsterdam: Elsevier; 2013.
40. Deslyper G, Colgan TJ, Cooper AJ, Holland CV, Carolan JC. A proteomic investigation of hepatic resistance to *Ascaris* in a murine model. PLoS Negl Trop Dis. 2016;10(8):e0004837. https://doi.org/10.1371/journal.pntd.0004837. PubMed PMID: 27490109
41. Adam-Vizi V. Production of reactive oxygen species in brain mitochondria: contribution by electron transport chain and non-electron transport chain sources. Antioxid Redox Signal. 2005;7(9–10):1140–9. https://doi.org/10.1089/ars.2005.7.1140. PubMed PMID: 16115017
42. Kowaltowski AJ, de Souza-Pinto NC, Castilho RF, Vercesi AE. Mitochondria and reactive oxygen species. Free Radic Biol Med. 2009;47(4):333–43. https://doi.org/10.1016/j.freeradbiomed.2009.05.004. PubMed PMID: 19427899

43. Holland CV, Crompton DW, Asaolu SO, Crichton WB, Torimiro SE, Walters DE. A possible genetic factor influencing protection from infection with *Ascaris lumbricoides* in Nigerian children. J Parasitol. 1992;78(5):915–6. PubMed PMID: 1403439
44. Williams-Blangero S, Subedi J, Upadhayay RP, Manral DB, Rai DR, Jha B, et al. Genetic analysis of susceptibility to infection with *Ascaris lumbricoides*. Am J Trop Med Hyg. 1999;60(6):921–6. PubMed PMID: 10403321
45. Williams-Blangero S, VandeBerg JL, Subedi J, Aivaliotis MJ, Rai DR, Upadhayay RP, et al. Genes on chromosomes 1 and 13 have significant effects on *Ascaris* infection. Proc Natl Acad Sci USA. 2002;99(8):5533–8. https://doi.org/10.1073/pnas.082115999. PubMed PMID: 11960011; PubMed Central PMCID: PMCPMC122804
46. Williams-Blangero S, Vandeberg JL, Subedi J, Jha B, Corrêa-Oliveira R, Blangero J. Localization of multiple quantitative trait loci influencing susceptibility to infection with *Ascaris lumbricoides*. J Infect Dis. 2008;197(1):66–71. https://doi.org/10.1086/524060. PubMed PMID: 18171287
47. Acevedo N, Mercado D, Vergara C, Sánchez J, Kennedy MW, Jiménez S, et al. Association between total immunoglobulin E and antibody responses to naturally acquired *Ascaris lumbricoides* infection and polymorphisms of immune system-related LIG4, TNFSF13B and IRS2 genes. Clin Exp Immunol. 2009;157(2):282–90. https://doi.org/10.1111/j.1365-2249.2009.03948.x. PubMed PMID: 19604268; PubMed Central PMCID: PMCPMC2730854
48. Peisong G, Yamasaki A, Mao XQ, Enomoto T, Feng Z, Gloria-Bottini F, et al. An asthma-associated genetic variant of STAT6 predicts low burden of *ascaris* worm infestation. Genes Immun. 2004;5(1):58–62. https://doi.org/10.1038/sj.gene.6364030. PubMed PMID: 14735150
49. Walker M, Hall A, Basáñez MG. Individual predisposition, household clustering and risk factors for human infection with *Ascaris lumbricoides*: new epidemiological insights. PLoS Negl Trop Dis. 2011;5(4):e1047. https://doi.org/10.1371/journal.pntd.0001047. PubMed PMID: 21541362; PubMed Central PMCID: PMCPMC3082514
50. Hall A, Anwar KS, Tomkins AM. Intensity of reinfection with *Ascaris lumbricoides* and its implications for parasite control. Lancet. 1992;339(8804):1253–7. PubMed PMID: 1349668
51. Holland CV, Behnke JM, Dold C. Larval Ascariasis: impact, significance, and model organisms. In: Holland CV, editor. *Ascaris*: the neglected parasite. Oxford: Newnes; 2013. p. 108–25.
52. Davaine J. Nouvelles recherches sur le développement et la propagation de l'ascaride lombricoide et du trichocéphale de l'homme. Compt Rend Soc Biol. 1863;3(4):261–5.
53. Stewart FH. On the development of *Ascaris lumbricoides* Lin. and *Ascaris suilla* Duj. in the rat and mouse. Parasitology. 1917;9(02):213–27. https://doi.org/10.1017/S0031182000006053.
54. Slotved HC, Eriksen L, Murrell KD, Nansen P. Early *Ascaris suum* migration in mice as a model for pigs. J Parasitol. 1998;84(1):16–8. PubMed PMID: 9488331
55. Murrell KD, Eriksen L, Nansen P, Slotved HC, Rasmussen T. *Ascaris suum*: a revision of its early migratory path and implications for human ascariasis. J Parasitol. 1997;83(2):255–60. PubMed PMID: 9105307
56. Douvres FW, Tromba FG. Comparative development of *Ascaris suum* in rabbits, guinea pigs, mice and swine in 11 days. Proc Helminthol Soc Wash. 1971;38:246–52.
57. Roepstorff A, Eriksen L, Slotved HC, Nansen P. Experimental *Ascaris suum* infection in the pig: worm population kinetics following single inoculations with three doses of infective eggs. Parasitology. 1997;115(Pt 4):443–52. PubMed PMID: 9364572
58. Mitchell GF, Hogarth-Scott RS, Lewers HM, Edwards RD, Cousins G, Moore T. Studies on immune response to parasite antigens in mice. I. *Ascaris suum* larvae numbers and antiphosphorylcholine responses in infected mice of various strains and hypothymic nu/nu mice. Int Arch Allergy Immunol. 1976;56:64–78.

59. Maung M. *Ascaris lumbricoides* Linné, 1758 and *Ascaris suum* Goeze, 1782: morphological differences between specimens obtained from man and pig. Southeast Asian J Trop Med Public Health. 1973;4(1):41–5. PubMed PMID: 4718127
60. Leles D, Gardner SL, Reinhard K, Iñiguez A, Araujo A. Are *Ascaris lumbricoides* and *Ascaris suum* a single species? Parasit Vectors. 2012;5:42. https://doi.org/10.1186/1756-3305-5-42. PubMed PMID: 22348306; PubMed Central PMCID: PMCPMC3293767
61. Peng W, Criscione CD. Ascariasis in people and pigs: new inferences from DNA analysis of worm populations. Infect Genet Evol. 2012;12(2):227–35. https://doi.org/10.1016/j.meegid.2012.01.012. PubMed PMID: 22306815
62. Ansel M, Thibaut M. Value of the specific distinction between *tascaris lumbricoïdes* Linnè 1758 and *Ascaris suum* Goeze 1782. Int J Parasitol. 1973;3(3):317–9. PubMed PMID: 4732028
63. Sprent JF. Anatomical distinction between human and pig strains of *Ascaris*. Nature. 1952;170(4328):627–8. PubMed PMID: 13002394
64. Cavallero S, Snabel V, Pacella F, Perrone V, D'Amelio S. Phylogeographical studies of *Ascaris* spp. based on ribosomal and mitochondrial DNA sequences. PLoS Negl Trop Dis. 2013;7(4):e2170. https://doi.org/10.1371/journal.pntd.0002170. PubMed PMID: 23593529; PubMed Central PMCID: PMCPMC3623706
65. Blouin MS. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. Int J Parasitol. 2002;32(5):527–31. PubMed PMID: 11943225
66. Blouin MS, Yowell CA, Courtney CH, Dame JB. Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. Mol Biol Evol. 1998;15(12):1719–27. PubMed PMID: 9866206
67. Stevenson LA, Chilton NB, Gasser RB. Differentiation of *Haemonchus placei* from *H. contortus* (Nematoda: *Trichostrongylidae*) by the ribosomal DNA second internal transcribed spacer. Int J Parasitol. 1995;25(4):483–8. PubMed PMID: 7635624
68. Nadler SA, Hudspeth DS. Phylogeny of the *Ascaridoidea* (Nematoda: *Ascaridida*) based on three genes and morphology: hypotheses of structural and sequence evolution. J Parasitol. 2000;86(2):380–93. https://doi.org/10.1645/0022-3395(2000)086[0380:POTANA]2.0.CO;2. PubMed PMID: 10780561
69. Zhu X, Chilton NB, Jacobs DE, Boes J, Gasser RB. Characterisation of *Ascaris* from human and pig hosts by nuclear ribosomal DNA sequences. Int J Parasitol. 1999;29(3):469–78. PubMed PMID: 10333331
70. Anderson TJ, Romero-Abal ME, Jaenike J. Genetic structure and epidemiology of *Ascaris* populations: patterns of host affiliation in Guatemala. Parasitology. 1993;107(Pt 3):319–34. PubMed PMID: 7901831
71. Liu GH, CY W, Song HQ, Wei SJ, MJ X, Lin RQ, et al. Comparative analyses of the complete mitochondrial genomes of *Ascaris lumbricoides* and *Ascaris suum* from humans and pigs. Gene. 2012;492(1):110–6. https://doi.org/10.1016/j.gene.2011.10.043. PubMed PMID: 22075400
72. Betson M, Nejsum P, Bendall RP, Deb RM, Stothard JR. Molecular epidemiology of ascariasis: a global perspective on the transmission dynamics of *Ascaris* in people and pigs. J Infect Dis. 2014;210(6):932–41. https://doi.org/10.1093/infdis/jiu193. PubMed PMID: 24688073; PubMed Central PMCID: PMCPMC4136802
73. Anderson TJ. Ascaris infections in humans from North America: molecular evidence for cross-infection. Parasitology. 1995;110(Pt 2):215–9. PubMed PMID: 7885739
74. Nejsum P, Parker ED, Frydenberg J, Roepstorff A, Boes J, Haque R, et al. Ascariasis is a zoonosis in Denmark. J Clin Microbiol. 2005;43(3):1142–8. https://doi.org/10.1128/JCM.43.3.1142-1148.2005. PubMed PMID: 15750075; PubMed Central PMCID: PMCPMC1081283
75. Arizono N, Yoshimura Y, Tohzaka N, Yamada M, Tegoshi T, Onishi K, et al. Ascariasis in Japan: is pig-derived *Ascaris* infecting humans? Jpn J Infect Dis. 2010;63(6):447–8. PubMed PMID: 21099099
76. Criscione CD, Anderson JD, Sudimack D, Peng W, Jha B, Williams-Blangero S, et al. Disentangling hybridization and host colonization in parasitic roundworms of humans and

pigs. Proc Biol Sci. 2007;274(1626):2669–77. https://doi.org/10.1098/rspb.2007.0877. PubMed PMID: 17725977; PubMed Central PMCID: PMCPMC2279219
77. Zhou C, Li M, Yuan K, Deng S, Peng W. Pig *Ascaris*: an important source of human ascariasis in China. Infect Genet Evol. 2012;12(6):1172–7. https://doi.org/10.1016/j.meegid.2012.04.016. PubMed PMID: 22561394
78. Anderson TJ. The dangers of using single locus markers in parasite epidemiology: *Ascaris* as a case study. Trends Parasitol. 2001;17(4):183–8. PubMed PMID: 11282508
79. Arnold ML. Natural hybridization and the evolution of domesticated, pest and disease organisms. Mol Ecol. 2004;13(5):997–1007. https://doi.org/10.1111/j.1365-294X.2004.02145.x. PubMed PMID: 15078439
80. Betson M, Nejsum P, Stothard JR. Chapter 10—from the twig tips to the deeper branches: new insights into evolutionary history and phylogeography of *Ascaris*. *Ascaris*: the neglected parasite. Amsterdam: Elsevier; 2013. p. 265–85.
81. Anderson TJ, Jaenike J. Host specificity, evolutionary relationships and macrogeographic differentiation among *Ascaris* populations from humans and pigs. Parasitology. 1997;115 (Pt 3):325–42. PubMed PMID: 9300471
82. Søe MJ, Kapel CM, Nejsum P. *Ascaris* from humans and pigs appear to be reproductively isolated species. PLoS Negl Trop Dis. 2016;10(9):e0004855. https://doi.org/10.1371/journal.pntd.0004855. PubMed PMID: 27583548; PubMed Central PMCID: PMCPMC5008633
83. Dold C, Holland CV. *Ascaris* and ascariasis. Microbes Infect. 2011;13(7):632–7. https://doi.org/10.1016/j.micinf.2010.09.012. PubMed PMID: 20934531
84. Geenen PL, Bresciani J, Boes J, Pedersen A, Eriksen L, Fagerholm HP, et al. The morphogenesis of *Ascaris suum* to the infective third-stage larvae within the egg. J Parasitol. 1999;85 (4):616–22. PubMed PMID: 10461940
85. Pilitt PA, Lichtenfels JR, Tromba FG, Madden PA. Differentiation of late fourth and early fifth stages of *Ascaris suum* Goeze, 1782 (Nematoda: Ascaridoidea) in swine. Proc Helminthol Soc Wash. 1987;48:1–7.
86. Olsen LS, Kelley GW, Sen HG. Longevity and egg-production of *Ascaris suum*. Trans Am Microscop Soc. 1958;77:380–3.
87. Anderson R, May R. Infectious diseases of humans: dynamics and control. Oxford: Oxford University Press; 1992.
88. Sinniah B. Daily egg production of *Ascaris lumbricoides*: the distribution of eggs in the faeces and the variability of egg counts. Parasitology. 1982;84(1):167–75. PubMed PMID: 7063252
89. Gaasenbeek CP, Borgsteede FH. Studies on the survival of *Ascaris suum* eggs under laboratory and simulated field conditions. Vet Parasitol. 1998;75(2–3):227–34. PubMed PMID: 9637224
90. Kagei N. Techniques for the measurement of environmental pollution by infective stage of soil-transmitted helminths. In: Yokogawa M, Hayashi S, Kobayashi A, Kagei N, Suzuki N, Kunii C, editors. Collected papers on the control of soil-transmitted helminthiases, vol. 11. Tokyo: Asian Parasite Control Organization; 1983. p. 27–46.
91. Crompton D. Biology of *Ascaris lumbricoides*. In: Crompton D, Nesheim M, Pawlowski Z, editors. *Ascariasis* and its prevention and control. London: Taylor & Francis; 1989. p. 9–44.
92. Hall A, Hewitt G, Tuffrey V, de Silva N. A review and meta-analysis of the impact of intestinal worms on child growth and nutrition. Matern Child Nutr. 2008;4(Suppl 1):118–236. https://doi.org/10.1111/j.1740-8709.2007.00127.x. PubMed PMID: 18289159
93. Wong MS, Bundy DA, Golden MH. The rate of ingestion of *Ascaris lumbricoides* and *Trichuris trichiura* eggs in soil and its relationship to infection in two children's homes in Jamaica. Trans R Soc Trop Med Hyg. 1991;85(1):89–91. PubMed PMID: 2068773
94. WHO. Control of Ascariasis (report of WHO Committee). Technical Report Series 1967. p. 1–39.
95. Bidinger PD, Crompton DW, Arnold S. Aspects of intestinal parasitism in villagers from rural peninsular India. Parasitology. 1981;83(Pt 2):373–80. PubMed PMID: 7329725

96. Kroeger A, Schulz S, Witte B, Skewes-Ramm R, Etzler A. Helminthiasis and cultural change in the Peruvian rainforest. J Trop Med Hyg. 1992;95(2):104–13. PubMed PMID: 1560477
97. Smyth J. Introduction to animal parasitology. 3rd ed. New York: Cambridge University Press; 1994.
98. Read AF, Skorping A. The evolution of tissue migration by parasitic nematode larvae. Parasitology. 1995;111(Pt 3):359–71. PubMed PMID: 7567104
99. Mulcahy G, O'Neill S, Fanning J, McCarthy E, Sekiya M. Tissue migration by parasitic helminths—an immunoevasive strategy? Trends Parasitol. 2005;21(6):273–7. https://doi.org/10.1016/j.pt.2005.04.003. PubMed PMID: 15922248
100. Jungersen G, Fagerholm HP, Nansen P, Eriksen L. Development of patent *Ascaris suum* infections in pigs following intravenous administration of larvae hatched in vitro. Parasitology. 1999;119(Pt 5):503–8. PubMed PMID: 10599082
101. de Silva NR, Guyatt HL, Bundy DA. Worm burden in intestinal obstruction caused by *Ascaris lumbricoides*. Trop Med Int Health. 1997;2(2):189–90. PubMed PMID: 9472304
102. Thein-Hlaing, Thane-Toe, Than-Saw, Myat-Lay-Kyin, Myint-Lwin. A controlled chemotherapeutic intervention trial on the relationship between *Ascaris lumbricoides* infection and malnutrition in children. Trans R Soc Trop Med Hyg. 1991;85(4):523–8. PubMed PMID: 1755063
103. Brooker S. Estimating the global distribution and disease burden of intestinal nematode infections: adding up the numbers—a review. Int J Parasitol. 2010;40(10):1137–44. https://doi.org/10.1016/j.ijpara.2010.04.004. PubMed PMID: 20430032; PubMed Central PMCID: PMCPMC3034165
104. de Silva NR, Guyatt HL, Bundy DA. Morbidity and mortality due to *Ascaris*-induced intestinal obstruction. Trans R Soc Trop Med Hyg. 1997;91(1):31–6. PubMed PMID: 9093623
105. de Silva NR, Chan MS, Bundy DA. Morbidity and mortality due to ascariasis: re-estimation and sensitivity analysis of global numbers at risk. Trop Med Int Health. 1997;2(6):519–28. PubMed PMID: 9236818
106. Stephenson L. Pathophysiology of intestinal nematodes. In: Holland C, Kennede M, editors. The Geohelminths: *Ascaris*, *Trichuris* and hookworm. New York: Springer; 2002.
107. O'Lorcain P, Holland CV. The public health importance of *Ascaris lumbricoides*. Parasitology. 2000;121(Suppl):S51–71. PubMed PMID: 11386692
108. Awasthi S, Pande VK. Six-monthly de-worming in infants to study effects on growth. Indian J Pediatr. 2001;68(9):823–7. PubMed PMID: 11669028
109. Ezeamama AE, McGarvey ST, Hogan J, Lapane KL, Bellinger DC, Acosta LP, et al. Treatment for *Schistosoma japonicum*, reduction of intestinal parasite load, and cognitive test score improvements in school-aged children. PLoS Negl Trop Dis. 2012;6(5):e1634. https://doi.org/10.1371/journal.pntd.0001634. PubMed PMID: 22563514; PubMed Central PMCID: PMCPMC3341324
110. Bundy DA, Kremer M, Bleakley H, Jukes MC, Miguel E. Deworming and development: asking the right questions, asking the questions right. PLoS Negl Trop Dis. 2009;3(1):e362. https://doi.org/10.1371/journal.pntd.0000362. PubMed PMID: 19172186; PubMed Central PMCID: PMCPMC2627944
111. Taylor-Robinson DC, Maayan N, Soares-Weiser K, Donegan S, Garner P. Deworming drugs for soil-transmitted intestinal worms in children: effects on nutritional indicators, haemoglobin, and school performance. Cochrane Database Syst Rev. 2015;(7):CD000371. https://doi.org/10.1002/14651858.CD000371.pub6. PubMed PMID: 26202783; PubMed Central PMCID: PMCPMC4523932
112. Jardim-Botelho A, Raff S, Rodrigues R DÁ, Hoffman HJ, Diemert DJ, Corrêa-Oliveira R, et al. Hookworm, *Ascaris lumbricoides* infection and polyparasitism associated with poor cognitive performance in Brazilian schoolchildren. Trop Med Int Health. 2008;13(8):994–1004. https://doi.org/10.1111/j.1365-3156.2008.02103.x. PubMed PMID: 18627581

113. Holliday MA. Body composition and energy needs during growth. In: Falkner F, Tanner JM, editors. Human growth: a comprehensive treatise, vol. 2. New York, NY: Plenum; 1986. p. 101–17.
114. Eppig C, Fincher CL, Thornhill R. Parasite prevalence and the worldwide distribution of cognitive ability. Proc Biol Sci. 2010;277(1701):3801–8. https://doi.org/10.1098/rspb.2010.0973. PubMed PMID: 20591860; PubMed Central PMCID: PMCPMC2992705
115. Medley G, Turner H, Baggaley R, Holland C, Hollingsworth T. The role of more sensitive helminth diagnostics in mass drug administration campaigns: elimination and health impacts. In: Anderson R, Basanez M, editors. Mathematical models for neglected tropical diseases: essential tools for control and elimination, Part B. Advances in parasitology, vol. 94. New York: Academic; 2016.
116. Stephenson LS, Pond WG, Nesheim MC, Krook LP, Crompton DW. *Ascaris suum:* nutrient absorption, growth, and intestinal pathology in young pigs experimentally infected with 15-day-old larvae. Exp Parasitol. 1980;49(1):15–25. PubMed PMID: 7350001
117. Villamizar E, Méndez M, Bonilla E, Varon H, de Onatra S. *Ascaris lumbricoides* infestation as a cause of intestinal obstruction in children: experience with 87 cases. J Pediatr Surg. 1996;31(1):201–4. discussion 4-5. PubMed PMID: 8632280
118. Khuroo MS, Zargar SA, Mahajan R. Hepatobiliary and pancreatic ascariasis in India. Lancet. 1990;335(8704):1503–6. PubMed PMID: 1972440
119. Pawloski Z, Arfaa F. Ascariasis. In: Warren K, Mahmoud A, editors. Tropical and geographical medicine. New York: McGraw Hill; 1984. p. 347–58.
120. Coles G. Allergy and immunopathology of ascariasis. In: Crompton D, Nesheim M, Pawloski Z, editors. Ascariasis and its public health significance. London and Philadelphia: Taylor and Francis; 1985. p. 167–84.
121. Pawłowski ZS. Ascariasis. Clin Gastroenterol. 1978;7(1):157–78. PubMed PMID: 627097
122. Loeffler W. Zur Differentialdiagnose der Lungen-infiltrierungen. II Ueber fluchtige Succedanininfiltrate (mit Eosinophilie). Beiträge zur Klinik der Tuberkulose. 1932;79:368–82.
123. Loeffler W. Transient lung infiltrations with blood eosinophilia. Int Arch Allergy Appl Immunol. 1956;8(1–2):54–9. PubMed PMID: 13331628
124. McSharry C, Xia Y, Holland CV, Kennedy MW. Natural immunity to *Ascaris lumbricoides* associated with immunoglobulin E antibody to ABA-1 allergen and inflammation indicators in children. Infect Immun. 1999;67(2):484–9. PubMed PMID: 9916049; PubMed Central PMCID: PMCPMC96345
125. Donnelly S, O'Neill SM, Sekiya M, Mulcahy G, Dalton JP. Thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. Infect Immun. 2005;73(1):166–73. https://doi.org/10.1128/IAI.73.1.166-173.2005. PubMed PMID: 15618151; PubMed Central PMCID: PMCPMC538930
126. Gourbal BE, Guillou F, Mitta G, Sibille P, Thèron A, Pointier JP, et al. Excretory-secretory products of larval *Fasciola hepatica* investigated using a two-dimensional proteomic approach. Mol Biochem Parasitol. 2008;161(1):63–6. https://doi.org/10.1016/j.molbiopara.2008.05.002. PubMed PMID: 18556074
127. Spolski RJ, Corson J, Thomas PG, Kuhn RE. Parasite-secreted products regulate the host response to larval *Taenia crassiceps*. Parasite Immunol. 2000;22(6):297–305. PubMed PMID: 10849309
128. Chehayeb JF, Robertson AP, Martin RJ, Geary TG. Proteomic analysis of adult *Ascaris suum* fluid compartments and secretory products. PLoS Negl Trop Dis. 2014;8(6):e2939. https://doi.org/10.1371/journal.pntd.0002939. PubMed PMID: 24901219; PubMed Central PMCID: PMCPMC4046973
129. Kennedy MW. *Ascaris*—antigens, allergens, immunogenetics, protein structures. In: Holland C, editor. *Ascaris*: the neglected parasite. Amsterdam: Elsevier; 2013. p. 51–79.
130. Antunes MF, Titz TO, Batista IF, Marques-Porto R, Oliveira CF, Alves de Araujo CA, et al. Immunosuppressive PAS-1 is an excretory/secretory protein released by larval and adult

worms of the ascarid nematode *Ascaris suum*. J Helminthol. 2015;89(3):367–74. https://doi.org/10.1017/S0022149X14000200. PubMed PMID: 24703095
131. Wang T, Van Steendam K, Dhaenens M, Vlaminck J, Deforce D, Jex AR, et al. Proteomic analysis of the excretory-secretory products from larval stages of *Ascaris suum* reveals high abundance of glycosyl hydrolases. PLoS Negl Trop Dis. 2013;7(10):e2467. https://doi.org/10.1371/journal.pntd.0002467. PubMed PMID: 24098821; PubMed Central PMCID: PMCPMC3789772
132. McGonigle S, Loschiavo M, Pearce EJ. 14-3-3 proteins in *Schistosoma mansoni*; identification of a second epsilon isoform. Int J Parasitol. 2002;32(6):685–93. PubMed PMID: 12062487
133. Jex AR, Liu S, Li B, Young ND, Hall RS, Li Y, et al. *Ascaris suum* draft genome. Nature. 2011;479(7374):529–33. https://doi.org/10.1038/nature10553. PubMed PMID: 22031327
134. Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol. 2003;3(9):733–44. https://doi.org/10.1038/nri1183. PubMed PMID: 12949497
135. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature. 2010;464(7293):1367–70. https://doi.org/10.1038/nature08900. PubMed PMID: 20200518; PubMed Central PMCID: PMCPMC2862165
136. Maizels RM, Hewitson JP, Smith KA. Susceptibility and immunity to helminth parasites. Curr Opin Immunol. 2012;24(4):459–66. https://doi.org/10.1016/j.coi.2012.06.003. PubMed PMID: 22795966; PubMed Central PMCID: PMCPMC3437973
137. Allen JE, Maizels RM. Diversity and dialogue in immunity to helminths. Nat Rev Immunol. 2011;11(6):375–88. https://doi.org/10.1038/nri2992. PubMed PMID: 21610741
138. van Liempt E, van Vliet SJ, Engering A, García Vallejo JJ, Bank CM, Sanchez-Hernandez M, et al. *Schistosoma mansoni* soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. Mol Immunol. 2007;44(10):2605–15. https://doi.org/10.1016/j.molimm.2006.12.012. PubMed PMID: 17241663
139. Yoshida A, Nagayasu E, Horii Y, Maruyama H. A novel C-type lectin identified by EST analysis in tissue migratory larvae of *Ascaris suum*. Parasitol Res. 2012;110(4):1583–6. https://doi.org/10.1007/s00436-011-2677-9. PubMed PMID: 22006188
140. Kondo M, Tamaoki J, Takeyama K, Nakata J, Nagai A. Interleukin-13 induces goblet cell differentiation in primary cell culture from Guinea pig tracheal epithelium. Am J Respir Cell Mol Biol. 2002;27(5):536–41. https://doi.org/10.1165/rcmb.4682. PubMed PMID: 12397012
141. Hasnain SZ, Evans CM, Roy M, Gallagher AL, Kindrachuk KN, Barron L, et al. Muc5ac: a critical component mediating the rejection of enteric nematodes. J Exp Med. 2011;208(5):893–900. https://doi.org/10.1084/jem.20102057. PubMed PMID: 21502330; PubMed Central PMCID: PMCPMC3092342
142. Herbert DR, Yang JQ, Hogan SP, Groschwitz K, Khodoun M, Munitz A, et al. Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. J Exp Med. 2009;206(13):2947–57. https://doi.org/10.1084/jem.20091268. PubMed PMID: 19995957; PubMed Central PMCID: PMCPMC2806463
143. Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, et al. RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. Proc Natl Acad Sci U S A. 2004;101(37):13596–600. https://doi.org/10.1073/pnas.0404034101. PubMed PMID: 15340149; PubMed Central PMCID: PMCPMC518800
144. Balic A, Harcus Y, Holland MJ, Maizels RM. Selective maturation of dendritic cells by *Nippostrongylus brasiliensis*-secreted proteins drives Th2 immune responses. Eur J Immunol. 2004;34(11):3047–59. https://doi.org/10.1002/eji.200425167. PubMed PMID: 15468056

145. MacDonald AS, Pearce EJ. Cutting edge: polarized Th cell response induction by transferred antigen-pulsed dendritic cells is dependent on IL-4 or IL-12 production by recipient cells. J Immunol. 2002;168(7):3127–30. PubMed PMID: 11907061
146. Phythian-Adams AT, Cook PC, Lundie RJ, Jones LH, Smith KA, Barr TA, et al. CD11c depletion severely disrupts Th2 induction and development in vivo. J Exp Med. 2010;207 (10):2089–96. https://doi.org/10.1084/jem.20100734. PubMed PMID: 20819926; PubMed Central PMCID: PMCPMC2947067
147. Masure D, Vlaminck J, Wang T, Chiers K, Van den Broeck W, Vercruysse J, et al. A role for eosinophils in the intestinal immunity against infective Ascaris suum larvae. PLoS Negl Trop Dis. 2013;7(3):e2138. https://doi.org/10.1371/journal.pntd.0002138. PubMed PMID: 23556022; PubMed Central PMCID: PMCPMC3605247
148. Rosenberg HF, Phipps S, Foster PS. Eosinophil trafficking in allergy and asthma. J Allergy Clin Immunol. 2007;119(6):1303–10. https://doi.org/10.1016/j.jaci.2007.03.048. quiz 11-2; PubMed PMID: 17481712
149. Malik A, Batra JK. Antimicrobial activity of human eosinophil granule proteins: involvement in host defence against pathogens. Crit Rev Microbiol. 2012;38(2):168–81. https://doi.org/10.3109/1040841X.2011.645519. PubMed PMID: 22239733
150. Ronéus O. Studies on the aetiology and pathogenesis of white spots in the liver of pigs. Acta Vet Scand. 1966;7(Suppl 16):1–112. PubMed PMID: 5949007
151. Javid G, Wani NA, Gulzar GM, Khan BA, Shah AH, Shah OJ, et al. *Ascaris*-induced liver abscess. World J Surg. 1999;23(11):1191–4. PubMed PMID: 10501884
152. Pérez J, García PM, Mozos E, Bautista MJ, Carrasco L. Immunohistochemical characterization of hepatic lesions associated with migrating larvae of *Ascaris suum* in pigs. J Comp Pathol. 2001;124(2–3):200–6. https://doi.org/10.1053/jcpa.2000.0455. PubMed PMID: 11222018
153. Jenne CN, Kubes P. Immune surveillance by the liver. Nat Immunol. 2013;14(10):996–1006. https://doi.org/10.1038/ni.2691. PubMed PMID: 24048121
154. Bertolino P, Bowen DG. Malaria and the liver: immunological hide-and-seek or subversion of immunity from within? Front Microbiol. 2015;6:41. https://doi.org/10.3389/fmicb.2015.00041. PubMed PMID: 25741320; PubMed Central PMCID: PMCPMC4332352
155. Nogueira DS, Gazzinelli-Guimarães PH, Barbosa FS, Resende NM, Silva CC, de Oliveira LM, et al. Multiple exposures to *Ascaris suum* induce tissue injury and mixed Th2/Th17 immune response in mice. PLoS Negl Trop Dis. 2016;10(1):e0004382. https://doi.org/10.1371/journal.pntd.0004382. PubMed PMID: 26814713; PubMed Central PMCID: PMCPMC4729520
156. Lewis R, Behnke JM, Cassidy JP, Stafford P, Murray N, Holland CV. The migration of *Ascaris suum* larvae, and the associated pulmonary inflammatory response in susceptible C57BL/6j and resistant CBA/Ca mice. Parasitology. 2007;134(Pt 9):1301–14. https://doi.org/10.1017/S0031182007002582. PubMed PMID: 17381887
157. Gazzinelli-Guimarães PH, Gazzinelli-Guimarães AC, Silva FN, Mati VL, Dhom-Lemos LC, Barbosa FS, et al. Parasitological and immunological aspects of early *Ascaris* spp. infection in mice. Int J Parasitol. 2013;43(9):697–706. https://doi.org/10.1016/j.ijpara.2013.02.009. PubMed PMID: 23665127
158. Enobe CS, Araújo CA, Perini A, Martins MA, Macedo MS, Macedo-Soares MF. Early stages of *Ascaris suum* induce airway inflammation and hyperreactivity in a mouse model. Parasite Immunol. 2006;28(9):453–61. https://doi.org/10.1111/j.1365-3024.2006.00892.x. PubMed PMID: 16916369
159. Hagel I, Lynch NR, Di Prisco MC, Rojas E, Pérez M, Alvarez N. *Ascaris* reinfection of slum children: relation with the IgE response. Clin Exp Immunol. 1993;94(1):80–3. PubMed PMID: 8403522; PubMed Central PMCID: PMCPMC1534372
160. Paterson JC, Garside P, Kennedy MW, Lawrence CE. Modulation of a heterologous immune response by the products of *Ascaris suum*. Infect Immun. 2002;70(11):6058–67. PubMed PMID: 12379682; PubMed Central PMCID: PMCPMC130290

161. Gounni AS, Lamkhioued B, Ochiai K, Tanaka Y, Delaporte E, Capron A, et al. High-affinity IgE receptor on eosinophils is involved in defence against parasites. Nature. 1994;367 (6459):183–6. https://doi.org/10.1038/367183a0. PubMed PMID: 8114916
162. Palmer DR, Hall A, Haque R, Anwar KS. Antibody isotype responses to antigens of Ascaris lumbricoides in a case-control study of persistently heavily infected Bangladeshi children. Parasitology. 1995;111(Pt 3):385–93. PubMed PMID: 7567106
163. Miquel N, Roepstorff A, Bailey M, Eriksen L. Host immune reactions and worm kinetics during the expulsion of *Ascaris suum* in pigs. Parasite Immunol. 2005;27(3):79–88. https://doi.org/10.1111/j.1365-3024.2005.00752.x. PubMed PMID: 15882234
164. Mantis NJ, Rol N, Corthésy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol. 2011;4(6):603–11. https://doi.org/10.1038/mi.2011.41. PubMed PMID: 21975936; PubMed Central PMCID: PMCPMC3774538
165. Turner JD, Faulkner H, Kamgno J, Cormont F, Van Snick J, Else KJ, et al. Th2 cytokines are associated with reduced worm burdens in a human intestinal helminth infection. J Infect Dis. 2003;188(11):1768–75. https://doi.org/10.1086/379370. PubMed PMID: 14639550
166. Cooper PJ, Chico ME, Sandoval C, Espinel I, Guevara A, Kennedy MW, et al. Human infection with *Ascaris lumbricoides* is associated with a polarized cytokine response. J Infect Dis. 2000;182(4):1207–13. https://doi.org/10.1086/315830. PubMed PMID: 10979919
167. Anthony RM, Rutitzky LI, Urban JF, Stadecker MJ, Gause WC. Protective immune mechanisms in helminth infection. Nat Rev Immunol. 2007;7(12):975–87. https://doi.org/10.1038/nri2199. PubMed PMID: 18007680; PubMed Central PMCID: PMCPMC2258092
168. Chen D, Luo X, Xie H, Gao Z, Fang H, Huang J. Characteristics of IL-17 induction by *Schistosoma japonicum* infection in C57BL/6 mouse liver. Immunology. 2013;139 (4):523–32. https://doi.org/10.1111/imm.12105. PubMed PMID: 23551262; PubMed Central PMCID: PMCPMC3719069
169. Matera G, Giancotti A, Scalise S, Pulicari MC, Maselli R, Piizzi C, et al. *Ascaris lumbricoides*-induced suppression of total and specific IgE responses in atopic subjects is interleukin 10-independent and associated with an increase of CD25(+) cells. Diagn Microbiol Infect Dis. 2008;62(3):280–6. https://doi.org/10.1016/j.diagmicrobio.2008.06.015. PubMed PMID: 18801637
170. Nascimento WC, Silva RP, Fernandes ES, Silva MC, Holanda GC, Santos PA, et al. Immunomodulation of liver injury by *Ascaris suum* extract in an experimental model of autoimmune hepatitis. Parasitol Res. 2014;113(9):3309–17. https://doi.org/10.1007/s00436-014-3994-6. PubMed PMID: 24951170
171. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol. 2008;180(9):5771–7. PubMed PMID: 18424693
172. Reina Ortiz M, Schreiber F, Benitez S, Broncano N, Chico ME, Vaca M, et al. Effects of chronic ascariasis and trichuriasis on cytokine production and gene expression in human blood: a cross-sectional study. PLoS Negl Trop Dis. 2011;5(6):e1157. https://doi.org/10.1371/journal.pntd.0001157. PubMed PMID: 21666788; PubMed Central PMCID: PMCPMC3110165
173. Hewitson JP, Maizels RM. Vaccination against helminth parasite infections. Expert Rev Vaccines. 2014;13(4):473–87. https://doi.org/10.1586/14760584.2014.893195. PubMed PMID: 24606541
174. Oshiro TM, Rafael A, Enobe CS, Fernandes I, Macedo-Soares MF. Comparison of different monoclonal antibodies against immunosuppressive proteins of *Ascaris suum*. Braz J Med Biol Res. 2004;37(2):223–6. PubMed PMID: 14762577
175. Nikolay B, Brooker SJ, Pullan RL. Sensitivity of diagnostic tests for human soil-transmitted helminth infections: a meta-analysis in the absence of a true gold standard. Int J Parasitol. 2014;44(11):765–74. https://doi.org/10.1016/j.ijpara.2014.05.009. PubMed PMID: 24992655; PubMed Central PMCID: PMCPMC4186778
176. WHO. Bench aids for the diagnosis of intestinal parasites. Geneva: World Health Organization; 1994.

177. Katz N, Chaves A, Pellegrino J. A simple device for quantitative stool thick-smear technique in Schistosomiasis mansoni. Rev Inst Med Trop Sao Paulo. 1972;14(6):397–400. PubMed PMID: 4675644
178. Levecke B, Behnke JM, Ajjampur SS, Albonico M, Ame SM, Charlier J, et al. A comparison of the sensitivity and fecal egg counts of the McMaster egg counting and Kato-Katz thick smear methods for soil-transmitted helminths. PLoS Negl Trop Dis. 2011;5(6):e1201. https://doi.org/10.1371/journal.pntd.0001201. PubMed PMID: 21695104; PubMed Central PMCID: PMCPMC3114752
179. WHO. In: WHO/CDS/IPI, editor. Bench Aids for the diagnosis of intestinal helminths. Geneva: World Health Organization; 1992.
180. WHO. Basic laboratory methods in medical parasitology. Geneva: World Health Organization; 1991.
181. Engels D, Nahimana S, De Vlas SJ, Gryseels B. Variation in weight of stool samples prepared by the Kato-Katz method and its implications. Trop Med Int Health. 1997;2(3):265–71. PubMed PMID: 9491106
182. Crompton D. Ascaris lumbricoides. In: Scott M, Smith G, editors. Parasitic and infectious diseases. London and New York: Academic; 1994. p. 175–96.
183. Hall A. Quantitative variability of nematode egg counts in faeces: a study among rural Kenyans. Trans R Soc Trop Med Hyg. 1981;75(5):682–7. PubMed PMID: 7330922
184. Walker M, Hall A, Anderson RM, Basáñez MG. Density-dependent effects on the weight of female *Ascaris lumbricoides* infections of humans and its impact on patterns of egg production. Parasit Vectors. 2009;2(1):11. https://doi.org/10.1186/1756-3305-2-11. PubMed PMID: 19208229; PubMed Central PMCID: PMCPMC2672930
185. Kotze AC, Kopp SR. The potential impact of density dependent fecundity on the use of the faecal egg count reduction test for detecting drug resistance in human hookworms. PLoS Negl Trop Dis. 2008;2(10):e297. https://doi.org/10.1371/journal.pntd.0000297. PubMed PMID: 18827883; PubMed Central PMCID: PMCPMC2553282
186. Guyatt HL, Bundy DA, Medley GF, Grenfell BT. The relationship between the frequency distribution of *Ascaris lumbricoides* and the prevalence and intensity of infection in human communities. Parasitology. 1990;101(Pt 1):139–43. PubMed PMID: 2235069
187. Goodman D, Haji HJ, Bickle QD, Stoltzfus RJ, Tielsch JM, Ramsan M, et al. A comparison of methods for detecting the eggs of *Ascaris*, *Trichuris*, and hookworm in infant stool, and the epidemiology of infection in Zanzibari infants. Am J Trop Med Hyg. 2007;76(4):725–31. PubMed PMID: 17426179
188. Ritchie LS. An ether sedimentation technique for routine stool examinations. Bull US Army Med Dep. 1948;8(4):326. PubMed PMID: 18911509
189. Garcia L. Diagnostic medical parasitology. Herndon, VA: American Society for Microbiology; 2007.
190. Cringoli G, Rinaldi L, Maurelli MP, Utzinger J. FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nat Protoc. 2010;5(3):503–15. https://doi.org/10.1038/nprot.2009.235. PubMed PMID: 20203667
191. Barda B, Cajal P, Villagran E, Cimino R, Juarez M, Krolewiecki A, et al. Mini-FLOTAC, Kato-Katz and McMaster: three methods, one goal; highlights from north Argentina. Parasit Vectors. 2014;7:271. https://doi.org/10.1186/1756-3305-7-271. PubMed PMID: 24929554; PubMed Central PMCID: PMCPMC4074144
192. Levecke B, De Wilde N, Vandenhoute E, Vercruysse J. Field validity and feasibility of four techniques for the detection of *Trichuris* in simians: a model for monitoring drug efficacy in public health? PLoS Negl Trop Dis. 2009;3(1):e366. https://doi.org/10.1371/journal.pntd.0000366. PubMed PMID: 19172171; PubMed Central PMCID: PMCPMC2621347
193. Maurelli MP, Rinaldi L, Alfano S, Pepe P, Coles GC, Cringoli G. Mini-FLOTAC, a new tool for copromicroscopic diagnosis of common intestinal nematodes in dogs. Parasit Vectors.

2014;7:356. https://doi.org/10.1186/1756-3305-7-356. PubMed PMID: 25095701; PubMed Central PMCID: PMCPMC4262189
194. Das CJ, Kumar J, Debnath J, Chaudhry A. Imaging of ascariasis. Australas Radiol. 2007;51 (6):500–6. https://doi.org/10.1111/j.1440-1673.2007.01887.x. PubMed PMID: 17958683
195. Wu S. Sonographic findings of *Ascaris lumbricoides* in the gastrointestinal and biliary tracts. Ultrasound Q. 2009;25(4):207–9. https://doi.org/10.1097/RUQ.0b013e3181c47a2d. PubMed PMID: 19956053
196. Hall A, Romanova T. *Ascaris lumbricoides*: detecting its metabolites in the urine of infected people using gas-liquid chromatography. Exp Parasitol. 1990;70(1):35–42. PubMed PMID: 2295325
197. Basuni M, Muhi J, Othman N, Verweij JJ, Ahmad M, Miswan N, et al. A pentaplex real-time polymerase chain reaction assay for detection of four species of soil-transmitted helminths. Am J Trop Med Hyg. 2011;84(2):338–43. https://doi.org/10.4269/ajtmh.2011.10-0499. PubMed PMID: 21292911; PubMed Central PMCID: PMCPMC3029194
198. Taniuchi M, Verweij JJ, Noor Z, Sobuz SU, Lieshout L, Petri WA, et al. High throughput multiplex PCR and probe-based detection with Luminex beads for seven intestinal parasites. Am J Trop Med Hyg. 2011;84(2):332–7. https://doi.org/10.4269/ajtmh.2011.10-0461. PubMed PMID: 21292910; PubMed Central PMCID: PMCPMC3029193
199. Chatterjee BP, Santra A, Karmakar PR, Mazumder DN. Evaluation of IgG4 response in ascariasis by ELISA for serodiagnosis. Trop Med Int Health. 1996;1(5):633–9. PubMed PMID: 8911447
200. Needham CS, Lillywhite JE, Beasley NM, Didier JM, Kihamia CM, Bundy DA. Potential for diagnosis of intestinal nematode infections through antibody detection in saliva. Trans R Soc Trop Med Hyg. 1996;90(5):526–30. PubMed PMID: 8944263
201. WHO. WHO model lists of essential medicines. Geneva: WHO; 2015.
202. WHO. WHO model lists of essential medicines for children. Geneva: WHO; 2015.
203. Lustigman S, Prichard RK, Gazzinelli A, Grant WN, Boatin BA, McCarthy JS, et al. A research agenda for helminth diseases of humans: the problem of helminthiases. PLoS Negl Trop Dis. 2012;6(4):e1582. https://doi.org/10.1371/journal.pntd.0001582. PubMed PMID: 22545164; PubMed Central PMCID: PMCPMC3335854
204. Basáñez MG, Pion SD, Churcher TS, Breitling LP, Little MP, Boussinesq M. River blindness: a success story under threat? PLoS Med. 2006;3(9):e371. https://doi.org/10.1371/journal.pmed.0030371. PubMed PMID: 17002504; PubMed Central PMCID: PMCPMC1576321
205. Lacey E. Mode of action of benzimidazoles. Parasitol Today. 1990;6(4):112–5. PubMed PMID: 15463312
206. Robertson AP, Bjørn HE, Martin RJ. Pyrantel resistance alters nematode nicotinic acetylcholine receptor single-channel properties. Eur J Pharmacol. 2000;394(1):1–8. PubMed PMID: 10771027
207. Bennett A, Guyatt H. Reducing intestinal nematode infection: efficacy of albendazole and mebendazole. Parasitol Today. 2000;16(2):71–4. PubMed PMID: 10652492
208. Vercruysse J, Albonico M, Behnke JM, Kotze AC, Prichard RK, McCarthy JS, et al. Is anthelmintic resistance a concern for the control of human soil-transmitted helminths? Int J Parasitol Drugs Drug Resist. 2011;1(1):14–27. https://doi.org/10.1016/j.ijpddr.2011.09.002. PubMed PMID: 24533260; PubMed Central PMCID: PMCPMC3913213
209. Kwa MS, Veenstra JG, Roos MH. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol Biochem Parasitol. 1994;63(2):299–303. PubMed PMID: 7911975
210. Silvestre A, Cabaret J. Mutation in position 167 of isotype 1 beta-tubulin gene of *Trichostrongylid* nematodes: role in benzimidazole resistance? Mol Biochem Parasitol. 2002;120(2):297–300. PubMed PMID: 11897135
211. Ghisi M, Kaminsky R, Mäser P. Phenotyping and genotyping of *Haemonchus contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in

nematodes. Vet Parasitol. 2007;144(3–4):313–20. https://doi.org/10.1016/j.vetpar.2006.10.003. PubMed PMID: 17101226
212. Barrère V, Alvarez L, Suarez G, Ceballos L, Moreno L, Lanusse C, et al. Relationship between increased albendazole systemic exposure and changes in single nucleotide polymorphisms on the β-tubulin isotype 1 encoding gene in Haemonchus contortus. Vet Parasitol. 2012;186(3–4):344–9. https://doi.org/10.1016/j.vetpar.2011.11.068. PubMed PMID: 22192770
213. Prichard R. Genetic variability following selection of *Haemonchus contortus* with anthelmintics. Trends Parasitol. 2001;17(9):445–53. PubMed PMID: 11530357
214. Diawara A, Halpenny CM, Churcher TS, Mwandawiro C, Kihara J, Kaplan RM, et al. Association between response to albendazole treatment and β-tubulin genotype frequencies in soil-transmitted helminths. PLoS Negl Trop Dis. 2013;7(5):e2247. https://doi.org/10.1371/journal.pntd.0002247. PubMed PMID: 23738029; PubMed Central PMCID: PMCPMC3667785
215. Diawara A, Drake LJ, Suswillo RR, Kihara J, Bundy DA, Scott ME, et al. Assays to detect beta-tubulin codon 200 polymorphism in *Trichuris trichiura* and *Ascaris lumbricoides*. PLoS Negl Trop Dis. 2009;3(3):e397. https://doi.org/10.1371/journal.pntd.0000397. PubMed PMID: 19308251; PubMed Central PMCID: PMCPMC2654341
216. Elias D, Wolday D, Akuffo H, Petros B, Bronner U, Britton S. Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guérin (BCG) vaccination. Clin Exp Immunol. 2001;123(2):219–25. PubMed PMID: 11207651; PubMed Central PMCID: PMCPMC1905995
217. Cooper PJ, Espinel I, Paredes W, Guderian RH, Nutman TB. Impaired tetanus-specific cellular and humoral responses following tetanus vaccination in human onchocerciasis: a possible role for interleukin-10. J Infect Dis. 1998;178(4):1133–8. PubMed PMID: 9806045
218. Sabin EA, Araujo MI, Carvalho EM, Pearce EJ. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with *Schistosoma mansoni*. J Infect Dis. 1996;173(1):269–72. PubMed PMID: 8537675
219. Cooper PJ, Chico M, Sandoval C, Espinel I, Guevara A, Levine MM, et al. Human infection with *Ascaris lumbricoides* is associated with suppression of the interleukin-2 response to recombinant cholera toxin B subunit following vaccination with the live oral cholera vaccine CVD 103-HgR. Infect Immun. 2001;69(3):1574–80. https://doi.org/10.1128/IAI.69.3.1574-1580.2001. PubMed PMID: 11179329; PubMed Central PMCID: PMCPMC98058
220. Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN, et al. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. Nat Med. 2000;6(5):536–42. https://doi.org/10.1038/75015. PubMed PMID: 10802709
221. Kullberg MC, Pearce EJ, Hieny SE, Sher A, Berzofsky JA. Infection with *Schistosoma mansoni* alters Th1/Th2 cytokine responses to a non-parasite antigen. J Immunol. 1992;148(10):3264–70. PubMed PMID: 1533656
222. Actor JK, Shirai M, Kullberg MC, Buller RM, Sher A, Berzofsky JA. Helminth infection results in decreased virus-specific CD8+ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. Proc Natl Acad Sci USA. 1993;90(3):948–52. PubMed PMID: 8094248; PubMed Central PMCID: PMCPMC45787
223. Abanyie F, Lamb TJ. Implications of *Ascaris* co-infection. In: Holland C, editor. *Ascaris*: the neglected parasite; 2013.
224. Modjarrad K, Zulu I, Redden DT, Njobvu L, Lane HC, Bentwich Z, et al. Treatment of intestinal helminths does not reduce plasma concentrations of HIV-1 RNA in coinfected Zambian adults. J Infect Dis. 2005;192(7):1277–83. https://doi.org/10.1086/444543. PubMed PMID: 16136473; PubMed Central PMCID: PMCPMC2730764
225. Elliott AM, Mawa PA, Joseph S, Namujju PB, Kizza M, Nakiyingi JS, et al. Associations between helminth infection and CD4+ T cell count, viral load and cytokine responses in HIV-1-infected Ugandan adults. Trans R Soc Trop Med Hyg. 2003;97(1):103–8. PubMed PMID: 12886815

226. Abossie A, Petros B. Deworming and the immune status of HIV positive pre-antiretroviral therapy individuals in Arba Minch, Chencha and Gidole hospitals, Southern Ethiopia. BMC Res Notes. 2015;8:483. https://doi.org/10.1186/s13104-015-1461-9. PubMed PMID: 26415705; PubMed Central PMCID: PMCPMC4585997
227. Walson JL, Otieno PA, Mbuchi M, Richardson BA, Lohman-Payne B, Macharia SW, et al. Albendazole treatment of HIV-1 and helminth co-infection: a randomized, double-blind, placebo-controlled trial. AIDS. 2008;22(13):1601–9. https://doi.org/10.1097/QAD.0b013e32830a502e. PubMed PMID: 18670219; PubMed Central PMCID: PMCPMC2637615
228. Wolday D, Mayaan S, Mariam ZG, Berhe N, Seboxa T, Britton S, et al. Treatment of intestinal worms is associated with decreased HIV plasma viral load. J Acquir Immune Defic Syndr. 2002;31(1):56–62. PubMed PMID: 12352151
229. Chachage M, Podola L, Clowes P, Nsojo A, Bauer A, Mgaya O, et al. Helminth-associated systemic immune activation and HIV co-receptor expression: response to albendazole/praziquantel treatment. PLoS Negl Trop Dis. 2014;8(3):e2755. https://doi.org/10.1371/journal.pntd.0002755. PubMed PMID: 24675895; PubMed Central PMCID: PMCPMC3967945
230. Bentwich Z, Kalinkovich A, Weisman Z. Immune activation is a dominant factor in the pathogenesis of African AIDS. Immunol Today. 1995;16(4):187–91. PubMed PMID: 7734046
231. Nacher M. Interactions between worms and malaria: good worms or bad worms? Malar J. 2011;10:259. https://doi.org/10.1186/1475-2875-10-259. PubMed PMID: 21910854; PubMed Central PMCID: PMCPMC3192711
232. Kirwan P, Jackson AL, Asaolu SO, Molloy SF, Abiona TC, Bruce MC, et al. Impact of repeated four-monthly anthelmintic treatment on *Plasmodium* infection in preschool children: a double-blind placebo-controlled randomized trial. BMC Infect Dis. 2010;10:277. https://doi.org/10.1186/1471-2334-10-277. PubMed PMID: 20858280; PubMed Central PMCID: PMCPMC3161357
233. Brutus L, Watier L, Briand V, Hanitrasoamampionona V, Razanatsoarilala H, Cot M. Parasitic co-infections: does *Ascaris lumbricoides* protect against *Plasmodium falciparum* infection? Am J Trop Med Hyg. 2006;75(2):194–8. PubMed PMID: 16896118
234. Wammes LJ, Hamid F, Wiria AE, de Gier B, Sartono E, Maizels RM, et al. Regulatory T cells in human geohelminth infection suppress immune responses to BCG and *Plasmodium falciparum*. Eur J Immunol. 2010;40(2):437–42. https://doi.org/10.1002/eji.200939699. PubMed PMID: 20063313
235. Hartgers FC, Obeng BB, Kruize YC, Dijkhuis A, McCall M, Sauerwein RW, et al. Responses to malarial antigens are altered in helminth-infected children. J Infect Dis. 2009;199(10):1528–35. https://doi.org/10.1086/598687. PubMed PMID: 19392626
236. Nacher M. Worms and malaria: noisy nuisances and silent benefits. Parasite Immunol. 2002;24(7):391–3. PubMed PMID: 12164826
237. Nacher M, Singhasivanon P, Silachamroon U, Treeprasertsu S, Krudsood S, Gay F, et al. Association of helminth infections with increased gametocyte carriage during mild *falciparum* malaria in Thailand. Am J Trop Med Hyg. 2001;65(5):644–7. PubMed PMID: 11716129
238. Nacher M, Singhasivanon P, Gay F, Silachomroon U, Phumratanaprapin W, Looareesuwan S. Contemporaneous and successive mixed *Plasmodium falciparum* and *Plasmodium vivax* infections are associated with *Ascaris lumbricoides*: an immunomodulating effect? J Parasitol. 2001;87(4):912–5. https://doi.org/10.1645/0022-3395(2001)087[0912:CASMPF]2.0.CO;2. PubMed PMID: 11534659
239. Cooper PJ, Chico ME, Rodrigues LC, Ordonez M, Strachan D, Griffin GE, et al. Reduced risk of atopy among school-age children infected with geohelminth parasites in a rural area of the tropics. J Allergy Clin Immunol. 2003;111(5):995–1000. PubMed PMID: 12743563
240. Scrivener S, Yemaneberhan H, Zebenigus M, Tilahun D, Girma S, Ali S, et al. Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study. Lancet. 2001;358(9292):1493–9. https://doi.org/10.1016/S0140-6736(01)06579-5. PubMed PMID: 11705561

241. Palmer LJ, Celedón JC, Weiss ST, Wang B, Fang Z, Xu X. *Ascaris lumbricoides* infection is associated with increased risk of childhood asthma and atopy in rural China. Am J Respir Crit Care Med. 2002;165(11):1489–93. https://doi.org/10.1164/rccm.2107020. PubMed PMID: 12045121
242. Buendía E, Zakzuk J, Mercado D, Alvarez A, Caraballo L. The IgE response to *Ascaris* molecular components is associated with clinical indicators of asthma severity. World Allergy Organ J. 2015;8(1):8. https://doi.org/10.1186/s40413-015-0058-z. PubMed PMID: 25780492; PubMed Central PMCID: PMCPMC4347909
243. Cooper PJ. Interactions between helminth parasites and allergy. Curr Opin Allergy Clin Immunol. 2009;9(1):29–37. https://doi.org/10.1097/ACI.0b013e32831f44a6. PubMed PMID: 19106698; PubMed Central PMCID: PMCPMC2680069
244. Hunninghake GM, Soto-Quiros ME, Avila L, Ly NP, Liang C, Sylvia JS, et al. Sensitization to *Ascaris lumbricoides* and severity of childhood asthma in Costa Rica. J Allergy Clin Immunol. 2007;119(3):654–61. https://doi.org/10.1016/j.jaci.2006.12.609. PubMed PMID: 17336615
245. Takeuchi H, Zaman K, Takahashi J, Yunus M, Chowdhury HR, Arifeen SE, et al. High titre of anti-*Ascaris* immunoglobulin E associated with bronchial asthma symptoms in 5-year-old rural Bangladeshi children. Clin Exp Allergy. 2008;38(2):276–82. https://doi.org/10.1111/j.1365-2222.2007.02890.x. PubMed PMID: 18070165
246. Hawlader MD, Ma E, Noguchi E, Itoh M, Arifeen SE, Persson L, et al. *Ascaris lumbricoides* infection as a risk factor for asthma and atopy in rural Bangladeshi children. Trop Med Health. 2014;42(2):77–85. https://doi.org/10.2149/tmh.2013-19. PubMed PMID: 25237284; PubMed Central PMCID: PMCPMC4139537
247. Takeuchi H, Khan AF, Yunus M, Hasan MI, Hawlader MD, Takanashi S, et al. Anti-*Ascaris* immunoglobulin E associated with bronchial hyper-reactivity in 9-year-old rural Bangladeshi children. Allergol Int. 2016;65(2):141–6. https://doi.org/10.1016/j.alit.2015.07.002. PubMed PMID: 26666493
248. WHO. Prevention and control of schistosomiasis and soil-transmitted helminthiasis. Geneva: World Health Organization; 2002.
249. Kirwan P, Asaolu SO, Abiona TC, Jackson AL, Smith HV, Holland CV. Soil-transmitted helminth infections in Nigerian children aged 0-25 months. J Helminthol. 2009;83(3):261–6. https://doi.org/10.1017/S0022149X08201252. PubMed PMID: 19356265
250. Urban JF, Tromba FG. An ultraviolet-attenuated egg vaccine for swine ascariasis: parameters affecting the development of protective immunity. Am J Vet Res. 1984;45(10):2104–8. PubMed PMID: 6497109
251. Zhan B, Beaumier CM, Briggs N, Jones KM, Keegan BP, Bottazzi ME, et al. Advancing a multivalent 'Pan-anthelmintic' vaccine against soil-transmitted nematode infections. Expert Rev Vaccines. 2014;13(3):321–31. https://doi.org/10.1586/14760584.2014.872035. PubMed PMID: 24392641; PubMed Central PMCID: PMCPMC3934375
252. Montresor A, Gabrielli AF, Savioli L. Approaches to control of STHs including Ascariasis. In: Holland C, editor. *Ascaris*: the neglected parasite. Amsterdam: Elsevier; 2013. p. 383–93.
253. Esrey SA, Potash JB, Roberts L, Shiff C. Effects of improved water supply and sanitation on ascariasis, diarrhoea, dracunculiasis, hookworm infection, schistosomiasis, and trachoma. Bull World Health Organ. 1991;69(5):609–21. PubMed PMID: 1835675; PubMed Central PMCID: PMCPMC2393264
254. Albonico M, Crompton DW, Savioli L. Control strategies for human intestinal nematode infections. Adv Parasitol. 1999;42:277–341. PubMed PMID: 10050275
255. Hollingsworth TD, Truscott JE, Anderson RM. Chapter 9—transmission dynamics of *Ascaris lumbricoides*—theory and observation A2—Holland, Celia. *Ascaris*: the neglected parasite. Amsterdam: Elsevier; 2013. p. 231–62.

Overview of Hookworm Infection in Humans

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Contents

Neglected Tropical Diseases

Neglected tropical diseases (NTDs) are a group of 13 diseases, which usually infect the poorest people around the world. Among them, the six most prevalent are caused by helminths: ascariasis, trichuriasis, ancylostomiasis, schistosomiasis, lymphatic filariasis, and oncocercosis [1].

For classification purposes, helminths are divided into two phyla: Nematoda (cylindrical worms) and Platyhelminthes (flat worms). Nematoda worms include the main intestinal worms (which spend part of their life cycle in soil) and worms that cause lymphatic filariasis and oncocercosis. Platyhelminthes worms include *Fasciola*, *Schistosoma*, and *Taenia* [2].

Neglected parasitic diseases are among the most common infections in rural and urban areas in low-income countries located in Asia, sub-Saharan Africa, and Latin America [3]. This group of diseases has a strong impact on the physical and mental

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health of infected people, especially schoolchildren. Moreover, NTDs commonly appear as polyparasitism, i.e., more than one species infecting a single person. When polyparasitism is observed, there is an even bigger impact on cognitive and intellectual development in children. It is estimated that there are losses of billions of dollars as a result of people in the workforce being infected with NTDs [3]. Together, those effects intensify poverty in endemic areas and require effective interventions every year from health authorities to try to solve the endemicity.

1 Soil-Transmitted Helminths: Introduction

Soil transmitted helminths (STHs) are a group of neglected diseases, especially in tropical areas. It is estimated that 2 billion people around the world are infected by STHs, with higher concentrations in areas where sanitation is not available or is inadequate [4]. Infection by STHs arises from ovum ingestion (*Ascaris lumbricoides* and *Trichuris trichiura*) or larva ingestion (*Ancylostoma duodenale*) via infected food or contaminated food utensils, or because of poor hand washing; or active penetration of bare skin by larvae present in contaminated soil (*Necator americanus* or *Ancylostoma duodenale*). Once the host is infected, an adult worm in the intestine can survive for years, and the host can contaminate other places if personal hygiene and sanitation are not present or are suboptimal [4].

STHs are more prevalent in communities where the per capita income is no more than US$1.25/day. They also strongly impact public health and social and economic activity in several countries. Young women and children, when infected, present with the highest morbidities. In children, infection can cause physical, mental, and cognitive impairment, resulting in educational impairment and, consequently, economic impairment [2]. It has also been noted that in preschool children, infection causes malnutrition due to poor nutrient absorption, and competition between the parasite and the host impacts the child's memory and fluency [5]. World mortality due to STHs varies from 12,000 to 135,000 deaths per year [6]. The symptoms caused by STH parasite infections are very broad and include abdominal pain, diarrhea, and weakness. Hookworm can also cause chronic intestinal bleeding, leading to anemia [4].

Intestinal parasites are endemic in the majority of South Asian countries. In 2001, a new resolution was passed (WHA54.19) that encourages countries with endemic intestinal parasites to strongly fight against STH, parasitic worm infections, and schistosomiasis. The aim is to control the morbidity caused by STH by using drug therapy for prevention of infection in endemic areas. The global aim is to stop morbidity in children by 2020, especially by conducting deworming programs in schools [4].

2 Pathogens

2.1 Ancylostomatidae

The Ancylostomatidae family (from Greek: *ankylos* = angle + *tomma* = mouth) has more than 100 species and, among them, three are able to cause important infections in humans. *Ancylostoma duodenale* (Dubini, 1843) and *Necator americanus* (Stiles, 1902) are responsible for the majority of human infections. Although *Ancylostoma ceylanicum* [5, 7, 8] can infect humans, this species commonly infects cats and dogs [9, 10]. The mouth and its parts are used to classify the Ancylostomatidae family. The subfamily Ancylostomatinae is composed of *A. duodenale* and *A. ceylanicum* with marginal teeth in the mouth [11]. Species with sharp plates around the mouth, such as *N. americanus*, belong to the subfamily Bunostominae. Mouth structures are important to these parasites since they are used to cut the gut mucosa to access blood during feeding [10].

Like other nematodes, hookworms have a direct biological cycle with only one host. During part of the life cycle, larval stage 1 (L1), L2, and L3 are free. A parasitic phase is started after L3 actively penetrates the skin [11, 12]. After the penetration, L3 reaches the bloodstream and migrates to the lungs, where it maturates. The maturated L3 is then coughed up and swallowed to enter the digestive system. In the small intestine, the larvae will develop into mature worms (females and males), feed on blood, mate, and produce eggs, which will be released into the environment in feces. The egg has a thin shell and is rounded, measuring about 60 μm × 40 μm [11]. The eggs will then hatch to release L1. The three species have similar life cycles but also have differences in behavior and climatic condition responses. It has been shown that *Necator* female worms can produce 9000 eggs/day and *Ancylostoma* female worms can produce up to 30,000 eggs/day. To hatch in the environment, it is necessary that the eggs be placed in locations with good oxygen rates, humidity over 90%, and high temperatures (21–27 °C for *A. duodenale* and 27–32 °C for *N. americanus*) [13].

If all of the necessary conditions are in place, the eggs could take 24–48 h to hatch stage 1 larvae (L1) [11]. Another 3–4 days are necessary for L1 to produce an internal cuticle and become stage 2 larvae (L2). Both stages of larvae (L1 and L2) are rhabditiforms, with serpin movements, and feed on organic material and microorganisms present in the soil. Five more days are necessary for them to develop an external cuticle and part of the mouth cavity. At this stage L2 turns into L3 and does not feed or move. L3-stage larvae are viable for days in humid, hot, and sandy soil until their stored lipids are used up as an energy source [11]. The L3 larval stage is the only infective stage for hookworm to invade a host [12].

Some *A. duodenale* infections can happen by L3 larval ingestion; however, the majority are due to active penetration through the host's skin, also called *transdermal transmission* or *percutaneous transmission*. During skin penetration, the parasite loses its external cuticle and there is activation of the larva due to thermal and chemical host effects. L3 larvae can produce and secrete collagenases and other

enzymes, which are responsible for digesting basic matrix and fundamental substances present in the derma [14].

After the larvae reach the bloodstream or lymphatic stream, they arrive in the heart and migrate through the pulmonary artery to the lungs. Via the pulmonary microcirculation, they perforate the alveoli and reach the trachea to be finally swallowed and arrive in the small intestine [10, 11].

Migration through the lungs takes 2–7 days, and during this period the larvae change their cuticle and develop buccal and reproductive systems; this is the L4 stage. In the small intestine, the L4 stage fixes in microvilli and starts feeding on blood. This fixation is deep with the anterior portion using teeth for *A. duodenale* and plates for *N. americanus* to help not only fixation but also tissue maceration to help feeding. Altogether, these aspects contribute to the host's anemia and malnutrition [9, 10]. There have been reports that *A. duodenale* can live for 1–3 years and *N. americanus* for 3–10 years once established in the host's small intestine [11].

In the host, L3 skin penetration can be harsh, and it can result in a cutaneous syndrome known as *ground itch*. This comprises a pruritic erythematous papulovesicular rash, most commonly on the hands and feet [15]. Zoonotic infection with *A. braziliense*, on the other hand, can cause *larva migrans* characterized by migration of the larvae in the skin, especially on the feet, gluteus, and abdomen [11].

Although *A. duodenale* and *N. americanus* have many similarities in their life cycles, there are also differences, which reflect their parasitism dynamics (Table 1). *A. duodenale* infections are associated with more intestinal blood loss than infections of other hookworms, and their transmission can happen via skin penetration, oral ingestion, and even breastfeeding.

N. americanus is smaller than *A. duodenale* and also produces fewer eggs per female than other species. Moreover, *N. americanus* ingests less blood per day than other species. This is considered a highly adapted parasitism since there is less prejudice to the host than with other species. Since *N. americanus* is also more

Table 1 Biological characteristics of *Necator americanus* and *Ancylostoma* duodenale in the human host (adapted from Hoagland and Schad (1978), Schad and Banwell (1984), Bundy (1990), Anderson and May (1991), Crompton (2000) from Brooker et al. [11]

Characteristic	*N. americanus*	*A. duodenale*
Male adult size (mm)	7–9	8–11
Female adult size (mm)	9–11	10–13
Rate of egg production (per day)	3000–6000	10,000–20,000
Maturation time in humans (days)	40–50	28–50
Life expectancy of infective larvae (days)	3–5	1
Life expectancy of adult worms (years)	3–10	1–3
Blood loss (mL/worm/day; mean [range])	0.03 [0.01–0.04]	0.15 [0.14–0.30]
Lactogenic transmission	No	Yes
Oral transmission	No	Yes
Arrested development	No	Yes

widespread around the world than other hookworm species, it is considered the most important hookworm species that infects humans [11].

2.1.1 Molecular Biological Aspects

Important molecules involved in the parasite–host relationship have been described. The majority of them are classified into three categories: proteins secreted by L3, proteins secreted by adult worms, and molecules that are present in the villi membrane covering the digestive channel in adult hookworms.

Proteins Secretion During the L3 Infective Larval Stage

After stimulation with the host's serum, L3 larvae secrete bioactive peptides in response to specific factors inducing activation, development, and maintenance of the larvae in the host's body. Among the main secreted molecules are MTP-1 (zinc metalloprotease from the astacin family) and two ASPs (acylation-stimulating proteins) rich in cysteine and related to the pathogenesis of the disease (PRP). Those proteins participate in the transition between the external environment and the host's internal environment and are important to the biological development of the larvae. Experimental model studies indicate that ASPs and MTP-1 are possible vaccine targets for hookworm disease [11, 16].

Protein Secretion by Adult Worms

Once in the small gut mucosa, adult hookworms secrete several molecules that act on the host's immunomodulation and impair their elimination. Adult worms secrete distinct types of ASPs, two of which have structural similarities to L3-secreted ASPs.

The most abundant protein secreted by adult worms is similar to mammalian TIMP (tissue inhibitor metalloproteinase). Although its role is still unknown, hookworm TIMP is present in biological processes of immunomodulation since it can induce lymphocyte B to produce interleukin (IL)-10 [17, 18].

There are other immunomodulatory proteins secreted by adult worms: NIF (neutrophil inhibitory factor), an antagonist to CD11b/CD18 integrin; eotaxin proteases; calreticulin, a protein that binds to complement protein C1q; retinol binding protein; collagen binding protein; C-lectin; acetylcholinesterase; glutathione-S-transferase; Cu/Zn superoxide dismutase; and T cell apoptosis activation proteins [11].

To promote tissue invasion, hookworms also secrete collagen hydrolases including the metalloproteinase MTP-2, which is very similar to L3 MTP-1; protease cysteinyl (CP-1); aspartic protease (APR-1); and hyaluronidase [11].

There are also secreted proteins involved in blood feeding. The serine protease anticoagulant inhibitors are NAPc2 and NAP5, responsible for VIIa and Xa factor inhibition, respectively. These molecules can also act in the release of a platelet inhibitor that binds glycoprotein (GP) integrins IIb/IIIa and Ia/IIa [11].

Molecules Present in the Villi Membrane Covering the Digestive Channel in Adult Hookworms

In the adult worm's digestive system, proteolytic enzymes such as MEP-1, APR-2, and cysteine protease have been found. Those molecules are related to the hemoglobulin degradation cascade, similar to the one found in trematodes and *Plasmodium* [11].

2.1.2 Genome

The parasitic helminth genome is large and complex. In general, nematodes, as well as platyhelminths, have genomes of between 50 and 500 Mb with up to 20,000 protein gene codes [2]. Just recently, the *N. americanus* genome was sequenced. *N. americanus* has 1948 differentially expressed genes, of which 36% are highly expressed in L3 larvae and 64% in adult worms. Among those L3 highly expressed genes, eight are associated with molecular functions such as signal transduction and transmembrane receptor activity. Those genes reflect the ability of L3 to infect the host and correctly adapt to a new environment [19]. On the other hand, adult worms highly express genes related to nutritional adaptation to cover the high protein demands of this life cycle form. Adult *N. americanus* express four times as many proteins from the SCP/TAPS family which are related to parasite–host interaction [19].

3 Pathogenesis and Immunity

3.1 Host–Pathogen Interaction

Like other helminth infections, hookworm infection induces predominately an inadequate and not protective T helper 2 (Th2) response [20]. The most pronounced immunological alterations due to hookworm infections are eosinophilia, mastocytosis, and high immunoglobulin E (IgE) production [2].

During the infection a vast number of antigens are presented to the immune system, and they vary throughout the worm's life cycle. There are also multiple antigen presentations at several anatomical sites. The immunomodulatory molecule secretions, especially those by adult worms, contribute to stopping, decreasing, or evading the host's immune system [10, 21]. Due to the worm's life cycle, antigen

diversification, and development at several anatomical sites, it is challenging to the immune system to stop the parasitism.

Secreted molecules polarize the immune system to a Th2 response. The most common cytokines found in response to infection are IL-4, IL-5, IL-9, IL-13, IL-25, IL-31, IL-33, TSLP (thymus stromal lymphopoietin), matrix metalloproteinases, and IgG and IgE. The host's immunosuppression is progressive and systemic, which explains the higher parasite burden in adult hosts than in children. One mechanism related to this is the high production of IL-10 and clonal expansion of T regulatory (Treg) cells (expressing FoxP3). IL-25 and IL-31 cytokines are important to drive the Th2 response during infection [21–23].

During reinfection with hookworm, Th2 response polarization can destroy some of the larvae during body migration, especially in the lung stage. It has been observed that IL-5 production is correlated with protection against *N. americanus* infection due to action on hematopoietic cells. The B cell response to IL-4 is important to CD4 Th2 cell interaction, IL-13 production, and antibody activation of mastocytes and basophils [21, 24].

IL-4 and IL-13, produced in the small intestine, stimulate intestinal caliciform cells to multiply and produce resistin type β (RELM β). RELM β binds to chemosensory neurons in the worms and stops their feeding [21]. IL-4 and IL-13 also stimulate enteric nerves, smooth muscle contraction, and mucus production, and then contribute to impairing intestinal crypt worm binding. Also, those cytokines stimulate alternative activated macrophages via IL-4Rα signaling to contribute to tissue repair and hookworm elimination [21, 24, 25].

It has been shown that innate lymphoid cells (ILCs) contribute to the antihelminth immune response by stimulating production of Th2 cytokines, especially IL-5, IL-9, and IL-13 [26]. High IL-9 production acts on eosinophils, mastocytes, and caliciform cells, and also promotes lung tissue repair, decreasing the effects of infection [21].

During hookworm infection, there is a specific antibody response with high presence of IgE, IgG1, and IgG4 [10, 11]. IgE production can be increased by 100 times during infection, but not all of this is used to stop the parasitism. IgE has an important role in activation and degranulation of mastocytes, basophils, and eosinophils. High serum IgE titers are followed by systemic and pulmonary eosinophilia [11].

4 Epidemiology

Hookworm infection is one of the most common STHs. It affects 740 million people living in tropical and subtropical areas. In South Asia and India, there are 130 million cases [5, 15, 27].

In Bangladesh, STH infections are prevalent and are one of the top ten causes of hospital morbidity. It has been shown that 65.6% of women of fertile age and children up to 14 years old had helminth infection in one rural area of Bangladesh.

Of these, 5.5% had hookworm disease [28]. The high STH infection rate in Bangladesh is due to the lack of adequate sewerage systems. Although 80% of Bangladesh's population has access to potable water, only 36% have sewerage systems. This problem creates difficulties in stopping STH transmission and conducting deworming programs, increasing the reinfection rates [29].

In India, it is estimated that 7% of the population is infected with hookworm [30]. However, some areas have higher infection rates, such as Tamil Nadu. In this area, of 143 samples evaluated between 2013 and 2015, 119 showed positive DNA for hookworm, wherein *N. americanus* was present in all positive samples [31].

Nepal has problems with water not suitable for drinking, lack of sewerage systems, ineffective drainage systems, and lack of animal transit control. Together these problems contribute to high STH infection rates in that country [32]. In 1996, 67.7% of schoolchildren were infected by hookworm [33]. However, in 2008 this same group showed a decrease in the infection rate. Depending on the children's age and access to hospital, hookworm infection rates were shown to be between 9.2% and 51.6% [32].

In 2005, children in Afghanistan were shown to have an STH infection rate of 47.2%. In that country, infection with *Ascaris lumbricoides* is more common and only 0.7% of the children showed hookworm infection [34].

In Pakistan, there is a correlation between hookworm infection and residual water use by farmers. It has been shown that this group had a 7.7% infection rate in adults and 6% in children. Among the parasites, hookworm was most prevalent in farmers, with a 4.6% infection rate. The hookworm infection rate is around 3.7% of the population [35].

In Sri Lanka, before deworming programs were put in place, the STH infection rate was about 90% in fertile women and children. After the deworming programs, the prevalence of STH disease decreased to 29%, and 4.7% was due to hookworm [36]. Another study showed that 5% of schoolchildren were infected with *N. americanus* in two rural areas [37]. An important relationship between infection and the environment was shown in Sri Lanka. There were three peaks in monthly infection rate measurements, which coincided with higher temperatures [38].

Hookworm disease morbidity affects 122 million people around the world per year [9, 39]. Hookworm infection can cause important iron loss deficiency and malnutrition. Although death by hookworm infection is not common, other aspects are important. It is estimated that 22 million life years are lost to hookworm disease after adjustment (disability-adjusted life-years (DALYs)) [20, 21, 40]. The morbidity is measured by the infection rate in a population during a period of time and adjusted for the worm burden/persons infected. The worm burden is measured by egg counting per gram of feces (EPG). The World Health Organization (WHO) has associated the EPG with morbidity, and the worm burden classification is light, moderate, or heavy. The affected communities are also classified according to the prevalence: I (high), II (moderate), and III (low) [2].

The epidemiology of STH disease involves several factors, including:

- *Environment:* STH disease is dependent on and influenced by the temperature and humidity of the soil and the altitude of the local area [41]. There is also a relationship between poverty and STH infection [3].
- *Parasite burden:* The heterogeneity of the individual parasite burden influences STH infection by concentration in endemic areas. The majority of the people who become infected or reinfected are in those areas. The proportion reported is that 10% of the people in the endemic area have 70% of the worms [42].
- *Age:* The intensity of STH infection changes with age. In general, children are more infected than adults. However, this relation is opposite for hookworm infection [2, 43].
- *Family group:* There is evidence that the distribution of *A. lumbricoides*-infected people is not random but grouped in families. The presence of people who are heavily infected in a family is correlated with genetic factors, inadequate immune response to the parasite, and intrafamilial infection, i.e., one family member infects another [44].
- *Genetics:* There is evidence that people with high IgE-producing cells are more resistant to infection than people incapable of producing high amounts of IgE.
- *Polyparasitism:* Several studies have shown that susceptible people are frequently infected with more than one helminth species. There is an association between infection with *A. lumbricoides* and *T. trichiura* [45]. In coinfections, parasites can act in an initial immune response in synergy or not. Although more studies are necessary in this area, a negative association between helminth infection and HIV and malaria infections has been demonstrated [45].

5 Clinical Features

In general, hookworm infection is asymptomatic, and this can be very dangerous to the host, since the person does not seek treatment if he or she does not have symptoms [46]. There are symptoms that can appear just after the infection, such as itchiness caused by an allergic reaction to larval penetration, which is very common in *N. americanus* infection [47]. Once in the respiratory tract, symptoms such as cough and pneumonitis can be seen. After arriving in the small intestine, worms can cause diarrhea, bellyache, and gastrointestinal discomfort [48]. The high morbidity associated with hookworm infection is due to intestinal blood loss, anemia caused by iron deficiency, and protein malabsorption [48]. Anemia appears as a consequence of adult worm blood feeding, since the worm needs to ingest blood to degrade hemoglobulin and access iron [49]. In the long term, anemia can cause facial and peripheral edema. Some patients also show eosinophilia due to iron deficiency caused by anemia [47].

Moderate and heavy infections cause important blood loss in the host. Once the iron supply is depleted, anemia develops. This can be even more significant if there

is a history of a low-iron diet [11, 49]. This severe anemia can cause fatigue, headaches, effort dyspnea, syncope, and edema. In rare cases, it can cause ischemic heart disease symptoms such as angina and claudication [11].

It is important to mention that hookworm disease causes an important public health problem not only in adults but also in children. Chronic hookworm infection can impair growth and intellectual and cognitive development in children, which can never be proper remedied [49]. Another important problem that needs more attention is the maternal–fetal consequences for both mother and child if the mother has hookworm disease [49].

6 Diagnosis and Treatment

The most common diagnostic method for hookworm disease is the Kato-Katz technique. This technique is recommended by the WHO also for other helminth diseases due to its low cost, noninvasiveness and easy methodology [50, 51]. However, it is well known that Kato-Katz has sensitivity limitations. Just one of the problems with it is that it can yield false positive results, as hookworm eggs are similar to other eggs that can be present in the feces [50, 51]. An alternative method is FLOTAC. FLOTAC has been shown to be more sensitive for egg hookworm detection even if human feces are chemically fixated [52–54].

Hookworm infection disease, caused by *A. duodenale* or *N. americanus*, is treated by antihelminth drug administration: mebendazole, albendazole, or pyrantel pamoate. It has been shown that single-dose administration of those drugs can be effective for hookworm disease, with a 72% cure rate (95% confidence interval (CI) 59–81%; 742 patients) for oral albendazole, a 15% cure rate (95% CI 1–27%; 853 patients) for mebendazole, and a 31% cure rate (95% CI 19–42%; 152 patients) for pyrantel pamoate [55]. Thus, this suggests that albendazole is a better drug choice for hookworm disease in the event that a single-dose treatment is used. Table 2 shows the correct dosage to be used in hookworm disease treatment [55]. Those drugs are aimed at G protein–coupled receptors (GPCRs), nuclear receptors (NRs), ligand-gated ionic channels (LGICs), kinases, and voltage-gated ionic channels (VGICs) [19].

Adverse effects of hookworm drug treatment are abdominal and epigastric pain, nausea, diarrhea (with or without blood present), fever, itchiness, and headache [56].

Table 2 Drug treatment for hookworm (adapted from Utzinger and Keiser [56])

Drug	Dose
Albendazole	400 mg (200 mg for children 1–2 years old)
Mebendazole	500 mg (or 2 × 100 mg/day for 3 days)
Pyrantel pamoate	10–40 mg/kg for 1–3 days

7 Prevention and Control

Hookworm disease prevention can be achieved by interruption of different parts of the life cycle [57]:

- Treatment of infected people to eliminate or reduce egg production and release
- Institution of sewerage systems to treat infected feces and stop soil contamination
- Wearing of closed shoes to stop larval contact with bare skin
- Educational programs, especially for school children, to change behavior and teach self-hygiene to stop transmission

Although these seem to be simple actions, control and elimination are hard to achieve for hookworm diseases in endemic areas due to the lack of proper sewerage system availability [11, 49].

Mass drug administration (MDA) is one strategy used for disease control, involving antihelminth drug administration in endemic areas, depending on the country's legislation. Although this strategy works for other STH diseases, no reduction in anemia prevalence associated with hookworm disease has been shown after MDA [21, 58, 59]. This was also corroborated by the Global Burden of Disease (GBD) study, which showed no reduction of infection prevalence after MDA in 2013. It needs to be taken into account that MDA is usually applied only to schoolchildren, excluding heavily infected adult groups from treatment [21, 60]. It must be pointed out that MDA does not eliminate the parasite from endemic areas, nor is the transmission cycle stopped due to drug efficacy against hookworm. Thus, reinfections are common in endemic areas, and drug resistance can also occur [58, 61–63].

The majority of vaccine development strategies are aimed at the larval hookworm stage [21] or reinfection prevention. The protein *N. americanus* ASP-2 (Na-ASP-2) has shown promising results in people from nonendemic areas with no hookworm exposure, since it induced a prolonged and efficient immune response against the parasite [64, 65]. Another strategy is to impair worm blood feeding. Aspartic protease 1 (APR-1) is a candidate that is present in the hemoglobin digestion cascade; when it is inactivated, the worm can no longer feed on blood and will die [66].

8 Conclusion

Hookworm infection is an important neglected disease that affects millions of poor people around the world. Although prevention is simple, it also demands an effort from the population to change some behaviors and investment from local government to deliver potable water and adequate sanitation. With these in place, it is possible to stop transmission and decrease the mortality and morbidity due to hookworm infection.

References

1. Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, Savioli L. Control of neglected tropical diseases. N Engl J Med. 2007;357:1018–27.
2. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Invest. 2008;118:1311–21.
3. Hotez P. Hookworm and poverty. Ann N Y Acad Sci. 2008;1136:38–44.
4. WHO (2016) What are intestinal worms (soil transmitted helminthiasis)? Available from: http://www.who.int/intestinal_worms/disease/en/. Accessed 15 Nov 2016.
5. Kvalsvig JD, Cooppan RM, Connolly KJ. The effects of parasite infections on cognitive processes in children. Ann Trop Med Parasitol. 1991;85:551–68.
6. Ngui R, Aziz S, Chua KH, Aidil RM, Lee SC, Tan TK, Sani MM, Arine AF, Rohela M, Lim YA. Patterns and risk factors of soil-transmitted helminthiasis among Orang Asli subgroups in peninsular Malaysia. Am J Trop Med Hyg. 2015;93:361–70.
7. Carroll SM, Grove DI. Resistance of dogs to reinfection with *Ancylostoma ceylanicum* following anthelmintic therapy. Trans R Soc Trop Med Hyg. 1985;79:519–23.
8. Inpankaew T, Schär F, Dalsgaard A, Khieu V, Chimnoi W, Chhoun C, Sok D, Marti H, Muth S, Odermatt P, Traub RJ. High prevalence of *Ancylostoma ceylanicum* hookworm infections in humans, Cambodia, 2012. Emerg Infect Dis. 2014;20:976–82.
9. Bungiro R, Cappello M. Hookworm infection: new developments and prospects for control. Curr Opin Infect Dis. 2004;17:421–6.
10. Loukas A, Constant SL, Bethony JM. Immunobiology of hookworm infection. FEMS Immunol Med Microbiol. 2005b;43:115–24.
11. Brooker S, Bethony J, Hotez PJ. Human hookworm infection in the 21st century. Adv Parasitol. 2004;58:197–288.
12. Hawdon JM, Hotez PJ. Hookworm: developmental biology of the infectious process. Curr Opin Genet Dev. 1996;6:618–23.
13. Neves DP. Human parasitology. 11th ed. Chapter 30. São Paulo: Atheneu; 2004.
14. Souadkia N, Brown A, Leach L, Pritchard DI. Hookworm (*Necator americanus*) larval enzymes disrupt human vascular endothelium. Am J Trop Med Hyg. 2010;83:549–58.
15. Brooker S, Hotez PJ, Bundy DA. Hookworm-related anaemia among pregnant women: a systematic review. PLoS Negl Trop Dis. 2008;2:e291.
16. Hotez PJ, Zhan B, Bethony JM, Loukas A, Williamson A, Goud GN, Hawdon JM, Dobardzic A, Dobardzic R, Ghosh K, Bottazzi ME, Mendez S, Zook B, Wang Y, Liu S, Essiet-Gibson I, Chung-Debose S, Xiao S, Knox D, Meagher M, Inan M, Correa-Oliveira R, Vilk P, Shepherd HR, Brandt W, Russell PK. Progress in the development of a recombinant vaccine for human hookworm disease: the Human Hookworm Vaccine Initiative. Int J Parasitol. 2003;33:1245–58.
17. Guedez L, Mansoor A, Birkedal-Hansen B, Lim MS, Fukushima P, Venzon D, Stetler-Stevenson WG, Stetler-Stevenson M. Tissue inhibitor of metalloproteinases 1 regulation of interleukin-10 in B-cell differentiation and lymphomagenesis. Blood. 2001;97:1796–802.
18. Zhan B, Badamchian M, Meihua B, Ashcom J, Feng J, Hawdon J, Shuhua X, Hotez PJ. Molecular cloning and purification of Ac-TMP, a developmentally regulated putative tissue inhibitor of metalloprotease released in relative abundance by adult *Ancylostoma* hookworms. Am J Trop Med Hyg. 2002;66:238–44.
19. Tang YT, Gao X, Rosa BA, Abubucker S, Hallsworth-Pepin K, Martin J, Tyagi R, Heizer E, Zhang X, Bhonagiri-Palsikar V, Minx P, Warren WC, Wang Q, Zhan B, Hotez PJ, Sternberg PW, Dougall A, Gaze ST, Mulvenna J, Sotillo J, Ranganathan S, Rabelo EM, Wilson RK, Felgner PL, Bethony J, Hawdon JM, Gasser RB, Loukas A, Mitreva M. Genome of the human hookworm *Necator americanus*. Nat Genet. 2014;46:261–9.
20. Quinnell RJ, Bethony J, Pritchard DI. The immunoepidemiology of human hookworm infection. Parasite Immunol. 2004;26:443–54.

21. Bartsch SM, Hotez PJ, Asti L, Zapf KM, Bottazzi ME, Diemert DJ, Lee BY. The global economic and health burden of human hookworm infection. PLoS Negl Trop Dis. 2016;10: e0004922.
22. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R, Muchamuel T, Hurst SD, Zurawski G, Leach MW, Gorman DM, Rennick DM. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001;15:985–95.
23. Quinnell RJ. Genetics of susceptibility to human helminth infection. Int J Parasitol. 2003;33:1219–31.
24. Urban JF, Noben-Trauth N, Schopf L, Madden KB, Finkelman FD. Cutting edge: IL-4 receptor expression by non-bone marrow-derived cells is required to expel gastrointestinal nematode parasites. J Immunol. 2001;167:6078–81.
25. Du L, Wei H, Li L, Shan H, Yu Y, Wang Y, Zhang G. Regulation of recombinant *Trichinella spiralis* 53-kDa protein (rTsP53) on alternatively activated macrophages via STAT6 but not IL-4Rα in vitro. Cell Immunol. 2014;288:1–7.
26. Cheever AW, Williams ME, Wynn TA, Finkelman FD, Seder RA, Cox TM, Hieny S, Caspar P, Sher A. Anti-IL-4 treatment of *Schistosoma mansoni*-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. J Immunol. 1994;153:753–9.
27. de Silva NR, Brooker S, Hotez PJ, Montresor A, Engels D, Savioli L. Soil-transmitted helminth infections: updating the global picture. Trends Parasitol. 2003;19:547–51.
28. Benjamin-Chung J, Nazneen A, Halder AK, Haque R, Siddique A, Uddin MS, Koporc K, Arnold BF, Hubbard AE, Unicomb L, Luby SP, Addiss DG, Colford JM. The interaction of deworming, improved sanitation, and household flooring with soil-transmitted helminth infection in rural Bangladesh. PLoS Negl Trop Dis. 2015;9:e0004256.
29. Bath JL, Eneh PN, Bakken AJ, Knox ME, Schiedt MD, Campbell JM. The impact of perception and knowledge on the treatment and prevention of intestinal worms in the Manikganj district of Bangladesh. Yale J Biol Med. 2010;83:171–84.
30. Jalihal U, Avinash B, Satyaprakash BS. Hookworm infestation in south India—are we moving away from microscope? Indian J Gastroenterol. 2013;32:62–3.
31. George S, Levecke B, Kattula D, Velusamy V, Roy S, Geldhof P, Sarkar R, Kang G. Molecular identification of hookworm isolates in humans, dogs and soil in a tribal area in Tamil Nadu, India. PLoS Negl Trop Dis. 2016;10:e0004891.
32. Mukhopadhyay C, Wilson G, Chawla K, Vs B, Shivananda PG. A 6 year geohelminth infection profile of children at high altitude in Western Nepal. BMC Public Health. 2008;8:98.
33. Khanal P, Walgate R. Nepal deworming programme ready to go worldwide. Bull World Health Organ. 2002;80:423–4.
34. Gabrielli AF, Ramsan M, Naumann C, Tsogzolmaa D, Bojang B, Khoshal MH, Connolly M, Stothard JR, Montresor A, Savioli L. Soil-transmitted helminths and haemoglobin status among Afghan children in World Food Programme assisted schools. J Helminthol. 2005;79 (4):381.
35. Ensink JH, van der Hoek W, Mukhtar M, Tahir Z, Amerasinghe FP. High risk of hookworm infection among wastewater farmers in Pakistan. Trans R Soc Trop Med Hyg. 2005;99:809–18.
36. Gunawardena K, Kumarendran B, Ebenezer R, Gunasingha MS, Pathmeswaran A, de Silva N. Soil-transmitted helminth infections among plantation sector schoolchildren in Sri Lanka: prevalence after ten years of preventive chemotherapy. PLoS Negl Trop Dis. 2011;5:e1341.
37. Fernando SD, Paranavitane SR, Rajakaruna J, Weerasinghe S, Silva D, Wickremasinghe AR. The health and nutritional status of school children in two rural communities in Sri Lanka. Tropical Med Int Health. 2000;5:450–2.
38. Gunawardena GS, Karunaweera ND, Ismail MM. Effects of climatic, socio-economic and behavioural factors on the transmission of hookworm (*Necator americanus*) on two low-country plantations in Sri Lanka. Ann Trop Med Parasitol. 2005;99:601–9.

39. McSorley HJ, Loukas A. The immunology of human hookworm infections. Parasite Immunol. 2010;32:549–59.
40. Chan MS. The global burden of intestinal nematode infections—fifty years on. Parasitol Today. 1997;13:438–43.
41. Mabaso ML, Appleton CC, Hughes JC, Gouws E. The effect of soil type and climate on hookworm (*Necator americanus*) distribution in KwaZulu-Natal, South Africa. Tropical Med Int Health. 2003;8:722–7.
42. Anderson RM, May RM. Helminth infections of humans: mathematical models, population dynamics, and control. Adv Parasitol. 1985;24:1–101.
43. Bethony J, Chen J, Lin S, Xiao S, Zhan B, Li S, Xue H, Xing F, Humphries D, Yan W, Chen G, Foster V, Hawdon JM, Hotez PJ. Emerging patterns of hookworm infection: influence of aging on the intensity of *Necator* infection in Hainan Province, People's Republic of China. Clin Infect Dis. 2002;35:1336–44.
44. Forrester JE, Scott ME, Bundy DA, Golden MH. Clustering of *Ascaris lumbricoides* and *Trichuris trichiura* infections within households. Trans R Soc Trop Med Hyg. 1988;82:282–8.
45. Faulkner H, Turner J, Behnke J, Kamgno J, Rowlinson MC, Bradley JE, Boussinesq M. Associations between filarial and gastrointestinal nematodes. Trans R Soc Trop Med Hyg. 2005;99:301–12.
46. Stoll NR. On endemic hookworm, where do we stand today? Exp Parasitol. 1962;12:241–52.
47. Hotez PJ, Brooker S, Bethony JM, Bottazzi ME, Loukas A, Xiao S. Hookworm infection. N Engl J Med. 2004;351:799–807.
48. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet. 2006;367:1521–32.
49. Hotez PJ, Bethony J, Bottazzi ME, Brooker S, Buss P. Hookworm: "the great infection of mankind". PLoS Med. 2005;2:e67.
50. Irisarri-Gutiérrez MJ, Muñoz-Antolí C, Acosta L, Parker LA, Toledo R, Bornay-Llinares FJ, Esteban JG. Hookworm-like eggs in children's faecal samples from a rural area of Rwanda. Afr Health Sci. 2016;16:83–8.
51. Knopp S, Mgeni AF, Khamis IS, Steinmann P, Stothard JR, Rollinson D, Marti H, Utzinger J. Diagnosis of soil-transmitted helminths in the era of preventive chemotherapy: effect of multiple stool sampling and use of different diagnostic techniques. PLoS Negl Trop Dis. 2008;2:e331.
52. Coulibaly JT, Ouattara M, Becker SL, Lo NC, Keiser J, N'Goran EK, Ianniello D, Rinaldi L, Cringoli G, Utzinger J. Comparison of sensitivity and faecal egg counts of Mini-FLOTAC using fixed stool samples and Kato-Katz technique for the diagnosis of *Schistosoma mansoni* and soil-transmitted helminths. Acta Trop. 2016;164:107–16.
53. Knopp S, Salim N, Schindler T, Karagiannis Voules DA, Rothen J, Lweno O, Mohammed AS, Singo R, Benninghoff M, Nsojo AA, Genton B, Daubenberger C. Diagnostic accuracy of Kato-Katz, FLOTAC, Baermann, and PCR methods for the detection of light-intensity hookworm and *Strongyloides stercoralis* infections in Tanzania. Am J Trop Med Hyg. 2014;90:535–45.
54. Utzinger J, Rinaldi L, Lohourignon LK, Rohner F, Zimmermann MB, Tschannen AB, N'goran EK, Cringoli G. FLOTAC: a new sensitive technique for the diagnosis of hookworm infections in humans. Trans R Soc Trop Med Hyg. 2008;102:84–90.
55. Keiser J, Utzinger J. Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis. JAMA. 2008;299:1937–48.
56. Utzinger J, Keiser J. Schistosomiasis and soil-transmitted helminthiasis: common drugs for treatment and control. Expert Opin Pharmacother. 2004;5:263–85.
57. Rey L. One century of experience with the control of ancylostomiasis. Rev Soc Bras Med Trop. 2001;34:61–7.
58. Bennett A, Guyatt H. Reducing intestinal nematode infection: efficacy of albendazole and mebendazole. Parasitol Today. 2000;16:71–4.

59. Smith JL, Brooker S. Impact of hookworm infection and deworming on anaemia in non-pregnant populations: a systematic review. Tropical Med Int Health. 2010;15:776–95.
60. Collaborators, G B o D S. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2015;386:743–800.
61. Albonico M, Bickle Q, Ramsan M, Montresor A, Savioli L, Taylor M. Efficacy of mebendazole and levamisole alone or in combination against intestinal nematode infections after repeated targeted mebendazole treatment in Zanzibar. Bull World Health Organ. 2003;81:343–52.
62. Albonico M, Smith PG, Ercole E, Hall A, Chwaya HM, Alawi KS, Savioli L. Rate of reinfection with intestinal nematodes after treatment of children with mebendazole or albendazole in a highly endemic area. Trans R Soc Trop Med Hyg. 1995;89:538–41.
63. Truscott JE, Turner HC, Anderson RM. What impact will the achievement of the current World Health Organisation targets for anthelmintic treatment coverage in children have on the intensity of soil transmitted helminth infections? Parasit Vectors. 2015;8:551.
64. Bethony JM, Simon G, Diemert DJ, Parenti D, Desrosiers A, Schuck S, Fujiwara R, Santiago H, Hotez PJ. Randomized, placebo-controlled, double-blind trial of the Na-ASP-2 hookworm vaccine in unexposed adults. Vaccine. 2008;26:2408–17.
65. Diemert DJ, Bethony JM, Hotez PJ. Hookworm vaccines. Clin Infect Dis. 2008;46:282–8.
66. Loukas A, Bethony JM, Mendez S, Fujiwara RT, Goud GN, Ranjit N, Zhan B, Jones K, Bottazzi ME, Hotez PJ. Vaccination with recombinant aspartic hemoglobinase reduces parasite load and blood loss after hookworm infection in dogs. PLoS Med. 2005a;2:e295.

Overview on Lymphatic Filariasis in South Asia

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Contents

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1 Introduction

Parasitic diseases are responsible for major health problems in developing and underdeveloped countries of tropical and subtropical regions. Among helminth parasitic infections, filariasis, ascariasis and hookworm infections contribute significantly to the global burden of infectious disease. Lymphatic filariasis (LF) is a neglected tropical disease and remains the second leading cause of permanent and long-term disability after mental illness [1]. Lymphatic filariasis also known as elephantiasis is a mosquito-borne disease of tropics and subtropics. LF is transmitted by a variety of mosquito species across the world and infection usually occurs in early childhood but typically takes a long time to manifest in the form of clinical disease but mostly remains asymptomatic in the vast majority of infected individuals. When the worms enter into the lymphatic system, the worms induce major alterations, which leads to lymph fluid accumulation and causes disfiguring swelling of limbs and genitals known as elephantiasis. LF disease is not fatal, but it causes deformity and disability and is the second leading parasitic cause of disability with DALYs (disability-adjusted life years) and a major impact on socioeconomic development [2].

2 Epidemiology

Lymphatic filariasis is one of the oldest and most debilitating diseases in the world. Globally, 947 million people in 54 countries of tropics and subtropics are estimated to be at the risk of infection, 67.88 million are infected including 36.45 million parasite carriers; 19.43 million people with hydrocele and almost 16.68 millions people with lymphedema or elephantiasis [3]. The WHO South-East Asia Region accounts for the highest burden of LF with endemicity in 9 out of its 11 member states. Over all in Southeast Asia region, approximately 501.1 million people are said to be at the risk of infection and 13.8 million people are with lymphedema and hydrocele [4]. Approximately one third of the population of India lives at risk of developing LF. In India, LF is endemic in 255 districts of 16 states and 5 Union Territories (UTs) of the country [5]. About 370 million people were exposed to the risk of infection and required massive drug administration [6]. 190 districts were not surveyed at any point of time to observe the prevalence of microfilaria [7]. The national average prevalence of microfilaria showed a declining trend from 1.24% in 2004 to 0.63% in 2008 (http://www.nvbdcp.gov.in/filariasis-new.html). In Bangladesh, 69 million people are living in endemic areas with 16.8% infected and 10.1% living with chronic disease [8]. In Nepal, 15 million people are at risk of infection [9].

3 Pathogen

Lymphatic filariasis is caused by infection with parasites classified as nematodes (roundworm), which belongs to the family of Filariodidea. Lymphatic filariasis is caused by *Wuchereria bancrofti, Brugia malayi* and *Brugia timori*. All three parasites are transmitted to humans by the bites of infective mosquitoes. The nematode parasite *W. bancrofti* is the major cause of filarial disease accounting for 90% of the cases and is prevalent in Sub-Saharan Africa, South and Southeast Asia. Based on periodicity of microfilariae circulate in the blood of infected patients, *W. bancrofti* can be divided in to 3 subtypes [10]: (1) The nocturnally periodic strains: Mf comes in to the circulation only during night, are transmitted by *Culex quinquefasciatus* in urban areas of Asia, East Africa and the Americas and by *Anopheles* mosquitoes in rural areas [11]; (2) Nocturnally subperiodic strains: Mf present in the circulation and become denser during midnight, are common in Thailand and in the Andaman and Nicobar Islands of India where *Ochlerotatus* (*Aedes*) *niveus* and related species serve as vectors [12, 13]; and (3) Diurnally subperiodic *W. bancrofti*: transmitted by *Aedes polynesiensis* group of day-biting mosquitoes, are widespread in the Pacific region east of Wallace's line [14]. In India, *W. bancrofti* and *B. malayi* are the filarial species causing filarial disease and for *W. bancrofti*, humans are the exclusive host. Even though certain strains of *B. malayi* can also infect some feline and monkey species, the life cycles in humans and in these other animals generally remain epidemiologically distinct, so that little overlap exists. *B. malayi* is found in tropical regions of South and Southeast Asia, occasionally overlapping with the range of *W. bancrofti* [15]. *B. malayi* is transmitted by *Mansonia* mosquitoes. These strains are mainly nocturnally subperiodic. In Southeast Asia, they are readily passed between humans and wild and domestic animal hosts [16]. Brugian parasites are limited to areas of East and South Asia, especially India, Malaysia, Indonesia, and Thailand. *B. timori* has the most restricted geographic range of the lymphatic dwelling filarial species. It is only found in Indonesia and Timor-Leste (Table 1).

Table 1 Filarial parasites infecting humans in Asia

	Mosquito vectors	Periodicity	Distribution	Primary pathology	Morphology
Wuchereria bancrofti	Culex, Anopheles, Aedes	Periodic and Non-periodic	Asia, Africa, Australia, Pacific, South America	Lymphatic and lung	Sheathed Mf with tail containing no nuclei
Brugia malayi	Mansonia, Anopheles, Aedes	Periodic and non-periodic	South-East Asia	Lymphatic and lung	Sheathed Mf with tail containing 2 nuclei
Brugia timori	Anopheles	Periodic	Indonesia and East Timor	Lymphatic and lung	Sheathed Mf with tail containing 2 nuclei

3.1 Lifecycle of Filarial Parasite

Two different host systems are needed to complete the life cycle of filarial parasites. Depending on the species of the infecting parasite, the definitive host can be either man or some animal, and the intermediate host is the mosquito. The three species of lymphatic dwelling filariae have a complex life cycle that alternates between the mosquito vector and the human host. The parasite's life cycle consists of dioecious male and female adult worms, the microfilaria stage, and four larval stages (L1–L4). The third larval stage (L3) is the infectious stage and is transmitted to humans via a mosquito intermediate host. Upon entry into the human host, the L3 larvae migrate to the nearest afferent lymphatics, and L3 larvae undergo the process of molting and develop in to fourth larval (L4) stage and then mature in to adult worms. Adult females produce thousands of sheathed microfilariae (mf) that migrate to and circulate in the bloodstream, usually in synchrony with diurnal mosquito feeding patterns [17]. During subsequent blood meals the mosquitoes would pick up the mf. In the abdomen of the mosquito the mf mature into the first larval stage (L1) and then undergo two molts becoming second larval stage (L2) and subsequently emerging as human infective L3 larvae [18]. From the beginning to end the duration of the *B. malayi* lifecycle is as follows: in the mosquito gut, the molt from L1 to L2 ranges from 6 to 10 days and the molt from L2 to L3 takes 1 to 3 days. Thus, it takes approximately 2 weeks for the infective L3 larvae to mature in the vector. Subsequent to a bite from an infective mosquito and the entry of L3s into human skin, the L3 would transform into L4 after 9 to 14 days in the human host, while the worm is migrating through the circulatory system to the lymphatics. The final molt, from the L4 stage to the adult worm, requires a minimum of 3 months but may last as long as 12 months and is localized in the lumen of dilated lymphatic vessels, the final site of the adult worm in the human body [18]. *W. bancrofti* adults are typically found in the lymphatic vessels of the lower extremities in females and the lymphatic vessels of the spermatic cord in males. Although the life span of adult worms is not precisely known, it is estimated that adult females can remain reproductively active on the order of 5 years [17] (Fig. 1).

3.2 Molecular Biological Aspects Including Details of Genome etc.

The control of any parasitic disease needs the understanding of molecular mechanisms on parasites. The first parasitic nematode to be sequenced fully was *B. malayi* because of its ability to be maintained in small laboratory animals. The genome study of *B. malayi* has helped us to understand phenotypic difference, drug resistance between related filarial species. Using shotgun sequencing the draft of the *B. malayi* 95 Mb accounted for 90% of the genome with nearly nine fold coverage [19]. Deep sequencing approaches and the combined Illumina/454 sequence

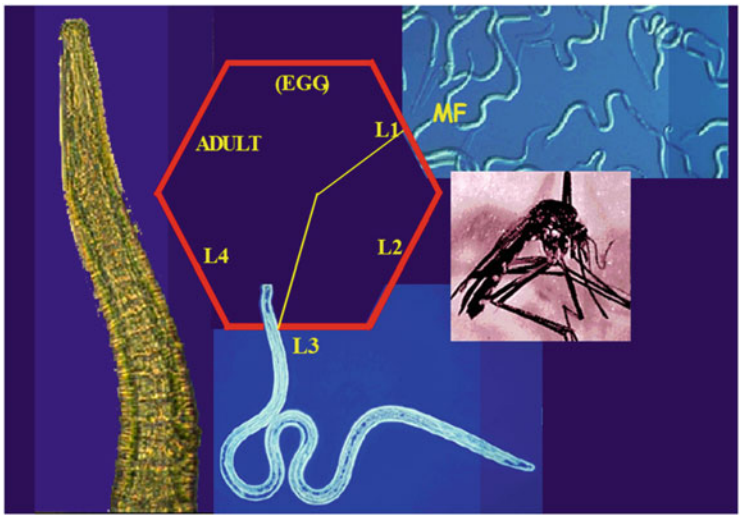

Fig. 1 Life cycle of the filarial parasite

enhanced the significant impact on resolution of the *B. malayi* genome resulting in the annotation of additional 6 Mb into scaffolds and the sequencing efforts also resulted in significant increase in the N50 of the scaffolds from 93 to 357 kb and decreased the number of scaffolds from 8236 to under 2900. The next build *B. malayi* genome will contain increased regulatory elements provided by proteomic studies [20].The rapidly increasing development of innovative and advanced genomic-related techniques, comprising "NextGen" DNA, RNA and protein sequencing, DNA capture methods, bioinformatics analysis methods, has accompanied in an in-depth analysis of the genomes of *Wolbachia* and their various invertebrate hosts and studies that pursue to reveal their symbiotic interactions [21], http://www.nematodes.org/nematodegenomes. The Filarial Genome Project, established in 1994, led to the rediscovery of nematode *Wolbachia*. WHO initiated the sequencing (genomic and cDNA sequencing) of *B. malayi*, which revealed that *Wolbachia* as the endosymbiont within the filarial nematode *Dirofilaria immitis* (dog heartworm) [22, 23]. *Wolbachia* in their hosts are maternally inherited and show a parasitic lifestyle related with reproductive manipulations, parthenogenesis, feminization and male killing [24]. *Wolbachia* is present in most filarial nematode species including *Brugia* spp. and *W. bancrofti*, and in *O. volvulus* and also found in all stages of life-cycle and the numbers vary among different stages [25]. α-proteobacterial endosymbiont *Wolbachia* present in most species of filarial nematodes tissues (Fig. 2).

A-WOL project is an international consortium created to distinguish anti-wolbachia compounds and they screened nearly 18,000 chemical library compounds using a *Wolbachia*-containing *Aedes albopictus* cell line (C6/36 Wp). A 16s rDNA quantitative PCR (qPCR) read-out quantifies *Wolbachia* copy number following treatment [26]. The genome sequences of *B. malayi* [19] and its

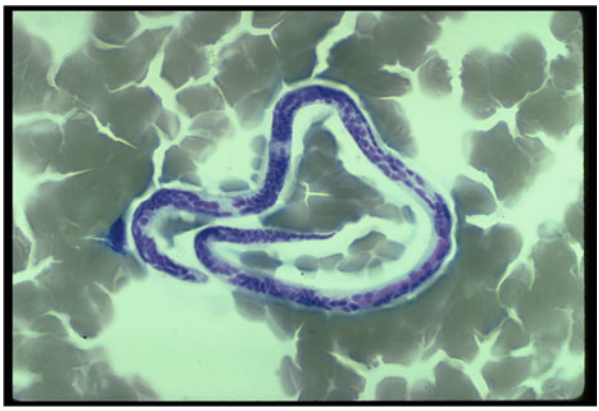

Fig. 2 Microfilaria of *W. bancrofti*

Wolbachia endosymbiont [27] together with transcriptomic approaches have proposed several possible candidates for the biochemical crosstalk. *Wolbachia* genomes have revealed the loss of multiple metabolic pathways, the abundance of repetitive DNA and the presence of a series of genes with potential roles in host interaction [27, 28]. *Wolbachia* proteins such as GroEL, WSP, and peptidoglycan-associated lipoprotein (PAL) are potent stimulants of immune responses in the mammalian host [29, 30]. Small et al. study provided the information on variable positions, demographic history and genes influenced by selection through whole genome sequencing of 13 *W. bancrofti* larvae [31]. Desjardins et al. studied on *L. loa* and *W. bancrofti* genome and envisaged 14,907 *L. loa* genes through RNA sequencing. They exhibited synteny between filarial nematodes but not with nonparasitic nematodes. Deficient of Wolbachia, *L. loa* did not undergo any new metabolic synthesis or transport capabilities while compared to *W. bancrofti* and *B. malayi*. This study suggests that the role of *Wolbachia* in filarial biology is more delicate and disclose marked differences between parasitic and nonparasitic nematodes [32].

Genetic studies help to identify the genetic basis for susceptibility to different species of filarial parasites. The identification of susceptibility loci can provide an important understanding about the mechanisms of protective immunity and pathogenesis and genetic epidemiology. The susceptibility genes associated with host that have shown significant results for the progression to chronic disease of lymphatic filariasis are TGFB1, TLR2, CTLA4, MBLA2, TNFR2, MBL2 and VEGFA [33–36]. Exosome-like vesicles (ELVs) of filarial parasites are enriched with microRNAs that are located between parasite and host, suggesting a potentially novel mechanism by which filarial worms can actively manipulate host gene expression [37].

4 Pathogenesis and Immunity

The most severe clinical manifestations of lymphatic filariasis are lymphedema and elephantiasis, which occur due to lymphatic dysfunction and/or obstruction. Lymphangiectasia and inflammatory reactions around the adult worms are the major components of LF disease. The adult filarial parasite within the lymphatic vessels of the host stimulates series of events, which affects the lymphatic integrity and function. The cells and the tissues present in the host provide the developmental signal to the parasites [38]. The lymphatic vascular system plays an important role in immune surveillance, tissue fluid homeostasis, and fat absorption [39, 40]. Perturbations in the maintenance and function of the lymphatic system can lead to a variety of pathological disorders, including lymphatic dilation and lymphedema [39, 41].

Vincent et al. first described the role of lymphatic damage in the Brugian animal infection. Normal or nude (lacking T cells) mice were infected with filarial parasites, which cause lymphatic dilatation and alterations in lymphatic flow in mice with acute and chronic inflammation [42]. The lymph-dwelling filariae can induce tissue scarring and fibrosis within and around the lymphatic vessels causing pathology manifested clinically by irreversible lymphedema or elephantiasis [43]. In patent infection, lymphangiectasia develops in the vicinity of adult worm nests [44]. Lymph node from Bancrofti-infected individuals demonstrates the presence of intact adult worms with slight or no associated inflammation [45, 46]. Lymphangiectasia is not constrained to the exact segment of lymphatics where the worms reside [47] suggesting that this process is mediated by soluble products excreted or secreted by the parasite that act on the lymphatic endothelial cells. It is also clear that with the advent of adaptive immunity, the host inflammatory response against the dead or dying worm and the subsequent release of parasite products and inflammatory mediators, a stage of irreversible lymphatic dysfunction ensues [43, 48, 49]. This then manifests clinically as progressive lymphedema. In addition, lymphatic dysfunction has been shown to predispose infected individuals to secondary bacterial and fungal infections and trigger inflammatory reactions in the skin and subcutaneous tissue that accelerates the progression of lymphedema and precipitates the development of elephantiasis [50, 51].

Nude mice with brugian infection show elevated levels of IL-1, IL-6, TNF-α and GM-CSF in lymph fluid [52], suggesting that pro-inflammatory cytokines of innate origin also appear to play an important role in the initiation of pathology in filarial-infected animal models. The importance of pro-inflammatory cytokines, possibly of innate origin, in the pathogenesis of lymphedema, has been further supported by several studies in humans in either the early or late stages or lymphedema. Studies have shown that individuals with chronic lymphatic pathology have elevated levels of C-reactive protein (an acute phase protein, indicating an acute inflammatory response) [53, 54], pro-inflammatory cytokines such as TNF-α, IL-6 and soluble TNF receptor [55, 56], endothelin-1 and IL-2 [57], as well as IL-8, MIP-1α, MIP-1β, MCP-1, TARC and IP-10 [58] in the peripheral circulation. Acute and

chronic manifestations of LF have shown elevated circulating levels of IL-6 and IL-8, while only those with chronic disease manifestations have elevated levels of sTNF receptors [58]. Babu et al. studied cytokine responses to various Toll ligands in patients with filarial disease and suggest an important role of TLR2 and TLR9 mediated induction of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-12 and IL-1β. In addition, activation of both the MAPK and NF-κB pathways was associated in the development of pathology in human lymphatic filariasis [59].

Very limited studies have examined the inflammatory milieu within the affected lymphatics; Olszewski et al. showed that elevated levels of gamma globulins, α-1 acid glycoprotein and IL-1β in the lymph fluid. The presence of circulating immune complexes (ICs), aggregates of antigens, immunoglobulin and complement components is a characteristic feature of human lymphatic filariasis [60]. An elevated level of complement activity is present in asymptomatic persons, while the reduced levels of complement activity in patients with chronic pathologic changes may aggravate disease morbidity [61]. Extra cellular matrix remodelling and progressive fibrosis are features of filarial pathology. Circulating levels of MMPs and TIMPs are increased in the filarial disease process and altered ratios of MMP/TIMP are an important factor in the pathogenesis of tissue fibrosis in filarial lymphatic disease. These levels were correlated with type 2 cytokines—IL-5, IL-13 and TGF-β, which are typically involved in fibrosis [62]. Pro fibrotic factors such as basic fibroblast growth factor (bFGF) and placental growth factor (PIGF) were increased in the filarial lymphedema patients [63].

The endothelium appears to be closely associated with pathogenesis of lymphatic disease and studies targeting the interaction between endothelial cells (vascular or lymphatic) and filarial parasites have been performed. The anatomical changes in the architecture of lymphatics that range from lymphangiectasia and granulomatous responses to the development of collaterals suggests that active lymphatic remodeling involving endothelial cell growth, migration and proliferation is an important feature of early disease [64, 65]. Differentiation of LEC into tube-like networks was found to be associated with significantly increased levels of matrix metalloproteinases (MMPs) and inhibition of their endogenous inhibitors—TIMPs (tissue inhibitors of MMPs) [65]. Other studies have shown that the vascular endothelial growth factor (VEGF) family associated with lymphangiogenesis [66, 67]. Other angiogenic factors such as angiopoietins-1 and -2 are also found at elevated levels in individuals with filarial-induced pathology [54, 63]. A major factor involved in the initiation of the pro-inflammatory response and the increased production of VEGF-A and -C might be the endosymbiont, *Wolbachia*, present in most filarial nematodes (including *W. bancrofti* and the 2 *Brugia* spp.) [66]. Recently, it has been demonstrated that the increased levels of VEGF-C and sVEGF-R3 (observed in lymphedema patients) were reduced following doxycycline treatment (a regimen that eliminates *Wolbachia*) and that there was improvement in lymphedema, suggesting that VEGF-C/VEGF-R3 interactions are the principal mechanism of lymphangiectasia in filarial infections [67]. Elevated levels of VEGF-A and endothelin-1 have been observed in the serum of filarial-infected individuals. VEGF-A has been implicated to play a role in the development of

hydrocele due to its ability to induce increased vascular density, enhance leucocyte adhesion and promote lymphangiogenesis [68].

Another important mechanism of immune activation in chronic infections is the occurrence of microbial translocation with elevations in the circulating levels of circulating microbial products, acute-phase proteins, and the so-called microbial translocation molecules [69]. Microbial translocation across the intestine or across the lymphatics could possibly contribute to inflammation and innate immune activation. Increased circulating levels of LPS (which serves as a marker for microbial translocation) and decreased levels of LPS-binding protein (LBP) are characteristic features of filarial pathology [62]. We have also demonstrated that this process is associated with development of an acute-phase response and the presence of markers of inflammation in plasma—CRP, alpha-2 macroglobulin, serum amyloid protein-A and haptoglobin. Increased serum levels of pro-inflammatory cytokines—IL-1β, IL-12, TNF-α and IL-6 are associated with progressive immune activation in filarial pathology [62]. Since filarial lymphedema is known to be associated with increased bacterial and fungal loads in the lymphatics, we postulate that microbial translocation across the damaged lymphatics in filarial lymphedema is a novel source of immune activation (Fig. 3).

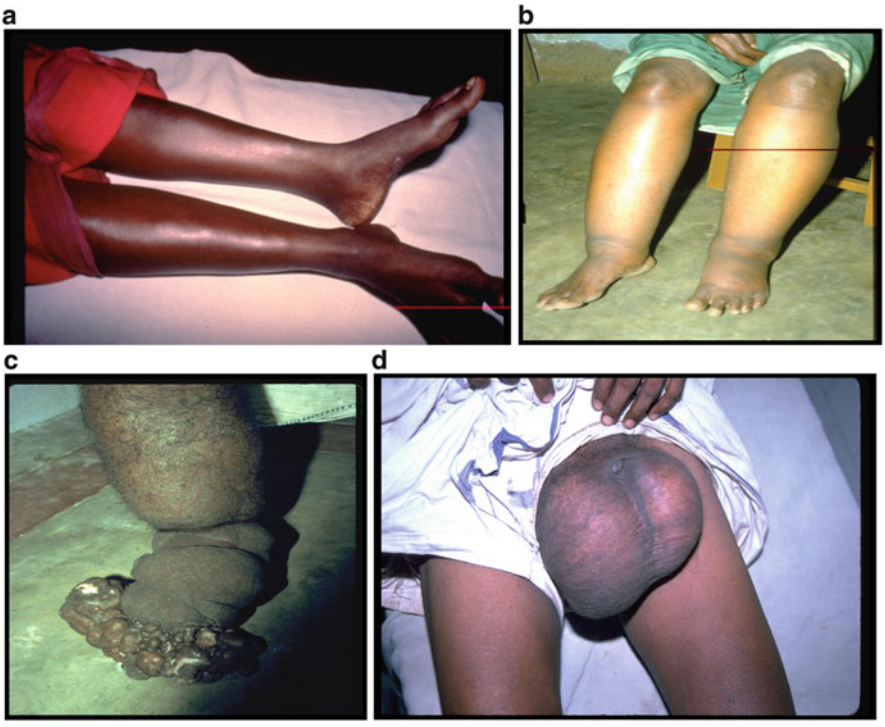

Fig. 3 (**a**) Early stage of lymphedema. (**b**) Advanced stage lymphedema. (**c**) Elephantiasis. (**d**) Hydrocele

There have been a large number of studies that have implicated a role for the adaptive immune systems in mediating pathology in lymphatic filariasis. Parasite antigens specifically down modulate CD4+ Th1 responses; live parasites appear to induce a global down regulation of both Th1 and Th2 responses in vitro [70]. Using multi-color flow cytometry, we have shown that the frequency of Th1 cells (CD4+ T cells expressing either IFNγ or IL-2 or TNF-α); Th9 cells (CD4+ T cells expressing IL-9 and IL-10); Th17 cells (CD4+ T cells expressing IL-17) and Th2 cells (CD4+ T cells expressing IL-22) is significantly enhanced in filarial pathology. This is accompanied by a concomitant decrease in the frequency of Th2 cells (CD4+ T cells expressing IL-4 or IL-5 or IL-13) both at homeostasis and following parasite antigen stimulation [71–75]. Th17 cells might also have an important role in the pathogenesis of disease. Since PBMC's from individuals with pathology (but not asymptomatic patients) express significantly higher levels of Th17 markers—IL-17A, IL-17F, IL-21 and IL-23 as well as the master transcription factor—RORC at the mRNA level [76]. Filarial pathology is also associated with increased frequencies of Th9 cells, CD4+ T cells that express both IL-9 and IL-10 but not IL-4 and this frequency exhibits a positive correlation with the severity of lymphedema in filarial infections [71]. A recent study revealed an important association between the expansion of Th17 and Th22 cells and the presence of lymphedema in filarial infections, and demonstrated that parasite specific inflammatory responses are driven by IL-1, IL-23, and TGF-β [75]. IL-10 family of cytokines play an important role in maintaining the integrity and homeostasis of tissues, modulating innate immune responses from tissues to limit the damage caused by viral and bacterial infections and enabling wound healing processes in infection and inflammation [77, 78]. IL-10 is known to play an important role in dampening inflammation. Among the IL-10 family of cytokines, IL-19 and IL-24 are associated with the regulation of immune responses in active filarial infection and potentially with protection against development of pathology, whereas IL-26 is predominantly associated with pathology in lymphatic filariasis [74]. Therefore, immunopathology in lymphatic filariasis appears to be mostly associated with poor regulation of effector CD4+ and CD8+ T cells that can release pro-inflammatory Th1, Th9 and Th17 type immune responses. How, these pro-inflammatory Th1, Th9 and Th17 cells interact with innate cells, endothelial cells and other target cells to initiate and propagate lymphatic damage and tissue fibrosis remains to be determined.

5 Clinical Features/Clinical Manifestations

The clinical manifestations of LF are varied, traditionally it has been accepted that people living in an endemic area can be classified into five groups: (1) Endemic Normals; (2) clinically asymptomatic, infected; (3) Acute clinical disease; (4) Chronic pathology and (5) Tropical pulmonary eosinophilia (TPE).

5.1 Endemic Normals

In an endemic area, a proportion of the population remains uninfected despite exposure to the parasite. This group has been termed as endemic normals.

5.2 Asymptomatic Patent Infection

In areas endemic for lymphatic filariasis, many individuals exhibit no symptoms of filarial infection and yet, on routine blood examinations, demonstrate the presence of significant numbers of parasites or the presence of circulating parasite antigen (a surrogate for viable adult worms). These individuals are carriers of infection. With the availability of better imaging techniques (e.g., ultrasound, lymphoscintigraphy, MRI, CT), it has become apparent that almost everyone with active infection (e.g., microfilarial positivity) has some degree of lymphatic abnormality that may include: dilatation and tortuosity of lymph vessels with collateralization, increased or abnormal patterns of lymph flow [79, 80] and urogenital lymphangiectasia [81] and microscopic hematuria and/or proteinuria [82]. At least half of all patients with lymphatic filariasis appear clinically asymptomatic. This asymptomatic presentation exists despite the presence of microfilariae in their blood and hidden damage to their lymphatics [83].

5.3 Acute Clinical Disease

Acute manifestations of lymphatic filariasis are periodic attacks of lymphadenitis and lymphangitis (pain in the affected part, tender red streaks) beside with fever, chills, headache and malaise. Over 90% of cases with chronic manifestations will give a history of acute attacks. During acute infection the microfilariae, larval forms and are transmitted by mosquitoes of various species. Occasionally the adult worms and their associated granulomatous reaction are showed as lumps in the subcutaneous tissue, breasts or testicles [83–85]. Acute filariasis is described by episodic incidence of inflammation in the lymph glands (lymphadenitis), inflammation of the lymph channels (lymphangitis) and subsequent swelling of the limbs or scrotum (lymphedema). Filarial fever is often seen with headache and chills, and will usually occur at the same time as lymphangitis. The lymph nodes commonly involved are the inguinal, axillary and epitrochlear nodes and, in addition, the lymphatic systems of the male genitals are frequently affected in *W. bancrofti* infection leading to funiculitis, epididymitis and/or orchitis [86]. The funiculo-epididymo-orchitis, lymphadenitis and retrograde lymphangitis has been termed acute dermatotolymphangitis, a process characterized by development of cutaneous or sub-cutaneous inflammation and accompanied by ascending lymphangitis and regional lymphadenitis. This manifestation is thought to result primarily from bacterial and fungal superinfections of the affected limbs [82].

Lymphadenitis and lymphangitis are distinctive features of *W. bancrofti* and *B. malayi* [87]. Lymphadenitis patients have the worm inside the lymph nodes of the body that induces immune reaction and inflammation. Adult worms block the lymph vessels and distrupt the lymphflow of the lymphatics and leads to the inflammation of lymph vessels. Inflammation of the lymph channels and lymph nodes along with a decreased draining efficiency leads to lymphedema. Lymphatic extremities areas are painful and the skin on the arms and legs may show red streaks from the infected lymphatics. The distal end of the affected limb becomes swollen during the attack and remains swollen for several days. Usually the swelling is initially limited to a single limb.

5.4 Chronic Pathology

The chronic pathology of lymphatic filariasis develops years after initial infection [88]. The most commonly affected nodes are in the femoral and epitrochlear regions. Abscess formation may occur at the nodes or anywhere along the distal vessel. Infection with *B. timori* appears to result in more abscesses than infection with *B. malayi* [89] or *W. bancrofti* [87]. The granulomas are characterized by macrophages (which develop into giant cells), with plasma cells, eosinophils, neutrophils and lymphocytes and with hyperplasia of the lymphatic endothelium, occur with repeated inflammatory episodes. The result is lymphatic damage and chronic leakage of protein-rich lymph in the tissues, thickening and verrucous changes of the skin, and chronic bacterial and fungal infections, which all contribute to the appearance of elephantiasis. *B. malayi* elephantiasis is more likely to affect the upper and lower limbs, with genital pathology and chyluria being rare. Progression of lymphoedema from one stage to the next in bancroftian filariasis is related with increased number of ADL attacks [90].

Stages of Lymphedema of the Leg (Stage I)

- Pitting edema, reversible on elevation of the affected limb
- Skin folds-Absent
- Appearance of Skin-Smooth, Normal

Stage II

- Pitting edema, does not reversible on elevation of the affected limb
- Skin folds-Absent
- Appearance of skin-Smooth, Normal

Stage III

- Non-pitting edema
- Swelling not reversible
- Skin folds-Shallow
- Appearance of skin-Smooth, Normal

Stage IV

- Non-pitting edema
- Swelling not reversible
- Skin folds-Shallow
- Appearance of skin—Irregular, Knobs, Nodules

Stage V

- Swelling not reversible at night
- Skin folds-Deep
- Appearance of skin—Smooth or Irregular

Stage VI

- Swelling not reversible at night
- Skin folds-Absent, Shallow, Deep
- Appearance of skin—Wart-like lesions on foot or top of the toes

Stage VII

- Swelling not reversible at night
- Skin folds-Deep
- Appearance of skin-Irregular
- Needs help for daily activities—Walking, bathing, using bathrooms, dependent on family or health care systems

Lymph scrotum is a less frequent manifestation of LF; it has important medical, psychological and socioeconomic repercussions for individuals who present with this condition. LF creates the greatest incapacitation among men [91]. Lymph scrotum or superficial scrotal lymphangiomatosis is a urogenital condition described by the presence of lymphatic vesicles on the surface of the scrotal skin that can easily rupture whitish secretion typical of the disease [91–93]. This secretion can act as culturing medium that support the recurrent bacterial infections. It may initiate the condition leads to lymphedema and scrotal elephantiasis, the advanced stages of the disease.

5.5 Tropical Pulmonary Eosinophilia

Tropical pulmonary eosinophilia (TPE) is a distinctive syndrome that patients infected with *W. bancrofti* and *B. malayi* [94, 95]. Tropical pulmonary eosinophilia is an extreme immune response to filarial infection. Features of TPE are high eosinophilia levels, asthma-like symptoms and restrictive lung disease, TPE observed in low frequency in endemic areas. Chest X-rays may be normal but generally show increased bronchovascular markings; diffuse miliary lesions or mottled opacities may be present in the middle and lower lung fields. Total serum

IgE levels (10,000–100,000 ng/mL) and antifilarial antibody titers are typically elevated.

6 Diagnosis and Treatment

6.1 Diagnosis of Lymphatic Filariasis

Diagnosis of LF was once an extremely challenging task but with the advent of recent antigen-detection techniques, such as ICT and FST card test and ELISA-based on the Og4C3 monoclonal antibody, diagnosis has become much easier. Molecular xenomonitoring (MX), which detects filarial DNA in mosquitoes by PCR, is a highly sensitive assay. Ultrasonography (USG) and lymphoscintigraphy also revolutionized the diagnosis of the disease and may be very helpful in monitoring the success of chemotherapy. In TPE, serum antibodies like IgG and IgE will be extremely high and the presence of IgG4 antibodies indicate active infection. In brief, here we have explained a few techniques used in diagnosis and monitoring.

1. New techniques for antigen detection represent the highest quality lab test for diagnosing infection by *W. bancrofti*. Very high levels of specific IgG4 antibody in microfilaraemic patients have also been considered as a good diagnostic marker.
2. PCR tests are also of high specificity and sensitivity, and detect parasite DNA in microfilariae in the blood in humans as well as in vectors in both bancroftian and brugian filariasis [96, 97]. Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), microsatellite marker method, Luminex xMAP-based technology (areas of multianalyte profiling), loop-mediated isothermal amplification (LAMP) have all been tested for molecular diagnosis of LF [98–100].
3. Immunochromatographic test (ICT) and Filaria Strip Test (FST), which are highly sensitive and specific filarial antigen detection assays, are available for the diagnosis of *W. bancrofti* infection [101, 102]. With these tests, the parasite antigens can be detected independent of the microfilariae's periodicity. It is rapid (1–10 min), and no such test exists for Brugian filariasis. ELISA-based assay using the Og4C3 monoclonal antibody is equally sensitive and specific for detecting antigen in bancroftian infections.
4. Basic parasitologic testing of peripheral blood for microfilariae remains a diagnostic standby, keeping the periodicity of the microfilariae in mind [97].
5. Ultrasonography using a 7.5 or 10 MHz probe has helped to locate and visualize the movements of living adult filarial worms of *W. bancrofti* principally in the scrotal lymphatics of asymptomatic males with microfilaraemia [47, 103–105].
6. Lymphoscintigraphy has been found useful in tracing lymphatic damage, dermal backflow after injecting radiolabeled proteins intradermally in both symptomatic and asymptomatic infections [79].

6.2 Treatment for Lymphatic Filariasis

6.2.1 Drugs for Lymphatic Filariasis

Remarkable advances in the treatment of LF have recently been achieved focusing not on individuals but on communities with infection, with the goal of reducing mf in the community, to levels below which successful transmission will not occur.

Drugs effective against filarial parasites

1. Diethyl Carbamazine citrate (DEC)
2. DEC-Fortified salt
3. Ivermectin
4. Albendazole
5. Levamisole hydrochloride
6. Moxidectin

Treatment of microfilaraemic patients may prevent transmission of infection and may be repeated every 6 months till mf and/or symptoms disappears. Albendazole (ALB) is a broad-spectrum anthelminthic drug against nematodes and cestodes that inhibits the polymerization of worm β-tubulin and microtubule formation [106]. Diethlycarbamazine attacks filarial parasites at all stages of the parasite life cycle. DEC is the most effective drug for human filarial infection. Studies have suggested that DEC has an indirect, host-mediated mode of action along with anti-inflammatory effects during treatments, inhibit the cyclooxygenase pathway (COX) and lipoxygenase pathways of parasites causing microfilariae death, and the drug inhibits nuclear transcription factor kappa B (NF-kB) activation, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2). DEC alters the host arachidonic acid and nitric oxide metabolic pathways [107–110]. Ivermectin a broad-spectrum anti filarial drug, which can paralysis MFs by interrelates with postsynaptic glutamate-gated chloride channels (GluCl). Its mechanism of action is thought to involve the activation of GABA (gamma-aminobutyric acid) pathways and chloride channel permeability. Ivermectin block the GluCl channels in nematodes, there by prevents the release of uterine microfilariae and immobilizes the skin and ocular microfilariae. Microfilariae are like this transported to the local lymph nodes, and effector cells kill the immobilized larvae [111].

6.3 Treatment and Prevention of ADL

The most distressing aspect of LF is the acute attacks of ADL, which result in considerable economic loss and deterioration of quality of life. In patients with late stages of oedema, long term antibiotic therapy using oral Penicillin or long acting parentral Benzathil Penicillin are sometimes used to prevent ADL.

6.3.1 Surgical Treatment

In the surgical aspect of lymphatic filariasis, grade I and grade II can be treated conservatively, whereas grade III and grade IV needs surgical correction together with regular antibiotics and chemotherapy with DEC. The old surgical techniques of excision and skin grafting is no more practiced as it gives poor cosmetic results, along with early recurrences. Thompson's, kondolean and Charles procedures are now given up and now more of newer techniques involving microvascular surgery like nodovenal shunt, lymphovenal shunt, with reduction and sculpturing is being carried out without skin grafting or a flap cover. Patient's local skin has been salvaged and made to a better quality by manual lymph drainage (MLD), bandaging and use the same skin for reconstitution [112]. The future of lymphedema surgery will be supra microvascular surgery like lymphatico-venus anastomosis at multiple levels or a free microvascular lymph node transfer to the affected areas. The anastomosis can be performed either by glue or by laser.

Chronic hydrocele is the accumulation of fluid around the testis leading to an increase in the volume of the scrotal contents. Chronic hydrocele has multiple etiologies, but irrespective of the cause, surgery is the standard form of treatment and this can be done using different surgical techniques. The prevalence of chronic hydrocele in filarial endemic areas is very high and represents the most common clinical manifestation. The surgeons' preference is for surgical techniques in which the hydrocele sac is opened, averted with or without partial resection of the sac, and the edges sutured behind the testis. To avoid hydrocele relapse, earlier recommendation was to a complete excision of hydrocele sac [81].

The Global Programme to Eliminate Lymphatic Filariasis (GPELF) is established to eliminate LF by interrupting transmission through mass drug administration, and morbidity control of lymphedema. The GPELF targets to provide access to a minimum package of care for every person associated chronic manifestations of lymphatic filariasis, thus reducing suffering and improving their quality of life. (http://www.who.int.ezproxy.nihlibrary.nih.gov/lymphatic_filariasis/disease/en/).

GPELF components includes

- Treatment for episodes of adenolymphangitis (ADL);
- Guidance in applying simple measures to manage lymphedema and hydrocele to prevent progression of lymphedema and debilitating, inflammatory episodes of ADL
- Surgery for hydrocele
- Treatment with anti-filarial medicines to destroy any remaining worms and microfilariae by preventive chemotherapy or individual treatment.
- Doxycycline for early stage lymphedema.

6.3.2 Washing

Good hygiene and treatment of entry lesions are important measures for managing lymphedema. Washing is a key component of lymphedema management, was outlined as careful washing, wiping and drying of the area affected by lymphedema.

- Four times a day with soap and water, wash the deep skin folds and the areas between the toes, followed by careful drying [44].
- Wet the leg with clean water at room temperature. Begin soaping at the highest point of swelling (usually around the knee).
- Repeat this careful washing until the rinse water is clean, and wash the other leg in the same way, even if it looks normal.

Check Skin for

- Entry lesions between the toes may cause itching. Avoid scratching to prevent further damage of the skin and can provoke an acute attack
- Toenails should be trimmed in such a way that the skin is not injured. Does not use sharp objects under nails to clean as these can cause entry lesions
- It is important to make sure to clean the skin every time when the leg is washed, since bacteria enters through the skin which causes entry lesions
- If entry lesions are found, they should be cleaned carefully

Dry the Skin

- Touch the area lightly with a clean towel.
- Carefully dry between the toes and between skin folds using a small cloth, gauze or cotton swab.
- Wet areas between the toes, skin folds and entry lesions promote bacterial and fungal growth leading to frequent acute attacks.

6.3.3 Elevation

Elevation helps avoid fluid accumulation in the leg by improving the flow. The knee should be slightly bent and a pillow placed under the knee for support. While sitting, elevate the foot as high as comfortable, preferably till the hip.

6.3.4 Exercise

- Exercise is useful for patients with lymphedema and in general, the more they exercise the better they are. Exercise supports by pumping the fluid and improving drainage. Though, patients should not exercise during acute attacks
- Walking short distances, standing up on toes, exercise, Stand with both feet slightly apart, holding on to a wall, a person or other support

- Rise on to the toes of both feet at the same time and then sink back down to flat feet, while sitting or lying down, point toes towards the floor
- Bend (extend) the toes upwards, while sitting move the foot in a circle to the right and to the left

6.3.5 Wearing Proper Footwear

Proper footwear protects feet from injury.

Other treatment options also provide relief and help to prevent further swelling.

- Wearing elasto-crepe bandage or stockings while traveling
- Elevation of the limb at night, after the bandage removal
- Exercising regularly of the affected limb
- Daily light massage of the limb especially in early oedema, to stimulate the lymphatics and to enhance the lymph flow towards larger patent vessels
- Heat therapy using either wet heat or hot ovens [113–116]

7 Immune Responses to Lymphatic Filariasis

Extensive studies have been conducted on human immune responses to filarial parasites. The host immune mechanisms to kill filarial parasite, are currently not well understood, and remain subtle. A key component of the immune system that has evolved to diminish the virulence of helminths is the type 2 (or T_H2) response [117]. The host immune response to filarial parasites is of T helper type 2 responses and characterized by the increased the cytokine production of IL-4, IL-5, IL-9, IL-10 and IL-13, the antibody isotypes—IgE and IgG4, and increased activation of accessory cells such as basophils, eosinophils, and mast cells [118]. Along with Th2 responses, regulatory T cells [119], alternatively activated (or M2) macrophages [120] and innate lymphoid cells are the key components involve during helminth infection. Chronic filarial infection exhibits a modified Th2 response and increased IL-10 reviewed by [121].

One of the major hallmarks of filarial infection is the elevated level of eosinophils and IgE following infection [122]. Most of the IgE produced is due to non-antigen specific stimulation of IgE producing B cells. IgE antibodies persist many years after the infection has been treated, indicating the presence of long-lived memory B cells or plasma cells in filarial infection [123]. IgG4 and IgG1 are elevated in chronic filarial infected individuals, IgG4 production depends on IL-4 and IL-10 [124]. IgE production both in mice and humans is absolutely dependent on IL-4 or IL-13 [125].

During early infection, innate immune cells such as eosinophils and neutrophils are recruited to the site of infection to activate the innate immune mechanism. The role of granulocytes in filariasis appears to be diverse. Either they promote protective immunity or help parasite establishment. During filarial infection, peripheral eosinophil counts may reach up to 75%, which can provoke tropical pulmonary eosinophil (TPE) in infected individuals [126]. During migration, the larva induces the cell degranulation in turn it binds to TLR receptors and activates the TLR dependent antibody dependent cell cytotoxicity (ADCC). Engagement of Fc receptors (FcR) results in the recruitment of innate immune cells, mainly eosinophils, macrophages and neutrophils. These cells release toxic granules after activation, which leads to parasite killing [127–129]. Release of granular proteins, such as ribonuclease (RNASE 2 and RNASE3), Eosinophil Cationic Protein (ECP), Major Basic Protein (MBP) and Eosinophil Peroxidase (EPO) by activated eosinophils imparts protective immunity. Knockout of EPO and MBP protein molecules from mice have showed that, in the absence of eosinophils together with its secretory granules, worms grow faster. This suggests that eosinophils play a role in Mf clearance during filarial infections [129]. Activated macrophages or granulocytes can release damaging nitrogen intermediates as well nitric oxide onto the surface of the parasites [38]. Basophils play a possible role in the initiation of Th2 immune response since they readily produce IL-4 with or without dependence of IgE [130]. Depletion of basophils in mice with chronic filarial infection showed a dramatic decrease in eosinophils and CD4+ T cell proliferation [131].

Macrophages are activated during filarial infection and characterized by enhanced expression of arginase 1, the secreted chitinase-like lectin Ym-1, resistin-like molecule α ((RELMα), mannose receptor C type 1 (MRC-1), macrophage galactose type C lectin (MGL) and chemokine ligand 18 (CCL18) [132]. Alternatively activated macrophages play a prominent role in wound healing and are thought to help limit tissue immunopathology [118]. Alternatively activated macrophages secrete cytokines, which regulate immune responses and support the survival of filarial nematodes via the release of regulatory cytokines IL-10, programmed cell death 1 ligand 2 (PDL2), and transforming growth factor (TGF)-β as a result leading to immunosuppression [118]. Live filarial parasites produce a monocyte phenotype that partly resembles the alternatively activated state seen in infected individuals [76]. Among the monocyte subsets, classical monocytes are capable in antigen-uptake in filarial infections [133]. A recent study showed that alveolar macrophages involved in the pathogenesis of TPE [134].

CD4+ helper T cells form the majority of T lymphocyte responses. T cells play a critical role in filarial immunity in both animal and human models. Animal studies showed that T cell deficient mice are vulnerable to filarial infection, demonstrating that T cells are important for elimination of infection [135, 136]. Upon antigenic exposure, naïve T cells are activated and differentiate into different effector Th1, Th2, Th17 and regulatory T cell subsets depending on the source of antigen and cytokine environment. T helper cells are controlled by T-bet (Th1), GATA-3 (Th2), RORγT (Th17) and Foxp3 (Tregs), respectively [137]. Lymphatic filarial disease is known to be associated with elevated Th1 responses and normal or diminished Th2

responses to parasite-specific antigens. Filarial Mf+ infected individuals exhibit T cell hypo-responsiveness and decreased production of IFN-γ and IL-2 [138].

During filarial infection, increased production of Foxp3+ Treg cells promotes the survival of filarial worm. Treg depletion resulted in decreased worm burden and enhanced immune mediated pathology [119]. Induction of Foxp3+ Treg, stimulate the differentiation and up-regulation of arginase-1 activity mediated by alternatively activated macrophages during filarial infection and plays an important role in immune regulation and tissue repair [120, 139]. Regulatory T cells have been proposed to play a role in the immune evasion mechanism during parasitic infection. IL-10 and TGFβ are produced in response to filarial infections and associated with regulatory mechanisms. Effector T cell responses can be modulated through by CTLA-4 and PD-1. Increased expression of CTLA-4 and PD-1 has been observed in human filarial infections, and blocking of CTLA-4 can restore partially a degree of immunological responsiveness in infected individuals [70, 140, 141]. - Filaria-associated Tregs were shown to be functional in suppressing proliferation and possibly Th2 cytokine responses to filarial specific antigen in infected but not in chronic pathology individuals [142]. Suppression of T cell functions in lymphatic filariasis is caused by microfilaria-modulated monocytes in an IL-10-dependent manner. Suppression of macrophages may contribute to the overall down-regulation of immune responses in infected patients [143].

8 Prevention and Control

In 1997 World Health Assembly (WHA) framed resolution and insisting all endemic countries to increase their efforts and determination to control and eliminate LF. In response, the WHO in 2000, the Global Program to Eliminate Lymphatic Filariasis (GPELF) was launched with the goal to eliminate the disease as a public health problem by 2020 by stopping the spread of infection through mass drug administration and alleviating suffering by managing morbidity and preventing disability. Even though significant progress has been made in the elimination of lymphatic filariasis, 18 countries completing interventions and on track to validate elimination (www.who.int/lymphatic_filariasis/global_progress/en/). The MDA approach is to give repeated, annual doses of albendazole (ALB) in combination with either diethylcarbamazine (DEC) or ivermectin (IVM) for the lifetime of adult worms (typically 5–7 years) [144]. In moderate and high endemic areas to accomplish the elimination of LF, based on mathematical models of LF transmission, the elimination will require high compliance (>70%) with MDA for 5–7 years with most potent drug combination (annual DEC + ALB [145]. El Setouhy et al. reported that microfilaricidal and macrofilaricidal effects are higher with ALB + DEC at 1 year for multiple doses [146]. ALB + DEC and ALB + IVM—strongly reduce the LF infection levels [147]. Community based study conducted in south India suggest that, single-dose combination (DEC + ALB)

mass treatment has an increased the effect against bancroftian filariasis in comparison to single-drug treatment [148].

The number of treatments varied from 2 to 12 and in most of the studies treatments were given at yearly or half-yearly interval dose of DEC + ivermectin [11] and ivermectin alone [149]. The Sri Lankan study showed nearly all regimens with IVM established a rapid kill rate of microfilaria with higher doses showing a greater diminution in microfilaria rates [147]. Bockarie et al. reported that either the combination of DEC and IVM or DEC alone, after four rounds of treatment (77–86% compliance rate), lymphoedema and hydrocoele were also significantly reduced in the population and the positivity of Mf infection were deceased by 86–98% [11]. Currently there are three regimens permitted for MDA, that is, DEC with ALB, IVM with ALB, and DEC-medicated salt (GAELF, "Progress to date," June 2009, http://www.filariasis.org/all_about_lf/disease.html#clinicalfeatures). Microfilaricide IVM to DEC + ALB may enhance microfilaria clearance and afford a more long-lasting effect than the 2-drug regimen. DEC-medicated cooking salt medicated with DEC has been used to aid mass treatment and has proved to be very effective and safe. DEC fortified salt has the ability to clear microfilaraemia without any adverse reactions since it is recommended for control programmes. A study from South India, after 10 years of annual MDA, on the efficiency of DEC (6 mg/kg, single dose) and IVM (400 μg/kg, single dose) has shown that DEC had the potential to interrupt the transmission of filariasis when compared with IVM [150]. Simulation modelling suggests that the triple-drug course of therapy has possible to speed up the eradication of lymphatic filariasis if high population treatment of mass drug administration can be achieved and if systematic non-adherence with mass drug administration is low [151].

The Global Program to Eliminate Lymphatic Filariasis (GPELF) recently released their progress report for 2014 [4]. The report summarized the work of the GPELF's first decade, which was focused on implementing mass drug administration (MDA) across all LF endemic regions. The report acknowledged that although MDA programme have been particularly successful in reducing infection within communities, efforts to reduce morbidity associated with LF remain lacking. Currently, only 24 of the 73 endemic countries have morbidity programs [9]. During 2000–2012, the MDA programme made remarkable achievements—a total of 6.37 billion treatments were offered and an estimated 4.45 billion treatments were consumed by the population living in endemic areas. Using a model based on empirical observations of the effects of treatment on clinical manifestations, it is estimated that 96.71 million LF cases, including 79.20 million microfilaria carriers, 18.73 million hydrocele cases and a minimum of 5.49 million lymphedema cases have been prevented or cured during this period. Consequently, the global prevalence of LF is calculated to have fallen by 59%, from 3.55% to 1.47%. The fall was highest for microfilaraemia prevalence (68%), followed by 49% in hydrocele prevalence and 25% in lymphedema prevalence. These programs focus on hygiene, skin care, hydrocele surgery, and exercises [152]. The GPELF plan for 2010–2020 highlights the need for the establishment of morbidity management programs in all endemic regions. In particular, the plan identifies the need for the development of metrics to monitor and report on the outcomes of these programs [9].

8.1 Vector Control

Vector-control strategies in the last century were based on chemical agents, current ecological and environmental protection standards do not allow such approaches because of the adverse effects of many insecticides on non-target species, including humans, their environmental impact, the contamination of soil and water and the development of selective processes and subsequent mosquito resistance to insecticides [153]. New strategies therefore had to be created to replace the use of insecticides. Genetic control methods have now arisen as promising alternative strategies, based on two approaches: the replacement of a vector population by disease-refractory mosquitoes and the release of mosquitoes carrying a lethal gene to suppress target populations. Genetically modified bacteria capable of colonizing a wide range of mosquito species may be a solution to this problem and another option for the control of these diseases. The paratransgenic approach, symbiotic bacteria are genetically modified and reintroduced in mosquitoes, where they express effector molecules. In this approach, genetic modified bacteria can act by: (a) causing pathogenic effects in the host; (b) interfering with the host's reproduction; (c) reducing the vector's competence; and (d) interfering with oogenesis and embryogenesis [102, 154].

8.2 Self Help Group or Social Helping Groups

Effective self-care implementation requires some degree of education, instruction or demonstration and the role of the educated health worker or trained volunteer cannot be ignored. Studies, which provided frequent monitoring, and support were associated with greater improvements than studies, which offered minimal, or no support services. A study by Akogun et al. [155], where one group was able to alter the program design to suit their immediate cultural and social constraints, reported good outcomes. A study by Wilson et al. [156] showed that basic self-care improved skin integrity and prevented new infections while limb stage remained the same. While a reduction in limb volume was reported in lymphedema, greater benefits were experienced among participants with early stages, suggesting that implementation of a self-care routine as soon as lymphedema is detected has the potential to curtail the number of cases that progress to advanced stages. A study by Bernhard et al. revealed that limb volume reduction was significantly greater in the self-treating group compared to the therapist treated group [157]. A review by Douglass et al. has supported the adoption of remedial exercises in the management of lymphedema and a greater emphasis on self-treatment practices for people with lymphedema [113].

8.3 Tools for Assessment of Elimination

Lymphatic filariasis (LF) is targeted for global elimination through treatment of entire at-risk populations with repeated annual mass drug administration (MDA). The end point of MDA can be assessed by transmission assessment survey (TAS), when transmission is reached accepted low level that it cannot be determined even in the absence of drug intervention and consequently MDA can be stopped. The decision to stop MDA is intricate and arrays of tools have been recommended to guide the decision. The first step is to define the parameter(s) that will be measured and the best diagnostic tool for assessing it. Multi center study was conducted for the evaluation of potential diagnostic tools for TAS [158]. In *W. bancrofti* and *Brugia* spp. endemic areas, the immunochromatographic (ICT) to test filarial antigen and the BmR1 antibody test (PanLF or Brugia Rapid) were recommended for TAS.

ICT Sensitive test to detect *W. bancrofti* antigen. Quick results can be obtained and it does not require laboratory equipment. Positive result indicates the presence of adult worm antigen. The rapid immunochromatographic test (ICT), detecting filarial antigen, is the selected tool for deciding when to stop MDA [158].

Brugia Rapid™ test Able to detect antibodies of *B. malayi* and *B. timori* and a more sensitive test. Quick results can be obtained and it does not require laboratory equipment. Positive result indicates the presence of anti filarial antibodies [159].

PanLF: BmR1 recombinant filarial antigen used to measure the antibodies in *Brugia* spp. Based on multi centric study it has similar sensitivity in all the countries [160, 161].

Bm14 and SXP Antibody detection tests like Bm14, BmR1, and Wb-SXP are useful tools which help program managers to decide in the perspective of filariasis elimination programs [162]. SXP and Bm14 have been developed as candidates for diagnostic assay. Bm14 has sensitivities of 85–90% to serum from mf carriers [101]. This antigen is equally sensitive for *B. malayi* or *W. bancrofti* sera [163].

Polymerase chain reaction (PCR) PCR assays are highly sensitive, rapidity and cost-effectiveness and specific for the detection of *Wb* DNA in individual human blood [164] and in mosquito [165]. qPCR pool-screening assay could be used in the population to scale filarial eradication monitoring and assessment survey [166].

Og4c3: The initiation of the filarial elimination programme has necessitated the use of highly sensitive and specific diagnostic tests. The Og4C3 monoclonal antibody-based ELISA test is highly specific and sensitive for the diagnosis of filariasis [167].

9 Conclusion

In this review, we have focused on the epidemiology of LF, clinical manifestations, pathogenesis of lymphatic filariasis and immune response, current strategy for control and treatment. In this review, while we may not have discussed all the mechanisms involved, we have endeavored to explain the role of major immune components and mechanism of killing parasite during infection. Complete elimination of parasites is rarely achieved apparently since sterilizing immunity might necessitate host deleterious immune responses. Therefore, immune-mediated pathology is often associated with disease manifestation in filarial infections. Rarely, due to a failure in an immune system-parasite homeostatic balance, the effects are of an overwhelming nature, due to dynamic host immune responses in the course of filarial infection. Filariasis continues to be one of the most debilitating diseases in the world despite the WHO's efforts to administration of mass drug administration and considerable success in reducing filarial incidence. Mosquito control is a supplemental strategy supported by WHO to reduce the transmission of lymphatic filariasis and other mosquito-borne infections. Depending on the parasite-vector species, measures such as insecticide-treated nets, indoor residual spraying or personal protection measures may help protect people from infection. Vector control has in select settings contributed to the elimination of lymphatic filariasis in the absence of large-scale preventive chemotherapy.

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References

1. Ottesen EA, Duke BO, Karam M, Behbehani K. Strategies and tools for the control/elimination of lymphatic filariasis. Bull World Health Organ. 1997;75(6):491–503.
2. Fenwick A. The global burden of neglected tropical diseases. Public Health. 2012;126(3): 233–6. https://doi.org/10.1016/j.puhe.2011.11.015.
3. Ramaiah KD, Ottesen EA. Progress and impact of 13 years of the global programme to eliminate lymphatic filariasis on reducing the burden of filarial disease. PLoS Negl Trop Dis. 2014;8(11):e3319. https://doi.org/10.1371/journal.pntd.0003319.
4. WHO. Global programme to eliminate lymphatic filariasis: progress report, 2015. Wkly Epidemiol Rec. 2016;39:441–60.
5. Dhariwal AC, Srivastava PK, Bhattacharjee J. J Indian Med Assoc. December 2015;113(12).
6. WHO (World Health Organization). Global programme to eliminate lymphatic filariasis: Wkly Epidemiol Rec. 2013;89:409–420.
7. Sabesan S, Raju KH, Subramanian S, Srivastava PK, Jambulingam P. Lymphatic filariasis transmission risk map of India, based on a geo-environmental risk model. Vector Borne Zoonotic Dis. 2013;13(9):657–65. https://doi.org/10.1089/vbz.2012.1238.
8. Hossain M. Elimination of lymphatic filariasis from bangladesh: current status. J Sci Found. 2016;14(1.) ISSN 1728-7855

9. Department of Health Services (DHS), Government of Nepal. Annual report F/Y 2071/72.
10. Sasa M. Human filariasis: a global survey of epidemiology and control. University of Tokyo Press; Japan: 1976. Filariasis due to *Wuchereria* and *Brugia*.
11. Bockarie MJ, Pedersen EM, White GB, Michael E. Role of vector control in the global program to eliminate lymphatic filariasis. Annu Rev Entomol. 2009;54:469–87. https://doi.org/10.1146/annurev.ento.54.110807.090626.
12. Dhamodharan R, Das MK, Hoti SL, Das PK, Dash AP. Genetic variability of diurnally sub-periodic Wuchereria bancrofti in Nicobarese tribe of Nicobar group of Islands, Andaman and Nicobar Islands, India. Parasitol Res. 2008;103(1):59–66. https://doi.org/10.1007/s00436-008-0927-2.
13. Pothikasikorn J, Bangs MJ, Boonplueang R, Chareonviriyaphap T. Susceptibility of various mosquitoes of Thailand to nocturnal subperiodic Wuchereria bancrofti. J Vector Ecol. 2008;33(2):313–20.
14. Moulia-Pelat JP, Glaziou P, Chanteau S, Nguyen-Ngoc L, Marcet Y, Gardines R, et al. Periodicity of Wuchereria bancrofti var. pacifica filariasis in French Polynesia. Trop Med Parasitol. 1993;44(2):83–5.
15. Michael E, Bundy DA. Global mapping of lymphatic filariasis. Parasitol Today. 1997;13(12):472–6.
16. Ash LR, Riley JM. Development of subperiodic Brugia malayi in the jird, Meriones unguiculatus, with notes on infections in other rodents. J Parasitol. 1970;56(5):969–73.
17. Keiser PB, Nutman TB. Update on lymphatic filarial infections. Curr Infect Dis Rep. 2002;4(1):65–9.
18. Bain O, Babayan S. Behaviour of filariae: morphological and anatomical signatures of their life style within the arthropod and vertebrate hosts. Filaria J. 2003;2(1):16. https://doi.org/10.1186/1475-2883-2-16.
19. Ghedin E, Wang S, Spiro D, Caler E, Zhao Q, Crabtree J, et al. Draft genome of the filarial nematode parasite Brugia malayi. Science. 2007;317(5845):1756–60. https://doi.org/10.1126/science.1145406.
20. Bennuru S, Meng Z, Ribeiro JM, Semnani RT, Ghedin E, Chan K, et al. Stage-specific proteomic expression patterns of the human filarial parasite Brugia malayi and its endosymbiont Wolbachia. Proc Natl Acad Sci USA. 2011;108(23):9649–54. https://doi.org/10.1073/pnas.1011481108.
21. Darby AC, Gill AC, Armstrong SD, Hartley CS, Xia D, Wastling JM, et al. Integrated transcriptomic and proteomic analysis of the global response of Wolbachia to doxycycline-induced stress. ISME J. 2014;8(4):925–37. https://doi.org/10.1038/ismej.2013.192.
22. Williams SA, Lizotte-Waniewski MR, Foster J, Guiliano D, Daub J, Scott AL, et al. The filarial genome project: analysis of the nuclear, mitochondrial and endosymbiont genomes of Brugia malayi. Int J Parasitol. 2000;30(4):411–9.
23. Sironi M, Bandi C, Sacchi L, Di Sacco B, Damiani G, Genchi C. Molecular evidence for a close relative of the arthropod endosymbiont Wolbachia in a filarial worm. Mol Biochem Parasitol. 1995;74(2):223–7.
24. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008;6(10):741–51. https://doi.org/10.1038/nrmicro1969.
25. Taylor MJ, Bandi C, Hoerauf A. Wolbachia bacterial endosymbionts of filarial nematodes. Adv Parasitol. 2005;60:245–84. https://doi.org/10.1016/S0065-308X(05)60004-8.
26. Taylor MJ, Hoerauf A, Townson S, Slatko BE, Ward SA. Anti-Wolbachia drug discovery and development: safe macrofilaricides for onchocerciasis and lymphatic filariasis. Parasitology. 2014;141(1):119–27. https://doi.org/10.1017/S0031182013001108.
27. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, et al. The Wolbachia genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode. PLoS Biol. 2005;3(4):e121. https://doi.org/10.1371/journal.pbio.0030121.
28. Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, et al. Phylogenomics of the reproductive parasite Wolbachia pipientis wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. 2004;2(3):E69. https://doi.org/10.1371/journal.pbio.0020069.

29. Turner JD, Langley RS, Johnston KL, Gentil K, Ford L, Wu B, et al. Wolbachia lipoprotein stimulates innate and adaptive immunity through toll-like receptors 2 and 6 to induce disease manifestations of filariasis. J Biol Chem. 2009;284(33):22364–78. https://doi.org/10.1074/jbc.M901528200.
30. Suba N, Shiny C, Taylor MJ, Narayanan RB. Brugia malayi Wolbachia hsp60 IgG antibody and isotype reactivity in different clinical groups infected or exposed to human bancroftian lymphatic filariasis. Exp Parasitol. 2007;116(3):291–5. https://doi.org/10.1016/j.exppara.2006.12.006.
31. Small ST, Reimer LJ, Tisch DJ, King CL, Christensen BM, Siba PM, et al. Population genomics of the filarial nematode parasite Wuchereria bancrofti from mosquitoes. Mol Ecol. 2016;25(7):1465–77. https://doi.org/10.1111/mec.13574.
32. Desjardins CA, Cerqueira GC, Goldberg JM, Dunning Hotopp JC, Haas BJ, Zucker J, et al. Genomics of Loa loa, a Wolbachia-free filarial parasite of humans. Nat Genet. 2013;45(5):495–500. https://doi.org/10.1038/ng.2585.
33. Debrah AY, Mand S, Toliat MR, Marfo-Debrekyei Y, Batsa L, Nurnberg P, et al. Plasma vascular endothelial growth factor-A (VEGF-A) and VEGF-A gene polymorphism are associated with hydrocele development in lymphatic filariasis. Am J Trop Med Hyg. 2007;77(4):601–8.
34. Junpee A, Tencomnao T, Sanprasert V, Nuchprayoon S. Association between toll-like receptor 2 (TLR2) polymorphisms and asymptomatic bancroftian filariasis. Parasitol Res. 2010;107(4):807–16. https://doi.org/10.1007/s00436-010-1932-9.
35. Idris ZM, Miswan N, Muhi J, Mohd TA, Kun JF, Noordin R. Association of CTLA4 gene polymorphisms with lymphatic filariasis in an East Malaysian population. Hum Immunol. 2011;72(7):607–12. https://doi.org/10.1016/j.humimm.2011.03.017.
36. Meyrowitsch DW, Simonsen PE, Garred P, Dalgaard M, Magesa SM, Alifrangis M. Association between mannose-binding lectin polymorphisms and Wuchereria bancrofti infection in two communities in North-Eastern Tanzania. Am J Trop Med Hyg. 2010;82(1):115–20. https://doi.org/10.4269/ajtmh.2010.09-0342.
37. Zamanian M, Fraser LM, Agbedanu PN, Harischandra H, Moorhead AR, Day TA, et al. Release of small RNA-containing exosome-like vesicles from the human filarial parasite Brugia malayi. PLoS Negl Trop Dis. 2015;9(9):e0004069. https://doi.org/10.1371/journal.pntd.0004069.
38. Rajan TV. A hypothesis for the tissue specificity of nematode parasites. Exp Parasitol. 1998;89(1):140–2. https://doi.org/10.1006/expr.1998.4264.
39. Witte MH, Bernas MJ, Martin CP, Witte CL. Lymphangiogenesis and lymphangiodysplasia: from molecular to clinical lymphology. Microsc Res Tech. 2001;55(2):122–45. https://doi.org/10.1002/jemt.1163.
40. Takahashi M, Macdonald RL. Vascular aspects of neuroprotection. Neurol Res. 2004;26(8):862–9. https://doi.org/10.1179/016164104X3815.
41. Saaristo A, Karkkainen MJ, Alitalo K. Insights into the molecular pathogenesis and targeted treatment of lymphedema. Ann N Y Acad Sci. 2002;979:94–110.
42. Vincent AL, Vickery AC, Lotz MJ, Desai U. The lymphatic pathology of Brugia pahangi in nude (athymic) and thymic mice C3H/HeN. J Parasitol. 1984;70(1):48–56.
43. Connor DH, Palmieri JR, Gibson DW. Pathogenesis of lymphatic filariasis in man. Z Parasitenkd. 1986;72(1):13–28.
44. Dreyer G, Noroes J, Figueredo-Silva J, Piessens WF. Pathogenesis of lymphatic disease in bancroftian filariasis: a clinical perspective. Parasitol Today. 2000;16(12):544–8.
45. Jungmann P, Figueredo-Silva J, Dreyer G. Bancroftian lymphadenopathy: a histopathologic study of fifty-eight cases from northeastern Brazil. Am J Trop Med Hyg. 1991;45(3):325–31.

46. Dreyer G, Noroes J, Addiss D, Santos A, Medeiros Z, Figueredo-Silva J. Bancroftian filariasis in a paediatric population: an ultrasonographic study. Trans R Soc Trop Med Hyg. 1999; 93(6):633–6.
47. Amaral F, Dreyer G, Figueredo-Silva J, Noroes J, Cavalcanti A, Samico SC, et al. Live adult worms detected by ultrasonography in human Bancroftian filariasis. Am J Trop Med Hyg. 1994;50(6):753–7.
48. Figueredo-Silva J, Noroes J, Cedenho A, Dreyer G. The histopathology of bancroftian filariasis revisited: the role of the adult worm in the lymphatic-vessel disease. Ann Trop Med Parasitol. 2002;96(6):531–41. https://doi.org/10.1179/000349802125001348.
49. von Lichtenberg F. The Wellcome Trust lecture. Inflammatory responses to filarial connective tissue parasites. Parasitology. 1987;94(Suppl):S101–22.
50. Olszewski WL, Jamal S, Manokaran G, Pani S, Kumaraswami V, Kubicka U, et al. Bacteriologic studies of skin, tissue fluid, lymph, and lymph nodes in patients with filarial lymphedema. Am J Trop Med Hyg. 1997;57(1):7–15.
51. Shenoy RK, Kumaraswami V, Suma TK, Rajan K, Radhakuttyamma G. A double-blind, placebo-controlled study of the efficacy of oral penicillin, diethylcarbamazine or local treatment of the affected limb in preventing acute adenolymphangitis in lymphoedema caused by brugian filariasis. Ann Trop Med Parasitol. 1999;93(4):367–77.
52. Rao UR, Vickery AC, Kwa BH, Nayar JK. Regulatory cytokines in the lymphatic pathology of athymic mice infected with Brugia malayi. Int J Parasitol. 1996;26(5):561–5.
53. Lal RB, Dhawan RR, Ramzy RM, Farris RM, Gad AA. C-reactive protein in patients with lymphatic filariasis: increased expression on lymphocytes in chronic lymphatic obstruction. J Clin Immunol. 1991;11(1):46–53.
54. Anuradha R, George JP, Pavankumar N, Kumaraswami V, Nutman TB, Babu S. Altered circulating levels of matrix metalloproteinases and inhibitors associated with elevated type 2 cytokines in lymphatic filarial disease. PLoS Negl Trop Dis. 2012;6(6):e1681. https://doi.org/10.1371/journal.pntd.0001681.
55. Das BK, Sahoo PK, Ravindran B. A role for tumour necrosis factor-alpha in acute lymphatic filariasis. Parasite Immunol. 1996;18(8):421–4.
56. Satapathy AK, Sartono E, Sahoo PK, Dentener MA, Michael E, Yazdanbakhsh M, et al. Human bancroftian filariasis: immunological markers of morbidity and infection. Microbes Infect. 2006;8(9–10):2414–23. https://doi.org/10.1016/j.micinf.2006.05.003.
57. el-Sharkawy IM, Haseeb AN, Saleh WA. Serum levels of endothelin-1 (ET-1), interleukin-2 (IL-2) and amino-terminal propeptide type III procollagen (PIII NP) in patients with acute and chronic filariasis. J Egypt Soc Parasitol. 2001;31(1):169–76.
58. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Chemokine receptors of T cells and of B cells in lymphatic filarial infection: a role for CCR9 in pathogenesis. J Infect Dis. 2005; 191(6):1018–26. https://doi.org/10.1086/427658.
59. Babu S, Anuradha R, Kumar NP, George PJ, Kumaraswami V, Nutman TB. Toll-like receptor- and filarial antigen-mediated, mitogen-activated protein kinase- and NF-kappaB-dependent regulation of angiogenic growth factors in filarial lymphatic pathology. Infect Immun. 2012;80(7):2509–18. https://doi.org/10.1128/IAI.06179-11.
60. Dixit V, Gupta AK, Bisen PS, Prasad GB, Harinath BC. Serum immune complexes as diagnostic and therapeutic markers in lymphatic filariasis. J Clin Lab Anal. 2007;21(2):114–8. https://doi.org/10.1002/jcla.20116.
61. Senbagavalli P, Anuradha R, Ramanathan VD, Kumaraswami V, Nutman TB, Babu S. Heightened measures of immune complex and complement function and immune complex-mediated granulocyte activation in human lymphatic filariasis. Am J Trop Med Hyg. 2011; 85(1):89–96. https://doi.org/10.4269/ajtmh.2011.11-0086.
62. Anuradha R, George PJ, Pavan Kumar N, Fay MP, Kumaraswami V, Nutman TB, et al. Circulating microbial products and acute phase proteins as markers of pathogenesis in lymphatic filarial disease. PLoS Pathog. 2012;8(6):e1002749. https://doi.org/10.1371/journal.ppat.1002749.

63. Bennuru S, Maldarelli G, Kumaraswami V, Klion AD, Nutman TB. Elevated levels of plasma angiogenic factors are associated with human lymphatic filarial infections. Am J Trop Med Hyg. 2010;83(4):884–90. https://doi.org/10.4269/ajtmh.2010.10-0039.
64. Witte MH, Way DL, Witte CL, Bernas M. Lymphangiogenesis: mechanisms, significance and clinical implications. EXS. 1997;79:65–112.
65. Bennuru S, Nutman TB. Lymphangiogenesis and lymphatic remodeling induced by filarial parasites: implications for pathogenesis. PLoS Pathog. 2009;5(12):e1000688. https://doi.org/10.1371/journal.ppat.1000688.
66. Pfarr KM, Debrah AY, Specht S, Hoerauf A. Filariasis and lymphoedema. Parasite Immunol. 2009;31(11):664–72. https://doi.org/10.1111/j.1365-3024.2009.01133.x.
67. Debrah AY, Mand S, Specht S, Marfo-Debrekyei Y, Batsa L, Pfarr K, et al. Doxycycline reduces plasma VEGF-C/sVEGFR-3 and improves pathology in lymphatic filariasis. PLoS Pathog. 2006;2(9):e92. https://doi.org/10.1371/journal.ppat.0020092.
68. Debrah AY, Mand S, Marfo-Debrekyei Y, Batsa L, Pfarr K, Lawson B, et al. Reduction in levels of plasma vascular endothelial growth factor-A and improvement in hydrocele patients by targeting endosymbiotic Wolbachia sp. in Wuchereria bancrofti with doxycycline. Am J Trop Med Hyg. 2009;80(6):956–63.
69. Brenchley JM, Douek DC. Microbial translocation across the GI tract. Annu Rev Immunol. 2012;30:149–73. https://doi.org/10.1146/annurev-immunol-020711-075001.
70. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Cutting edge: diminished T cell TLR expression and function modulates the immune response in human filarial infection. J Immunol. 2006;176(7):3885–9.
71. Anuradha R, George PJ, Hanna LE, Chandrasekaran V, Kumaran P, Nutman TB, et al. IL-4-, TGF-beta-, and IL-1-dependent expansion of parasite antigen-specific Th9 cells is associated with clinical pathology in human lymphatic filariasis. J Immunol. 2013;191(5):2466–73. https://doi.org/10.4049/jimmunol.1300911.
72. Anuradha R, George PJ, Hanna LE, Chandrasekaran V, Kumaran PP, Nutman TB, et al. Parasite-antigen driven expansion of IL-5(−) and IL-5(+) Th2 human subpopulations in lymphatic filariasis and their differential dependence on IL-10 and TGFbeta. PLoS Negl Trop Dis. 2014;8(1):e2658. https://doi.org/10.1371/journal.pntd.0002658.
73. Anuradha R, George PJ, Hanna LE, Kumaran P, Chandrasekaran V, Nutman TB, et al. Expansion of parasite-specific CD4+ and CD8+ T cells expressing IL-10 superfamily cytokine members and their regulation in human lymphatic filariasis. PLoS Negl Trop Dis. 2014;8(4):e2762. https://doi.org/10.1371/journal.pntd.0002762.
74. Anuradha R, George PJ, Kumaran P, Nutman TB, Babu S. Interleukin-10- and transforming growth factor beta-independent regulation of CD8(+) T cells expressing type 1 and type 2 cytokines in human lymphatic filariasis. Clin Vaccine Immunol. 2014;21(12):1620–7. https://doi.org/10.1128/CVI.00598-14.
75. Anuradha R, George PJ, Chandrasekaran V, Kumaran PP, Nutman TB, Babu S. Interleukin 1 (IL-1)- and IL-23-mediated expansion of filarial antigen-specific Th17 and Th22 cells in filarial lymphedema. Clin Vaccine Immunol. 2014;21(7):960–5. https://doi.org/10.1128/CVI.00257-14.
76. Babu S, Bhat SQ, Pavan Kumar N, Lipira AB, Kumar S, Karthik C, et al. Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells. PLoS Negl Trop Dis. 2009;3(4):e420. https://doi.org/10.1371/journal.pntd.0000420.
77. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol. 2011;29:71–109. https://doi.org/10.1146/annurev-immunol-031210-101312.
78. Sabat R. IL-10 family of cytokines. Cytokine Growth Factor Rev. 2010;21(5):315–24. https://doi.org/10.1016/j.cytogfr.2010.11.001.

79. Freedman DO, de Almeida Filho PJ, Besh S, Maia e Silva MC, Braga C, Maciel A. Lymphoscintigraphic analysis of lymphatic abnormalities in symptomatic and asymptomatic human filariasis. J Infect Dis. 1994;170(4):927–33.
80. Freedman DO, de Almeido Filho PJ, Besh S, Maia e Silva MC, Braga C, Maciel A, et al. Abnormal lymphatic function in presymptomatic bancroftian filariasis. J Infect Dis. 1995; 171(4):997–1001.
81. Noroes J, Addiss D, Amaral F, Coutinho A, Medeiros Z, Dreyer G. Occurrence of living adult Wuchereria bancrofti in the scrotal area of men with microfilaraemia. Trans R Soc Trop Med Hyg. 1996;90(1):55–6.
82. Dreyer G, Ottesen EA, Galdino E, Andrade L, Rocha A, Medeiros Z, et al. Renal abnormalities in microfilaremic patients with Bancroftian filariasis. Am J Trop Med Hyg. 1992;46(6): 745–51.
83. Kabatereine NB, Malecela M, Lado M, Zaramba S, Amiel O, Kolaczinski JH. How to (or not to) integrate vertical programmes for the control of major neglected tropical diseases in sub-Saharan Africa. PLoS Negl Trop Dis. 2010;4(6):e755. https://doi.org/10.1371/journal.pntd.0000755.
84. Narain JP, Dash AP, Parnell B, Bhattacharya SK, Barua S, Bhatia R, et al. Elimination of neglected tropical diseases in the South-East Asia Region of the World Health Organization. Bull World Health Organ. 2010;88(3):206–10. https://doi.org/10.2471/BLT.09.072322.
85. Al-Shaham AA, Sood S. Recurrent furunculosis as a cause of isolated penile lymphedema: a case report. J Med Case Rep. 2010;4:196. https://doi.org/10.1186/1752-1947-4-196.
86. Pani SP, Vanamail P, Yuvaraj J. Limb circumference measurement for recording edema volume in patients with filarial lymphedema. Lymphology. 1995;28(2):57–63.
87. Okon OE, Iboh CI, Opara KN. Bancroftian filariasis among the Mbembe people of Cross River state. Nigeria J Vector Borne Dis. 2010;47(2):91–6.
88. Partono F. The spectrum of disease in lymphatic filariasis. CIBA Found Symp. 1987;127: 15–31.
89. Edeson JF. The epidemiology and treatment of infection due to Brugia malayi. Bull World Health Organ. 1962;27(4–5):529–41.
90. Pani SP, Srividya A. Clinical manifestations of bancroftian filariasis with special reference to lymphoedema grading. Indian J Med Res. 1995;102:114–8.
91. DeVries CR. The role of the urologist in the treatment and elimination of lymphatic filariasis worldwide. BJU Int. 2002;89(Suppl 1):37–43.
92. Belokar WK, De Sa O, Amonkar DP, Dharwadkar AM, Priolkar RP. Uncommon manifestations of filariasis. J Postgrad Med. 1983;29(3):170–4B.
93. Dreyer G, Addiss D, Santos A, Figueredo-Silva J, Noroes J. Direct assessment in vivo of the efficacy of combined single-dose ivermectin and diethylcarbamazine against adult Wuchereria bancrofti. Trans R Soc Trop Med Hyg. 1998;92(2):219–22.
94. Ong RK, Doyle RL. Tropical pulmonary eosinophilia. Chest. 1998;113(6):1673–9.
95. Ottesen EA, Nutman TB. Tropical pulmonary eosinophilia. Annu Rev Med. 1992;43:417–24. https://doi.org/10.1146/annurev.me.43.020192.002221.
96. McCarthy JS, Lustigman S, Yang GJ, Barakat RM, Garcia HH, Sripa B, et al. A research agenda for helminth diseases of humans: diagnostics for control and elimination programmes. PLoS Negl Trop Dis. 2012;6(4):e1601. https://doi.org/10.1371/journal.pntd.0001601.
97. McCarthy JS, Zhong M, Gopinath R, Ottesen EA, Williams SA, Nutman TB. Evaluation of a polymerase chain reaction-based assay for diagnosis of Wuchereria bancrofti infection. J Infect Dis. 1996;173(6):1510–4.
98. Dunbar SA. Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. Clin Chim Acta. 2006;363(1–2):71–82. https://doi.org/10.1016/j.cccn.2005.06.023.
99. Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in

clinical diagnosis of infectious diseases. Rev Med Virol. 2008;18(6):407–21. https://doi.org/10.1002/rmv.593.
100. Temperley ND, Webster LM, Adam A, Keller LF, Johnson PC. Cross-species utility of microsatellite markers in trichostrongyloid nematodes. J Parasitol. 2009;95(2):487–9. https://doi.org/10.1645/GE-1624.1.
101. Weil GJ, Lammie PJ, Weiss N. The ICT Filariasis Test: a rapid-format antigen test for diagnosis of bancroftian filariasis. Parasitol Today. 1997;13(10):401–4.
102. Wilke AB, Marrelli MT. Paratransgenesis: a promising new strategy for mosquito vector control. Parasit Vectors. 2015;8:342. https://doi.org/10.1186/s13071-015-0959-2.
103. Anitha K, Shenoy RK. Treatment of lymphatic filariasis: current trends. Indian J Dermatol Venereol Leprol. 2001;67(2):60–5.
104. Mand S, Marfo-Debrekyei Y, Dittrich M, Fischer K, Adjei O, Hoerauf A. Animated documentation of the filaria dance sign (FDS) in bancroftian filariasis. Filaria J. 2003;2(1):3.
105. Netto MJ, Bonfim C, Brandao E, Aguiar-Santos AM, Medeiros Z. Burden of lymphatic filariasis morbidity in an area of low endemicity in Brazil. Acta Trop. 2016;163:54–60. https://doi.org/10.1016/j.actatropica.2016.07.006.
106. Horton J. Albendazole: a review of anthelmintic efficacy and safety in humans. Parasitology. 2000;121(Suppl):S113–32.
107. Maizels RM, Denham DA. Diethylcarbamazine (DEC): immunopharmacological interactions of an anti-filarial drug. Parasitology. 1992;105(Suppl):S49–60.
108. Peixoto CA, Silva BS. Anti-inflammatory effects of diethylcarbamazine: a review. Eur J Pharmacol. 2014;734:35–41. https://doi.org/10.1016/j.ejphar.2014.03.046.
109. Geary TG, Maule AG. Neuropeptide systems as targets for parasite and pest control. Preface. Adv Exp Med Biol. 2010;692:v–vi.
110. Geary TG. Nonpeptide ligands for peptidergic G protein-coupled receptors. Adv Exp Med Biol. 2010;692:10–26.
111. Geary TG, Moreno Y. Macrocyclic lactone anthelmintics: spectrum of activity and mechanism of action. Curr Pharm Biotechnol. 2012;13(6):866–72.
112. Tan IC, Maus EA, Rasmussen JC, Marshall MV, Adams KE, Fife CE, et al. Assessment of lymphatic contractile function after manual lymphatic drainage using near-infrared fluorescence imaging. Arch Phys Med Rehabil. 2011;92(5):756–64.e1. https://doi.org/10.1016/j.apmr.2010.12.027.
113. Douglass J, Graves P, Gordon S. Self-care for management of secondary lymphedema: a systematic review. PLoS Negl Trop Dis. 2016;10(6):e0004740. https://doi.org/10.1371/journal.pntd.0004740.
114. Yahathugoda TC, Wickramasinghe D, Weerasooriya MV, Samarawickrema WA. Lymphoedema and its management in cases of lymphatic filariasis: the current situation in three suburbs of Matara, Sri Lanka, before the introduction of a morbidity-control programme. Ann Trop Med Parasitol. 2005;99(5):501–10. https://doi.org/10.1179/136485905X46450.
115. Vaqas B, Ryan TJ. Lymphoedema: pathophysiology and management in resource-poor settings – relevance for lymphatic filariasis control programmes. Filaria J. 2003;2(1):4.
116. Mathieu E, Dorkenoo AM, Datagni M, Cantey PT, Morgah K, Harvey K, et al. It is possible: availability of lymphedema case management in each health facility in Togo. Program description, evaluation, and lessons learned. Am J Trop Med Hyg. 2013;89(1):16–22. https://doi.org/10.4269/ajtmh.12-0453.
117. Pulendran B, Artis D. New paradigms in type 2 immunity. Science. 2012;337(6093):431–5. https://doi.org/10.1126/science.1221064.
118. Allen JE, Maizels RM. Diversity and dialogue in immunity to helminths. Nat Rev Immunol. 2011;11(6):375–88. https://doi.org/10.1038/nri2992.
119. Taylor MD, van der Werf N, Maizels RM. T cells in helminth infection: the regulators and the regulated. Trends Immunol. 2012;33(4):181–9. https://doi.org/10.1016/j.it.2012.01.001.
120. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity. 2010;32(5):593–604. https://doi.org/10.1016/j.immuni.2010.05.007.

121. Babu S, Nutman TB. Immunopathogenesis of lymphatic filarial disease. Semin Immunopathol. 2012;34(6):847–61. https://doi.org/10.1007/s00281-012-0346-4.
122. Hussain R, Hamilton RG, Kumaraswami V, Adkinson NF Jr, Ottesen EA. IgE responses in human filariasis. I. Quantitation of filaria-specific IgE. J Immunol. 1981;127(4):1623–9.
123. Mitre E, Nutman TB. IgE memory: persistence of antigen-specific IgE responses years after treatment of human filarial infections. J Allergy Clin Immunol. 2006;117(4):939–45. https://doi.org/10.1016/j.jaci.2005.12.1341.
124. Ottesen EA, Skvaril F, Tripathy SP, Poindexter RW, Hussain R. Prominence of IgG4 in the IgG antibody response to human filariasis. J Immunol. 1985;134(4):2707–12.
125. Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. Nat Rev Immunol. 2003;3(9):721–32. https://doi.org/10.1038/nri1181.
126. Capron M, Capron A, Torpier G, Bazin H, Bout D, Joseph M. Eosinophil-dependent cytotoxicity in rat schistosomiasis. Involvement of IgG2a antibody and role of mast cells. Eur J Immunol. 1978;8(2):127–33. https://doi.org/10.1002/eji.1830080211.
127. Saint Andre A, Blackwell NM, Hall LR, Hoerauf A, Brattig NW, Volkmann L, et al. The role of endosymbiotic Wolbachia bacteria in the pathogenesis of river blindness. Science. 2002;295(5561):1892–5. https://doi.org/10.1126/science.1068732.
128. Dakshinamoorthy G, Samykutty AK, Munirathinam G, Reddy MV, Kalyanasundaram R. Multivalent fusion protein vaccine for lymphatic filariasis. Vaccine. 2013;31(12):1616–22. https://doi.org/10.1016/j.vaccine.2012.09.055.
129. Specht S, Saeftel M, Arndt M, Endl E, Dubben B, Lee NA, et al. Lack of eosinophil peroxidase or major basic protein impairs defense against murine filarial infection. Infect Immun. 2006;74(9):5236–43. https://doi.org/10.1128/IAI.00329-06.
130. Mitre E, Taylor RT, Kubofcik J, Nutman TB. Parasite antigen-driven basophils are a major source of IL-4 in human filarial infections. J Immunol. 2004;172(4):2439–45.
131. Torrero MN, Hubner MP, Larson D, Karasuyama H, Mitre E. Basophils amplify type 2 immune responses, but do not serve a protective role, during chronic infection of mice with the filarial nematode Litomosoides sigmodontis. J Immunol. 2010;185(12):7426–34. https://doi.org/10.4049/jimmunol.0903864.
132. Nair MG, Gallagher IJ, Taylor MD, Loke P, Coulson PS, Wilson RA, et al. Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. Infect Immun. 2005;73(1):385–94. https://doi.org/10.1128/IAI.73.1.385-394.2005.
133. Semnani RT, Keiser PB, Coulibaly YI, Keita F, Diallo AA, Traore D, et al. Filaria-induced monocyte dysfunction and its reversal following treatment. Infect Immun. 2006;74(8):4409–17. https://doi.org/10.1128/IAI.01106-05.
134. Sharma P, Sharma A, Vishwakarma AL, Agnihotri PK, Sharma S, Srivastava M. Host lung immunity is severely compromised during tropical pulmonary eosinophilia: role of lung eosinophils and macrophages. J Leukoc Biol. 2016;99(4):619–28. https://doi.org/10.1189/jlb.4A0715-309RR.
135. Nelson FK, Greiner DL, Shultz LD, Rajan TV. The immunodeficient scid mouse as a model for human lymphatic filariasis. J Exp Med. 1991;173(3):659–63.
136. Babu S, Shultz LD, Klei TR, Rajan TV. Immunity in experimental murine filariasis: roles of T and B cells revisited. Infect Immun. 1999;67(6):3166–7.
137. Kara EE, Comerford I, Fenix KA, Bastow CR, Gregor CE, McKenzie DR, et al. Tailored immune responses: novel effector helper T cell subsets in protective immunity. PLoS Pathog. 2014;10(2):e1003905. https://doi.org/10.1371/journal.ppat.1003905.
138. Semnani RT, Nutman TB. Toward an understanding of the interaction between filarial parasites and host antigen-presenting cells. Immunol Rev. 2004;201:127–38. https://doi.org/10.1111/j.0105-2896.2004.00196.x.
139. Broadhurst MJ, Leung JM, Lim KC, Girgis NM, Gundra UM, Fallon PG, et al. Upregulation of retinal dehydrogenase 2 in alternatively activated macrophages during retinoid-dependent

type-2 immunity to helminth infection in mice. PLoS Pathog. 2012;8(8):e1002883. https://doi.org/10.1371/journal.ppat.1002883.
140. King CL, Mahanty S, Kumaraswami V, Abrams JS, Regunathan J, Jayaraman K, et al. Cytokine control of parasite-specific anergy in human lymphatic filariasis. Preferential induction of a regulatory T helper type 2 lymphocyte subset. J Clin Invest. 1993;92(4):1667–73. https://doi.org/10.1172/JCI116752.
141. Babu S, Nutman TB. Immunology of lymphatic filariasis. Parasite Immunol. 2014;36(8):338–46. https://doi.org/10.1111/pim.12081.
142. Wammes LJ, Hamid F, Wiria AE, Wibowo H, Sartono E, Maizels RM, et al. Regulatory T cells in human lymphatic filariasis: stronger functional activity in microfilaremics. PLoS Negl Trop Dis. 2012;6(5):e1655. https://doi.org/10.1371/journal.pntd.0001655.
143. O'Regan NL, Steinfelder S, Venugopal G, Rao GB, Lucius R, Srikantam A, et al. Brugia malayi microfilariae induce a regulatory monocyte/macrophage phenotype that suppresses innate and adaptive immune responses. PLoS Negl Trop Dis. 2014;8(10):e3206. https://doi.org/10.1371/journal.pntd.0003206.
144. Tisch DJ, Michael E, Kazura JW. Mass chemotherapy options to control lymphatic filariasis: a systematic review. Lancet Infect Dis. 2005;5(8):514–23. https://doi.org/10.1016/S1473-3099(05)70192-4.
145. Michael E, Malecela-Lazaro MN, Kazura JW. Epidemiological modelling for monitoring and evaluation of lymphatic filariasis control. Adv Parasitol. 2007;65:191–237. https://doi.org/10.1016/S0065-308X(07)65003-9.
146. El Setouhy M, Ramzy RM, Ahmed ES, Kandil AM, Hussain O, Farid HA, et al. A randomized clinical trial comparing single- and multi-dose combination therapy with diethylcarbamazine and albendazole for treatment of bancroftian filariasis. Am J Trop Med Hyg. 2004;70(2):191–6.
147. de Kraker ME, Stolk WA, van Oortmarssen GJ, Habbema JD. Model-based analysis of trial data: microfilaria and worm-productivity loss after diethylcarbamazine-albendazole or ivermectin-albendazole combination therapy against Wuchereria bancrofti. Tropical Med Int Health. 2006;11(5):718–28. https://doi.org/10.1111/j.1365-3156.2006.01606.x.
148. Rajendran R, Sunish IP, Mani TR, Munirathinam A, Arunachalam N, Satyanarayana K, et al. Community-based study to assess the efficacy of DEC plus ALB against DEC alone on bancroftian filarial infection in endemic areas in Tamil Nadu, south India. Tropical Med Int Health. 2006;11(6):851–61. https://doi.org/10.1111/j.1365-3156.2006.01625.x.
149. Yuvaraj J, Pani SP, Vanamail P, Ramaiah KD, Das PK. Impact of seven rounds of mass administration of diethylcarbamazine and ivermectin on prevalence of chronic lymphatic filariasis in south India. Tropical Med Int Health. 2008;13(5):737–42. https://doi.org/10.1111/j.1365-3156.2008.02044.x.
150. Ramaiah KD, Das PK, Vanamail P, Pani SP. Impact of 10 years of diethylcarbamazine and ivermectin mass administration on infection and transmission of lymphatic filariasis. Trans R Soc Trop Med Hyg. 2007;101(6):555–63. https://doi.org/10.1016/j.trstmh.2006.12.004.
151. Irvine MA, Stolk WA, Smith ME, Subramanian S, Singh BK, Weil GJ, et al. Effectiveness of a triple-drug regimen for global elimination of lymphatic filariasis: a modelling study. Lancet Infect Dis. 2017;17(4):451–8. https://doi.org/10.1016/S1473-3099(16)30467-4.
152. Dreyer G, Dreyer P, Noroes J. Recommendations for the treatment of bancroftian filariasis in symptomless and diseased patients. Rev Soc Bras Med Trop. 2002;35(1):43–50.
153. Dorta DM, Vasuki V, Rajavel A. Evaluation of organophosphorus and synthetic pyrethroid insecticides against six vector mosquitoe species. Rev Saude Publica. 1993;27(6):391–7.
154. Carvalho DO, Costa-da-Silva AL, Lees RS, Capurro ML. Two step male release strategy using transgenic mosquito lines to control transmission of vector-borne diseases. Acta Trop. 2014;132(Suppl):S170–7. https://doi.org/10.1016/j.actatropica.2013.09.023.
155. Akogun OB, Badaki JA. Management of adenolymphangitis and lymphoedema due to lymphatic filariasis in resource-limited North-eastern Nigeria. Acta Trop. 2011;120(Suppl 1):S69–75. https://doi.org/10.1016/j.actatropica.2010.10.006.

156. Wilson SF, Guarner J, Valme AL, Louis-Charles J, Jones TL, Addiss DG. Histopathologic improvement with lymphedema management, Leogane, Haiti. Emerg Infect Dis. 2004; 10(11):1938–46. https://doi.org/10.3201/eid1011.040548.
157. Bernhard L, et al. Management of patients with lymphoedema caused by filariasis in Northeastern Tanzania. Physiotherapy 2013;89(12):743–9.
158. Gass K, Beau de Rochars MV, Boakye D, Bradley M, Fischer PU, Gyapong J, et al. A multicenter evaluation of diagnostic tools to define endpoints for programs to eliminate bancroftian filariasis. PLoS Negl Trop Dis. 2012;6(1):e1479. https://doi.org/10.1371/journal.pntd.0001479.
159. Dewi RM, Tuti S, Ganefa S, Anwar C, Larasati R, Ariyanti E, et al. Brugia rapid antibody responses in communities of Indonesia in relation to the results of 'transmission assessment surveys' (TAS) for the lymphatic filariasis elimination program. Parasit Vectors. 2015;8:499. https://doi.org/10.1186/s13071-015-1093-x.
160. Noordin R, Itoh M, Kimura E, Abdul Rahman R, Ravindran B, Mahmud R, et al. Multicentre evaluations of two new rapid IgG4 tests (WB rapid and panLF rapid) for detection of lymphatic filariasis. Filaria J. 2007;6:9. https://doi.org/10.1186/1475-2883-6-9.
161. Abdul Rahman R, Hwen-Yee C, Noordin R. Pan LF-ELISA using BmR1 and BmSXP recombinant antigens for detection of lymphatic filariasis. Filaria J. 2007;6:10. https://doi.org/10.1186/1475-2883-6-10.
162. Lammie PJ, Weil G, Noordin R, Kaliraj P, Steel C, Goodman D, et al. Recombinant antigen-based antibody assays for the diagnosis and surveillance of lymphatic filariasis – a multicenter trial. Filaria J. 2004;3(1):9. https://doi.org/10.1186/1475-2883-3-9.
163. Rao KV, Eswaran M, Ravi V, Gnanasekhar B, Narayanan RB, Kaliraj P, et al. The Wuchereria bancrofti orthologue of Brugia malayi SXP1 and the diagnosis of bancroftian filariasis. Mol Biochem Parasitol. 2000;107(1):71–80.
164. Fischer P, Boakye D, Hamburger J. Polymerase chain reaction-based detection of lymphatic filariasis. Med Microbiol Immunol. 2003;192(1):3–7. https://doi.org/10.1007/s00430-002-0152-z.
165. Williams SA, Laney SJ, Bierwert LA, Saunders LJ, Boakye DA, Fischer P, et al. Development and standardization of a rapid, PCR-based method for the detection of Wuchereria bancrofti in mosquitoes, for xenomonitoring the human prevalence of bancroftian filariasis. Ann Trop Med Parasitol. 2002;96(Suppl 2):S41–6. https://doi.org/10.1179/000349802125002356.
166. Plichart C, Lemoine A. Monitoring and evaluation of lymphatic filariasis interventions: an improved PCR-based pool screening method for high throughput Wuchereria bancrofti detection using dried blood spots. Parasit Vectors. 2013;6:110. https://doi.org/10.1186/1756-3305-6-110.
167. Hoti SL, Elango A, Radjame K, Yuvaraj J, Pani SP. Detection of day blood filarial antigens by Og4C3 ELISA test using filter paper samples. Natl Med J India. 2002;15(5):263–6.

Leprosy

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1 Introduction

Leprosy is a complex, chronic disease of low infectivity caused by *Mycobacterium leprae* (*M. leprae*). The clinical presentation of leprosy is varied and polymorphous; clinical features range from a small single hypopigmented or erythematous skin patch to diffuse infiltration of skin involvement of the peripheral nerve trunk and other organs like the eyes, spleen, liver, testis, bones, and joints. The disease course in leprosy is interspersed with lepra reactions, which are hypersensitivity reactions and are the main cause of morbidity in leprosy. Leprosy is also considered one of the leading causes of infectious peripheral neuropathy. It is associated with both sensory and motor impairment leading to deformities and disabilities, often causing significant social stigma.

Leprosy has been known to man since time immemorial. DNA taken from the shrouded remains of a man from the Old City of Jerusalem shows him to be the earliest human proven to have suffered from leprosy. The remains were dated by radiocarbon methods to 1–50 AD. The origin of leprosy has always been a matter of uncertainty, and an Indian or African origin for the disease has often been assumed based on historical sources that support an initial spread of the disease from Asia to Europe. Dr. Robbins and colleagues reported a case of leprosy in a skeleton showing changes associated with leprosy buried around 2000 BC in Rajasthan, India, at the site of Balathal [1].

Leprosy was considered incurable, and this was one of the other causes of stigma associated with the disease. The discovery of dapsone in 1940 and then the introduction of multidrug therapy in 1984 were responsible for reducing the case burden of leprosy. Early detection and initiating prompt treatment with multidrug therapy (MDT) remains the key strategy to reduce global burden of leprosy and to prevent associated disabilities and deformities.

Leprosy is one of the least infectious diseases because majority of the population have natural immunity against it. The only natural reservoir of *M. leprae* is man,

and the most important source of infection is an untreated case of *multibacillary* leprosy, although all untreated cases should be considered as potential sources of infection. Leprosy is also found in animals like wild armadillos, chimpanzees, sooty mangabey monkeys, and cynomolgus macaque. Leprosy is considered a zoonosis in the southern United States, and epidemiological studies from the United States and Brazil have implicated contact with armadillos as a risk factor for leprosy infection [2, 3].

2 Epidemiology

Leprosy is no longer an ancestral plague that it has been, and globally leprosy has been almost eliminated or it is no longer a public health problem as the number of registered cases has decreased from 5.4 million in the year 1985 to 171,948 in 2016, and the prevalence rate per 10,000 fell from 21.1 to 0.23 over the last three decades [4] (Table 1).

As per "Global leprosy update, 2015: time for action, accountability and inclusion," published by WHO in September 2017, the leprosy statistics revealed the following [4]:

- 214,783 newly diagnosed patients were reported in 2016 (detection rate of 2.9 per 100,000 population).
- 94–96% of leprosy patients reported in 2016 were from 22 countries; India reported 135,485 new cases, accounting for 63% of the global new leprosy cases; Brazil reported 25,218 new cases, representing 13% of the global new cases; and Indonesia reported 16,826 new cases, 8% of the global case load.
- 39.2% of patients were females and 8.5% of patients were children.
- 59.18% of patients reported were of multibacillary (MB) type.
- 3039 cases of relapse were reported from 46 countries.
- With 12,437 new G2D cases, the proportion of new G2D cases was 12% lower than 2015, and it corresponds to a detection rate of 1.7 per million population.

The global burden of leprosy necessitated a massive and combined effort of various organizations like the World Health Organization (WHO), various NGOs,

Table 1 Registered prevalence of leprosy in different regions 1st quarter of 2017 [4]

WHO region	No. of case registered	Prevalence (number of case/10,000 population)
Africa	21,465	0.3
America	26,365	0.31
Southeast Asia	115,180	0.6
Eastern Mediterranean	3102	0.01
Western Pacific	5820	0.03
Total	171,948	0.23

governmental bodies, and healthcare professionals to tackle it together. Multidrug therapy (MDT), first conceived in 1982, has been the main weapon against leprosy. However, as seen in the past few years, new cases continue to occur in most endemic countries, and high-burden pockets exist against a low-burden background.

The WHO launched Global Leprosy Strategy 2016–2020 with the following aims [5]:

- Zero grade 2 disability (G2D) among pediatric leprosy patients
- Reduction of new leprosy cases with G2D to less than one case per million population
- Zero countries with legislation allowing discrimination on basis of leprosy

Leprosy continues to be an important infectious disease as is evident by (a) a relatively stable new case detection over the last decade, (b) a growing number of treated leprosy patients with recurrent reactions, and (c) a long-term neurologic dysfunction and disability as a result of irreversible peripheral nerve injury. We may have eliminated leprosy as a public health problem at a global level, but there are some issues that remain unanswered, and if we don't find a solution or answer to these, we may be faced with the problem of leprosy resurgence. The challenges or unsolved issues are lack of clarity about (a) the precise mode and route of transmission; (b) environmental, socioeconomic, and behavioral factors that promote its transmission; and (c) strategies for early diagnosis and prevention of neurologic impairment to reduce the disability burden among newly identified cases and among treated cases who continue to endure long-term disability [6].

3 Situation of Leprosy in India [7, 8]

– A total of 135,485 new cases were detected during the year 2016–2017, which gives an annual new case detection rate (ANCDR) of 10.2 per 100,000 population, as against 127,326 cases in 2015–2016. This showed increased in ANCDR in 2016–2017 (Table 2).
– A total of 88,166 cases were on record as of 1 April 2017, giving a prevalence rate (PR) of 0.66 per 10,000 population, as against 86,147 cases in 1 April 2016. This showed increase in PR in 2016–2017. This increase is due to active case finding under Leprosy Case Detection Campaigns (LCDC) organized all over the country.
– Among new leprosy cases detected, the proportion was as follows: MB (49.57%), female (39.17%), child (8.65%), grade 2 deformity (3.87%).
– A total of 5245 cases with grade 2 disability were detected among the new leprosy cases during 2016–2017, indicating the grade 2 disability rate of 3.87 per million.

Table 2 Situation of leprosy in India (2014–2015)

	2015–2016	2016–2017 [9]
Registered prevalence	86,147	88,166
Number of new case detected	127,326	135,485
ANCDR	9.71/100,000	10.2/100,000
Number of new cases of MB leprosy	65,337 (51.48%)	67,160 (49.57%)
Number of females among the new cases	46,845 (36.91%)	53,072 (39.17%)
Number of new cases among children	12,043 (9.49%)	11,792 (8.65%)
Number of new cases with grade 2 deformity	5851 (4.6%)	5245 (3.87%)

– A total of 11,792 child cases were recorded, indicating a child case rate of 0.86/100,000 population. This was less than the figure of 2015–2016.

4 Targets Under Program Implementation Plan (PIP) of NLEP to Be Achieved by 2016–2017 [8]

The 12th Five Year Plan for National Leprosy Eradication Programme (NLEP) for the period 2012–2013 to 2016–2017 was approved by the Government of India.

The objectives under PIP or 12th Plan were:

(a) Elimination of leprosy, i.e., prevalence of less than 1 case per 10,000 population in all districts of the country
(b) Strengthening of Disability Prevention and Medical Rehabilitation of persons affected by leprosy
(c) Reduction in the level of stigma associated with leprosy

To achieve the objectives of the plan, the main strategies to be followed include:

- Integrated leprosy services through general healthcare system
- Early detection and complete treatment of new leprosy cases
- Carrying out of household contact survey for early detection of cases
- Involvement of Accredited Social Health Activist (ASHA) in the detection and completion of treatment of leprosy cases on time
- Strengthening of Disability Prevention and Medical Rehabilitation (DPMR) services and Information, Education and Communication (IEC) activities in the community to improve self-reporting to Primary Health Centre (PHC) and reduction of stigma
- Intensive monitoring and supervision at block Primary Health Centre/Community Health Centre

The targets under PIP include decrease prevalence rate $< 1/10{,}000$ in 100% of districts, new ANCDR of less than 10 per 100,000 population in 100% districts, and decrease of grade 2 disability rate by 35%. The targets are summarized in Table 3.

Table 3 Targets to be achieved by 2016–2017 under PIP for 12th Plan [8]

Sr. no.	Indicators	Baseline (2011–2012)	Targets (by March 2017)
1	Prevalence rate (PR) < 1/10,000	543 districts (84.6%)	642 districts (100%)
2	(ANCDR) <10/100,000 population	445 districts (69.3%)	642 districts (100%)
3	Cure rate of multibacillary leprosy cases (MB)	90.56%	>95%
4	Cure rate of paucibacillary leprosy cases (PB)	95.28%	>97%
5	Grade 2 disability rate in percentage of new cases	3.04%	35% reduction 1.98%
6	Stigma reduction	Percentage reported (NSS 2010–2011)	50% reduction over the percentage reported by NSS

5 Etiology

M. leprae is a non-motile, non-spore-forming, acid-fast, gram-positive, microaerophilic obligate intracellular bacillus that shows tropism for cells of the reticuloendothelial system and peripheral nervous system (notably Schwann cells). It is a slow-growing bacillus and takes 12–14 days to divide into two. It is present in large numbers in lesions of lepromatous leprosy, often grouped together and arranged like bundles of cigars. Under the electron microscope, the bacillus appears to be polymorphous with the commonest form being a slightly curved filament 0.3–1 μm in width and 1–8 μm in length. Studies in animal models indicate that *M. leprae* grows best at 27 °C to 30 °C, correlating with its predilection to affect cooler areas of the body (the skin, nerve segments close to the skin, and the mucous membranes of the upper respiratory tract) [10, 11].

The inability of *M. leprae* to grow and survive at elevated temperatures is probably due to its inability to mount a protective heat stress response. *M. leprae* grows extensively in the nine-banded armadillo (*Dasypus novemcinctus*), which has a core body temperature of 34 °C [12].

The genome of *M. leprae* has been fully sequenced, and it was observed that *M. leprae* has less than half of the functional genes of *M. tuberculosis*. It contains an extraordinary number of pseudogenes, and genes for key enzymes of many essential metabolic pathways are missing [13, 14]. The *M. leprae* genome is highly conserved, but, using a combination of single-nucleotide polymorphisms and variable number tandem repeats, the major strain types can be reasonably discriminated [15].

6 *M. lepromatosis*

Han and Quintanilla reported this organism which is very similar to *M. leprae* but with some distinct differences in the DNA sequence for 16S RNA [16]. A total of 64 cases of leprosy caused singly by *M. lepromatosis* have been reported so far from Mexico or in patients of Mexican origin. Cases reported from other parts of the world are five cases from Singaporean Chinese, one case of a native Canadian, and two cases in native Costa Ricans. There are reports of coinfection with *M. leprae* and *M. lepromatosis* in patients from Mexico. Initially the bacterium was isolated form cases of diffuse lepromatous leprosy or Lucio leprosy, and subsequent reports show a more variable clinical presentation similar to *M. leprae* [16]. Limited data is available regarding *M. lepromatosis*; it has not yet been cultured, and its ability to infect nerves and other basic aspects of its biology is still unknown. Limited clinical experience with this isolate indicates that it presents with the same clinical features, responds well to same anti-mycobacterial drugs, and has same prognosis as *M. leprae* infection [17–19]. The identification of *M. lepromatosis* may be of epidemiological importance in the current scenario.

7 Pathogenesis of Leprosy

The clinical manifestations of leprosy are related to *M. leprae* survival and depend upon the interplay of innate and acquired immune responses involving interactions of the bacterial proteins with immune components of the host. These interactions may either prevent the invasion and infection or promote their growth and development of pathology. The immune system has evolved primarily to combat infection, but in leprosy, the immune response is responsible for the broad clinical spectrum of the disease and, similar to an autoimmune disease, seems to trigger further complications such as nerve damage [20].

8 Genetic Determinants of Host Response

Even after sustained exposure to *M. leprae*, only a subset of individuals develop clinical leprosy as majority of the population is immune to leprosy. Based on early studies on familial aggregation of leprosy cases to the most recent genome-wide association studies on leprosy-associated genetic polymorphisms, there is a strong evidence that human genetic factors influence the acquisition of leprosy and its further clinical course [21]. The polarization concept in leprosy has been studied by both DNA-independent analyses (familial correlations, twin and segregation studies) and DNA-based analyses (linkage and association studies). The first genome-wide linkage study of leprosy was performed in India in 2001. The sample included

224 families comprising 245 sibling pairs affected by leprosy [22]. A significant linkage hit ($p < 2 \times 10^{-5}$) for leprosy was observed with genetic markers located on chromosomal region 10p13. However, the study population had 98% PB cases; hence, it was not possible to decide whether the mapped locus influenced leprosy per se or was specific for the PB form. Overall, the results of the linkage studies in Indian and Vietnamese leprosy patients suggest that the 10p13 and 20p12 regions are differentially implicated according to the subtype considered [23, 24].

The various genes and genetic polymorphisms that have been studied for association with leprosy subtypes are (Box 1):

Box 1. Genetic Polymorphisms Associated with Leprosy [21]

Validated studies from different parts of the world	Genes that have been studied once from a particular region
Toll-like receptor 2 (TLR2) from Ethiopia and Malawi	IFN-γ and HLA-G in Brazil
TNF-α gene from India and Thailand	TLR4 and leukotriene A4 hydrolase (LTA4H) in Ethiopia
Mannose-binding lectin 2 (MBL2) in Brazil and Nepal	Vitamin D receptor (VDR), killer cell immunoglobulin-like receptor, two Ig domains and short cytoplasmic tail 3 (KIR2DS3), heat shock protein 1A (HSPA1A), and IL-23R from India
Mannose receptor C type 1 (MRC1) from Brazil and Vietnam	
Interleukin-10 (IL-10) from Brazil	
MHC class I chain-related gene A (MICA) from China and Brazil	

9 Transmission

The skin and the nasal mucosa are the major exit routes of *M. leprae* from the human body. Lepromatous cases harbor large numbers of organisms deep in the dermis, and sometimes they may also be found in the stratum corneum. These organisms from the superficial keratin layer could exit through the skin by exfoliation. However, transmission through this route has still not been definitely proven [25].

The main portal of entry of *M. leprae* is through the nasal mucosa. Whether this exposure results in infection or not depends largely on genetic susceptibility, immunological response of the individual, and the bacillary load. Hematological dissemination of the bacilli is considered to be the route of spread and widespread clinical pattern of the disease. A successful immune response or innate immunity aborts the further invasion, and *M. leprae* is eliminated in majority of the individuals. However, in a minority, *M. leprae* evades immunological defenses and

continues to multiply in macrophages and the Schwann cells surrounding peripheral nerves and subsequently in the skin and other tissues. Studies of household contacts who do not manifest leprosy have detected disseminated subclinical autonomic neuropathy in them, as evident by abnormal vasomotor reflexes—that might be like the Ghon's focus in the lung seen after exposure to *Mycobacterium tuberculosis* [26].

Environmental factors such as soil and water exposure, insect vectors, and the free-living amoebae (e.g., *Acanthamoeba* spp.) may also play an important role in the transmission of leprosy [6, 27, 28]. These environmental factors may also participate in the environmental viability of leprosy in some biotopes [27]. However some of the experts believe that most of these reports are on PCR-based studies, and although leprosy bacilli may remain viable in certain cell-free environments for a variable period, it does not mean that they remain infectious or they can replicate; moreover, with an abbreviated genome, it is most unlikely that *M. leprae* can replicate in any extracellular environment [29].

Zoonotic transmission of leprosy from natural infection of armadillos in the Southeast United States has been confirmed as responsible for the majority of autochthonous (indigenous or not from other regions/migrants) transmission in this area. It is likely that animals like armadillos may also play an important role in the transmission of leprosy in some areas of Latin America such as in Colombia, Venezuela, Mexico, and Brazil. Understanding how environmental factors influence host-pathogen interactions in complex natural systems, where multiple feedbacks between biotic and abiotic factors take place, is especially important in the context of environmentally persistent pathogens such as *M. leprae* [6, 30, 31].

10 Immunopathogenesis

In leprosy the clinical phenotype that the patient develops depends on the immunological response of the host. When *M. leprae* is first encountered, the monocytes may phagocytose all bacilli; however, in tuberculoid leprosy, the organisms may be totally destroyed, while in lepromatous leprosy, microvacuolated monocytes (phagocytes) with bacillary debris and live bacilli may persist [20, 32]. Although the precise mechanisms are unclear, the level of cell-mediated immunity or Th1 vs Th2 response to infection with *M. leprae* in the host determines the progression of disease toward tuberculoid or the lepromatous spectrum. Toll-like receptors on innate immune cells may recognize mycobacterial lipoproteins, generating cytokines that mediate specific responses in a Th1 or Th2 direction. A robust CMI or Th1 response either aborts the infection in the initial stages or contains it as is seen in the tuberculoid leprosy. Nonresponsiveness toward *M. leprae* seems to correlate with a Th2 cytokine profile and development of lepromatous leprosy [25]. In addition to Th1 and Th2, the concept of T cell plasticity is also seen, and various subsets like Th17, Th22, and T-Reg cells have also been described in the

pathogenesis of leprosy, and sometimes the distribution of these subsets depends on the level of antigenic stimulation or infection [32].

11 Pathogenesis of Nerve Involvement in Leprosy

All patients with leprosy have some degree of nerve involvement. Perineural inflammation is the histopathologic hallmark of leprosy, and this localization may reflect a vascular route of entry of *M. leprae* into nerves. Axonal atrophy may occur early in this process; ultimately, affected nerves undergo segmental demyelination [33]. The invasion of Schwann cells by *M. leprae* is the first step in the induction of nerve damage. The neurotropism of *M. leprae* is due to its affinity for the G-domain of laminin-alpha 2, an extracellular matrix protein that is present in the basal lamina of Schwann cells. *M. leprae*/laminin-alpha 2 complexes bind to alpha/beta dystroglycan complexes expressed on the Schwann cell surface. The bacterial components involved in the interaction are ML-LBP21, PDIM, and PGL-1. Recent in vitro work has suggested that early *M. leprae*-induced nerve damage is mediated via ErbB2 receptor tyrosine kinase signaling, which results in early nerve demyelination [25]. Another in vitro study suggested that early molecular pathways of nerve damage originate from *M. leprae*-induced excessive Schwann cell survival, which triggers glial cell proliferation and the inflammatory response [25, 33, 34]. Nerve damage may also be mediated by inflammatory and immune-mediated processes, as well as due to edema and mechanical processes.

Nerve damage due to leprosy can be divided into two phases:

1. Initial phase: This phase is common to both tuberculoid and lepromatous ends. The hallmark of this phase is the absence of inflammatory cells. It is due to early biochemical changes in the axonal compartment and axonal atrophy and occurs before structural changes in myelinated fibers; these changes are seen more in small, poorly myelinated or unmyelinated nerve fibers [33].
2. Later phase: It is characterized by the presence of inflammatory response which ranges from well-organized granulomatous response in tuberculoid disease that aggressively affects the nerve to a disorderly chronic inflammation in lepromatous patients which eventually destroys the nerve and the surrounding tissue.

12 Stages of Nerve Involvement [25]

Five stages of nerve involvement can be recognized, the first two being identifiable only by histological scrutiny while the later three are clinical:

1. Stage of parasitization: *M. leprae* found within nerves.
2. Stage of tissue response: inflammatory response to the presence of bacilli.
3. Stage of clinical infection: nerve thickening with no apparent nerve function impairment.

4. Stage of nerve damage: apparent nerve function impairment. This stage is reversible.
5. Stage of nerve destruction: nerve fibers are totally destroyed and collagenized.

13 Risk Factors for Leprosy

Poor socioeconomic status and unhygienic living conditions like contaminated water, inadequate housing, malnutrition, and diseases compromising the immune function are usually the risk factors for leprosy. HIV infection has not been reported to increase the susceptibility to leprosy, although initiation of antiretroviral therapy can either activate subclinical leprosy or exacerbate pre-existing lesions due to immune reconstitution inflammatory syndrome [25].

Results of various studies suggest that susceptibility to leprosy is multigenic, with a high degree of heterogeneity among different populations studied. HLA-DR2 and the Taq1 polymorphism of the vitamin D receptor gene, alleles in the PARK2 and PACRG, and NOD2 have been found to be associated with susceptibility to infection with *M. leprae* [35, 36].

14 Incubation Period of Leprosy

Leprosy has a relatively long incubation period with the average incubation period varying between 3 and 10 years. However it is difficult to measure the accurate period because of the paucity of adequate immunological tools and slow onset of disease [25].

15 Clinical Features of Leprosy

Leprosy patients have skin lesions varying from ill- to well-defined macules, patches, nodules, and plaques to diffuse involvement which is often difficult to distinguish from the normal skin. However, in most leprosy cases, the lesions are in the form of a hypoesthetic, hypopigmented, or erythematous patches, but papular, annular, nodular, and plaque types of lesions occur in variable numbers and are distributed on various parts of the body. Most of these lesions have diminished or absent sensations, impairment of sweating, and reduced hair density.

Leprosy is diagnosed when at least one of the cardinal signs is manifested [37]:

- A definite loss of sensation in a pale (hypopigmented) or reddish skin patch
- A thickened or enlarged peripheral nerve, with loss of sensation and/or weakness of the muscle supplied by that nerve
- The presence of acid-fast bacilli in a slit skin smear [37]

16 Classification of Leprosy

The classification of a disease is used to identify and understand the different aspects of disease presentation and linking them to the underlying immunopathogenesis which is helpful for the treatment decisions, prognosis, and research. Classification of leprosy allows the risk of complications to be predicted; for example, leprosy patients with borderline leprosy are at a much higher risk of developing reactions than patients with tuberculoid disease. The first system for classification of leprosy was proposed at an international meeting in Manila in 1931. This was followed by systems proposed in Cairo in 1938, Rio de Janeiro in 1946, Havana in 1948, and Madrid in 1953, followed by an Indian classification in 1955 [38] (Box 2). The initial classifications were predominantly based on clinical features with some support from histological and lepromin testing. They classified leprosy into tuberculoid and lepromatous poles and borderline, dimorphous, or intermediate categories.

Ridley-Jopling classification is the most widely used classification system in leprosy. They classified leprosy into five types: lepromatous leprosy (LL), borderline lepromatous leprosy (BL), borderline leprosy (BB), borderline tuberculoid leprosy (BT), tuberculoid leprosy (TT), and indeterminate. This classification of leprosy is recognized to be an expression of the patient's resistance to infection or the immunity and the spectrum ranges from a form with a robust immune response and very few organisms (tuberculoid or paucibacillary) to a form with a weaker immune response and a higher burden of organisms (lepromatous or multibacillary). The classification is based on the cutaneous, neurologic, and biopsy findings, all of which correlate with immunological capability of the individual. The categories also correlate with the number of acid-fast bacilli present in the dermis [39, 40].

1. **Tuberculoid form (TT):** Characterized by a single lesion (maximum up to three lesions). The lesions are usually large in size, well-defined, and in the form of erythematous plaques with raised clear-cut edges (Fig. 1). The surface of lesion (s) looks dry, scaly, and turgid. Sensations as well as hair are usually absent on the lesion. Lepromin test is strongly positive (++++). On histopathological examination, large epithelioid cells are found to be arranged in compact

Box 2. The Indian Classification System

1. Tuberculoid (T)
2. Lepromatous (L)
3. Maculoanesthetic (MA)
4. Polyneuritic (P)
5. Borderline (B)
6. Indeterminate (I)

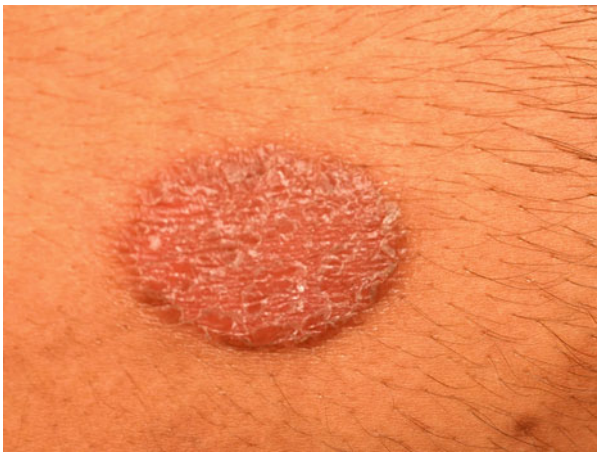


Fig. 1 Well defined erythematous plaque of tuberculoid leprosy (TT)

granulomas along neurovascular bundles, with dense peripheral lymphocytic infiltrate. Langhans giant cells are usually absent. Dermal nerves may be obliterated and destroyed by dense lymphocyte cuffs. Acid-fast bacilli are rarely found even in nerves. The granuloma usually reaches and may even erode the epidermis [41].

2. **Borderline tuberculoid (BT):** The lesions are few (maximum of ten). The lesions are well defined and variable in size. Many lesions may slope outward or fade into surrounding skin. Few satellite lesions may sometimes be seen (Fig. 2). The surface of lesions is dry, scaly, and infiltrated. Sensations and hair over the lesion are usually absent, just like in TT. AFBs when seen are scanty. Lepromin reactivity is positive (++ or +). Histology shows Langhans giant cells, which are variable in number and not large in size. Granulomas along the superficial vascular plexus are frequent, but they do not infiltrate up into the epidermis (grenz zone). Nerve invasion and obliteration are typical. BI ranges from 0 to 2+.
3. **Borderline borderline (BB)**: Several lesions (10–30) may have an inverted saucer appearance, where the outer margin is sloping and the inner margin is punched out (Fig. 3). They may be dull or shiny. Sensations and hair over the lesions are moderately diminished. Lepromin test is negative or weakly positive (+ or −). Histology shows a grenz zone; macrophages are activated into epithelioid cells, but there are only few distinct granulomas. Lymphocytes are scanty. There are no giant cells. BI ranges from 3 to 4+.
4. **Borderline lepromatous (BL):** The lesions are numerous and tend to be symmetrical. Size may vary from small to large (Fig. 4). Most lesions are ill-defined, although few may show a better defined edge. The surface of lesions is shiny and sensations and hair are only mildly affected. Numerous AFBs are found in the lesion and lepromin test is negative. Histopathology shows a grenz zone and prominent lymphocytic infiltrate, with activation of macrophages to form poorly to moderately defined granulomas. There is perineural fibroblast proliferation, forming an "onion peel" in cross section. Foamy cells are present but not prominent, and BI ranges from 4 to 5+.

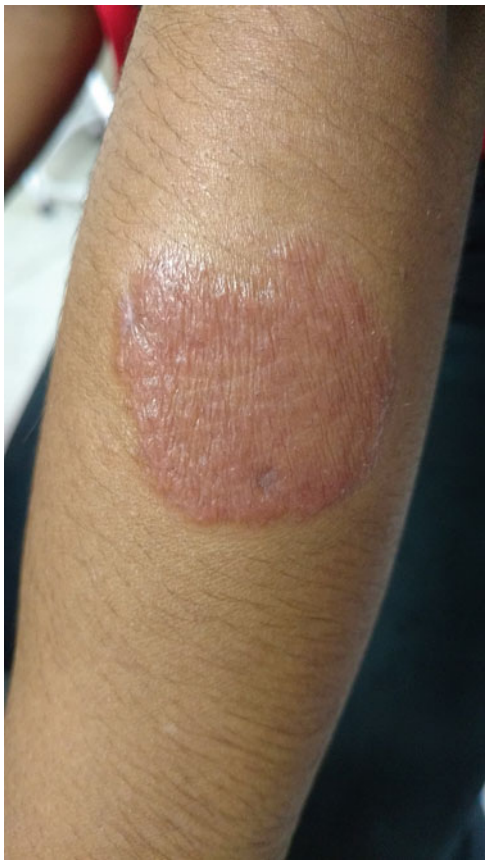


Fig. 2 Erythematous plaque with pseudopodia and few satellite lesions in borderline tuberculoid (BT) leprosy

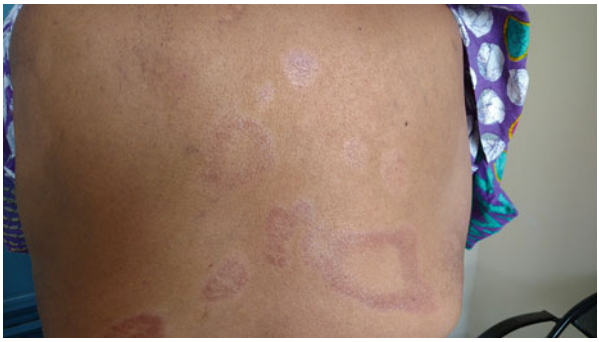


Fig. 3 Inverted saucer appearance of lesions in mid borderline (BB) leprosy

5. **Lepromatous leprosy (LL):** The lesions are innumerable and skin may even be diffusely infiltrated (Fig. 5a). The lesions are ill-defined, small, shiny plaques that are distributed symmetrically. In the early stage of the disease, sensations are not affected and overlying hairs are normally present. AFBs are plentiful, some forming "globi." Lepromin test is negative. Histopathological examination

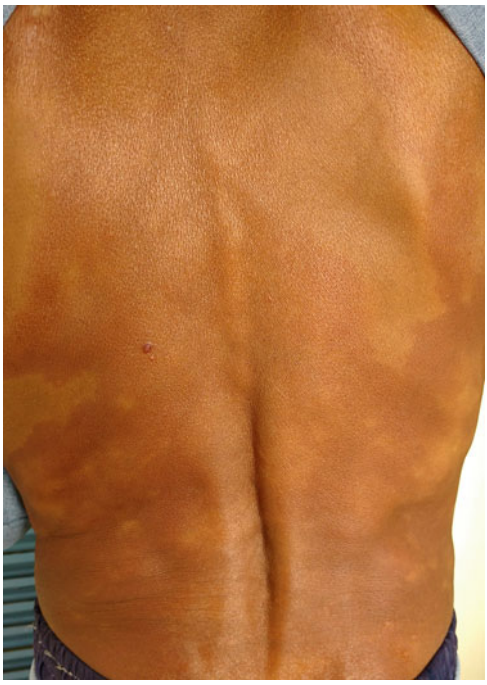


Fig. 4 Multiple hypopigmented plaques in borderline lepromatous (BL) leprosy

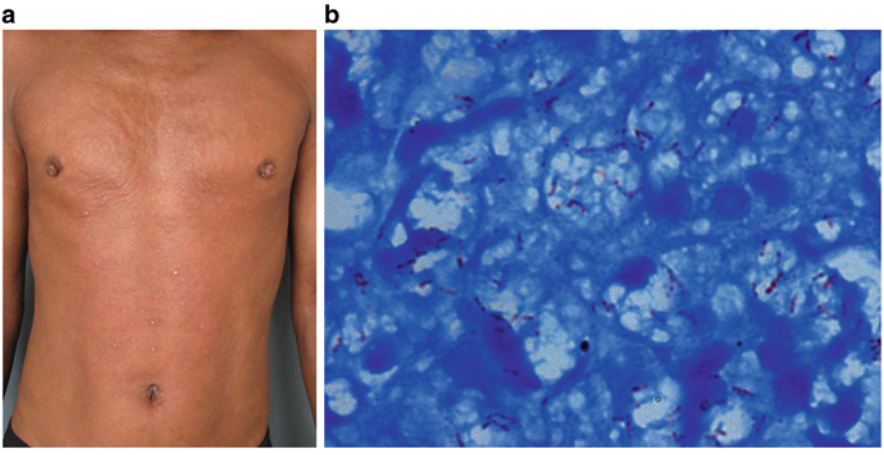


Fig. 5 (**a**) Diffuse infiltration with papules and nodules in lepromatous leprosy (LL). (**b**) Fite-Faraco Stain: numerous foamy macrophages and lymphocytes, containing many acid fast bacilli

shows extensive cellular infiltrate separated from the flattened epidermis by a narrow grenz zone, causing destruction of the cutaneous appendages and may extend even into the subcutaneous fat. Macrophages have abundant eosinophilic

cytoplasm and contain a mixed population of solid and fragmented bacilli. Foamy cells, granulomas, and globi are seen (Fig. 5b).

6. **Polar and subpolar forms**: LL may be polar LL (LLp) or subpolar LL (LLs). LLp is immunologically stable and starts and stays as lepromatous throughout. LLs is unstable, and it evolves from downgrading of the borderline group and may have some lesions of borderline leprosy as evidence of having downgraded. Similarly, TT may also be polar TT (TTp) or subpolar TT (TTs). TTp originates as tuberculoid and hardly ever downgrades, while TTs is the unstable form, usually downgrades to borderline leprosy, and may rarely upgrade from borderline spectrum [41].

17 Other Forms of Leprosy

1. **Indeterminate**: Characterized by ill-defined hypopigmented scaly macules or patches (Fig. 6). The lesions are few in number, and slight impairment of sensation may be present. There is mild lymphocytic and macrophage accumulation around neurovascular bundles, sweat glands, and erector pili muscle. Diagnosis should only be made on finding one or more acid-fast bacilli at the sites of predilection: in the nerve, in the erector pili muscle, just under the epidermis, or in a macrophage around a vessel [41].

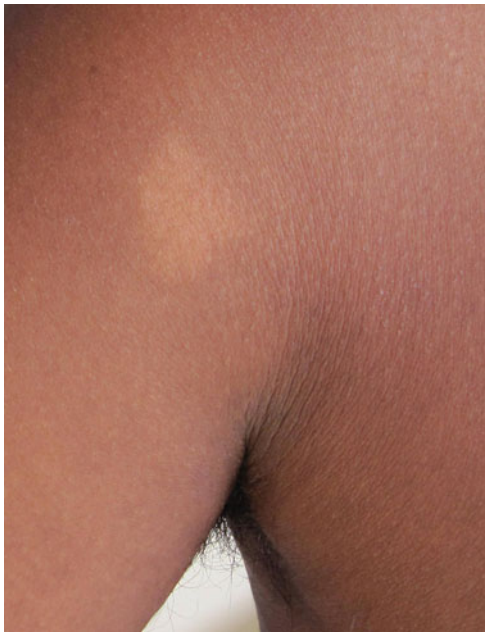


Fig. 6 Ill defined hypopigmented patch of indeterminate leprosy

2. **Lucio Leprosy**: Lucio leprosy (lepra bonita), first described by Lucio and Alvarado in 1852, is characterized by a diffuse shiny infiltration of all body skin with widespread sensory loss. As the disease progresses, the eyelids become thickened, giving the patients a sleepy and sad appearance. Madarosis is often the first sign. Patients may complain of numbness of the hands and feet, nasal congestion and epistaxis, hoarseness of the voice, and edema of the feet. Bacteriological smears are highly positive (BI 6+) and lepromin reaction is negative. Lucio leprosy is considered to be the most anergic of all the immunological spectrum of leprosy [41]. Endothelial cell injury appears to be the main event in the pathogenesis of diffuse leprosy of Lucio and Latapí. Once *M. leprae* has entered the endothelial cell, the microorganism damages the blood vessels, leading to the specific changes seen in this variety of lepromatous leprosy [42].
3. **Histoid Leprosy**: Histoid leprosy is a special manifestation of lepromatous leprosy and was first described by Wade in 1963 [43]. It is characterized by papules and sharply demarcated, firm nodules. Histoid leprosy has been generally reported to manifest in patients after long-term dapsone monotherapy and irregular or inadequate therapy. However, there are also reports of disease developing as relapse after successful treatment or even appearing de novo without a prior history of any antileprosy treatment. Clinically the histoid lesions appear as smooth, shiny, hemispherical, dome-shaped, nontender soft to firm nodules which may be superficial, subcutaneous, or deep dermal nodules and plaques or pads appearing on otherwise normal-looking skin (Fig. 7). The lesions are usually located on the face, back, buttocks, and extremities and over bony prominences, especially around the elbows and knees. On histological examination, spindle-shaped cells containing *M. leprae* are found. Bacteriological smears are highly positive (BI 6+) and the lepromin reaction is negative [44].

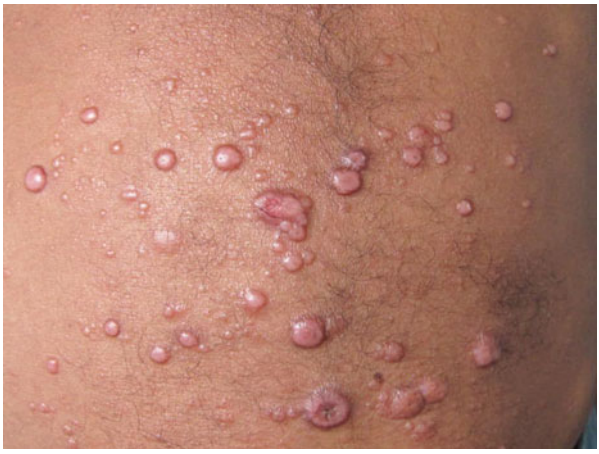


Fig. 7 Papules and sharply demarcated firm nodules on apparently normal appearing skin in histoid leprosy

4. **Pure Neuritic Leprosy**: There is thickening of the peripheral nerve trunk with sensory loss in the area of its distribution, with or without associated motor paralysis, primarily in the absence of any skin patch regardless of clinical evidence of reaction involving the nerve. It accounts for around 4–6% of leprosy cases in the Indian subcontinent. Commonly affected nerves are ulnar, median, radial, lateral popliteal, posterior tibial, sural, facial, and sometimes trigeminal. Mononeuritis or mononeuritis multiplex is the most common presentation [45]. Slit skin smear or a skin biopsy usually does not reveal changes of leprosy. Nerve conduction studies, FNAC, nerve biopsy, PCR, etc. may be used for diagnosis. All other causes of peripheral neuropathy should be excluded, and the clinician should have a high index of suspicion [46].

WHO Classification
The World Health Organization (WHO) classification system was designed for use in situations in which there is little or no clinical expertise or laboratory support; it is based upon the number of skin lesions present [47]:

- Paucibacillary (PB) leprosy is defined as five or fewer skin lesions without detectable bacilli on skin smears.
- Multibacillary (MB) leprosy is defined as six or more lesions and may be skin smear positive. Counting skin lesions alone may lead to misclassification of many patients with PB leprosy rather than MB leprosy, leading to undertreatment in some cases [37].

18 Neuropathy in Leprosy

Classical Leprosy Neuropathy This involves nerves of the extremities (both upper and lower), which may present as:

1. Thickening of nerve trunks
2. Mononeuritis
3. Mononeuritis multiplex
4. Polyneuropathy with a “glove and stocking”-type distribution
5. Cranial neuropathies
6. Peripheral autonomic dysfunctions (anhidrotic dry skin, compromised sudomotor and vasomotor responses, and trophic ulcers) [47]

Acute Neuritis This is one of the most common and dramatic presentations in leprosy and generally occurs during the leprosy reactions. It often starts with spontaneous nerve pain, paresthesias, and nerve tenderness. These symptoms are followed by nerve function impairment with objective sensory-motor loss. Recognition of the symptoms at onset is crucial, as initiation of steroids reduces long-term damage [26].

Silent Neuropathy The patient has only neurological deficit which is mostly progressive in the absence of nerve pain and tenderness with no evidence of reaction. The lack of spontaneous subjective nerve impairment makes this condition detectable only when specifically assessed with standardized monofilament testing for sensory impairment and voluntary muscle strength for motor impairment [48].

Subclinical Neuropathy Subclinical neuropathy appears to be more prevalent in leprosy than was previously believed [47]. Testing using monofilaments and other sensitive methods has demonstrated that functional nerve impairment occurs earlier in the course of lepromatous disease than in tuberculoid disease, even though patients with tuberculoid disease may be aware of numbness or weakness earlier in the course of their illness than patients with lepromatous disease [49]. In a prospective study of early neuropathy diagnosis in leprosy, sensory nerve conduction and warmth perception were the most frequently and earliest affected tests; in a large proportion of patients, these became abnormal $\geq$3 months or more before other abnormalities were identified using monofilaments [50].

Chronic Neuropathic Pain It is diagnosed when the patient presents with pain and new nerve function impairment in the absence of leprosy reaction after completion of MDT. The pain is described as continuous burning type with glove and stocking distribution. It can also manifest as paresthesias, dysesthesias, hyperesthesia, and allodynia along the nerve and in its area of distribution. Different pathophysiological mechanisms possibly leading to leprosy-related neuropathic pain are small fiber neuropathy and persistent intraneural inflammation due to residual bacterial antigens or persisters in the Schwann cells. We should rule out pain due to extraneural pathology such as osteitis, periosteitis, and osteomyelitis in all these cases. Management is difficult and tricyclic antidepressants, anticonvulsants, and opioids have been tried [51].

Childhood Leprosy

Childhood leprosy is a significant indicator of control programs in the society as the detection of new cases in children under 15 years of age reveals an active circulation of bacilli, continued transmission, and lack of disease control by the health system. According to the NLEP report of 2017, children constituted 8.65% of the newly detected leprosy cases in India [7]. Among patients under 15 years of age, the most affected age group is children between 10 and 14 years of age, although there are case reports of patients younger than 1 year as well. Household contacts are the primary source of infection. Various studies have reported rates varying from 8.7% to 38.8% [52, 53]. Paucibacillary forms of the disease are more common, especially borderline tuberculoid leprosy, with a single lesion in exposed areas of the body representing the main clinical manifestation. Lepra reactions are rare, although some authors have reported high frequencies of type 1 lepra reaction. Peripheral nerve involvement has been described at very high rates in some studies, which increases the chance of deformities, a serious problem, especially if one considers the age of these patients. High disability rates have been reported in studies from

India ranging from 0.5% to 24%, which could be due to delay in diagnosis or detection of leprosy in these cases. If we have to bring this figure to zero by 2020, then we will have to initiate the school health surveys and household contact surveys again [54].

Physical Examination

The diagnosis of leprosy should be considered in patients with skin lesions and/or enlarged nerve(s) accompanied by sensory loss or motor weakness. Leprosy should be suspected in the setting of the following symptoms:

- Hypopigmented or reddish patch(es) on the skin
- Diminished sensation or loss of sensation within skin patch(es)
- Paresthesias (tingling or numbness in the hands or feet)
- Painless wounds or burns on the hands or feet
- Lumps or swelling on the earlobes or face
- Tender, enlarged peripheral nerves [55]

Late findings include weakness of the hands with claw fingers, foot drop, facial paralysis or lagophthalmos, lack of eyebrows and eyelashes, collapsed nose, or perforated nasal septum. Clinical findings correlate with the extent of nerve involvement, classification of disease, and presence of the immunologic complications known as reactions.

The examination should include evaluation of skin lesions and palpation of peripheral nerves for enlargement and/or tenderness, including the ulnar nerve at the elbow, median and superficial radial cutaneous nerve at the wrist, great auricular nerve in the neck, and common peroneal nerve at the popliteal fossa (Fig. 8). A sensory examination of skin lesions, distal extremities, and motor evaluation should also be performed. Eyes should be examined by simple inspection of the conjunctiva and cornea, as well as assessment of corneal sensation.

Ocular involvement is also commonly seen in leprosy and is estimated to be present in about 70–75% of all cases of leprosy. About 10–50% of leprosy patients suffer from severe ocular symptoms (potential sight-threatening complications/involvement), and blindness occurs in about 5% of patients [56]. Complications like lagophthalmos, corneal hypoanesthesia, neurotrophic or infectious keratitis, iridocyclitis, and cataract formation are considered potentially sight-threatening complications and should be managed urgently. Completion of multidrug treatment does not guarantee the prevention from ocular complications which may continue to occur years after completion of MDT [57].

Rheumatologic Manifestations of Leprosy

Musculoskeletal involvement is the third most common manifestation in leprosy following the cutaneous and neural manifestations but is less frequently reported.

Different patterns of musculoskeletal presentation can occur in leprosy patients. Joint involvement can be monoarticular, oligoarticular, or polyarticular. It can present as acute symmetrical polyarthritis or chronic symmetrical polyarthritis mimicking rheumatoid arthritis. Enthesitis and tenosynovitis are also common presentations in leprosy.

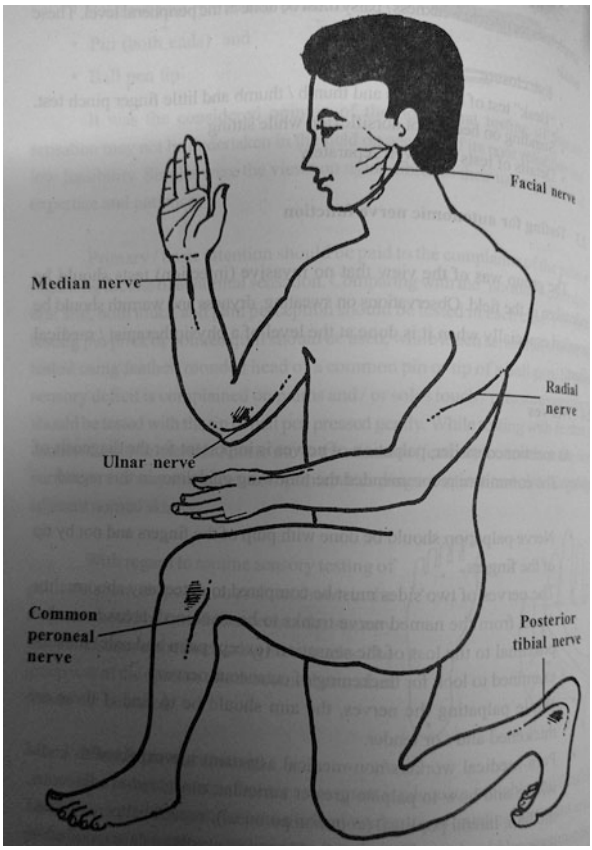


Fig. 8 The peripheral nerves that should be examined in a case of leprosy

Chauhan et al. classified the arthritis in leprosy into the following groups: (1) Charcot arthropathy secondary to peripheral sensory neuropathy, (2) swollen hands and feet syndrome, (3) acute polyarthritis of lepra reaction, and (4) chronic arthritis from direct infiltration of the synovium by lepra bacilli [58, 59]. Hence it is important to include leprosy in the list of possible differential diagnosis of arthritis, mainly in those countries where leprosy is prevalent or those patients who have traveled from endemic areas.

Differential Diagnosis

The clinical presentation of leprosy is highly variable and it can mimic a variety of other dermatological and neurological disorders.

Dermatological Disorders [60]

Macular hypopigmented lesions	Annular lesions	Infiltrated plaques
Vitiligo, pityriasis versicolor, nevus anemicus, nevus		Lupus vulgaris, sarcoidosis, discoid lupus

(continued)

Macular hypopigmented lesions	Annular lesions	Infiltrated plaques
depigmentosus, and pityriasis alba	Granuloma annulare, tinea corporis, lichen planus, syphilis, and granuloma multiforme	erythematosus, cutaneous leishmaniasis

Box 3. Differential diagnosis of enlarged peripheral nerves

1. Hereditary motor and sensory neuropathy
2. Neurofibromatosis
3. Refsum's disease
4. Perineuroma/localized hypertrophic neuropathy
5. Nerve tumors
6. Amyloidosis

Neurological Disorders

Neurological disorders that need to be differentiated from leprosy include hereditary neuropathy, mono- or polyneuritis, nutritional and alcoholic neuropathy, lead poisoning, nerve damage after trauma, entrapment neuropathy, poliomyelitis, syringomyelia, Guillain-Barré syndrome, and neurogenic muscular atrophy [61]. Leprosy has to be differentiated from other causes presenting as thickened peripheral nerves (Box 3). Because leprosy does not affect the central nervous system, the presence of signs such as loss of tendon reflexes, pathological reflexes, and nystagmus will exclude leprosy. Hence a detailed neurological examination is mandatory when the diagnosis of leprosy is in doubt.

19 Laboratory Diagnosis

Although leprosy is considered a clinical diagnosis, laboratory diagnostic tests such as slit skin smear (SSS) and histopathology of involved tissues provide the necessary information to make the diagnosis in almost all cases. Serology, polymerase chain reaction, and bacteriologic, histopathologic, and immunologic studies provide evidence to support the clinical diagnosis and are more important for research.

19.1 Slit Skin Smear (SSS)

Slit skin smear examination is helpful in classification and management of disease, as well as in following up of patients for response to treatment and detecting

relapse. The diagnostic specificity of skin smears is almost 100%; however, its sensitivity is rarely more than 50% because smear-positive patients represent only 10–50% of all cases. SSS is prepared by scraping the sides of slit(s) made in the skin over lesion(s) and other sites like earlobes, forehead, knees, elbows, and fingers. Bacterial index (BI) and morphological index (MI) are calculated for follow-up. The density of bacilli in smears is known as the bacteriological index (BI) and includes both living and dead bacilli. It is reported in a scale from 1 to 6 depending upon the number of bacilli in a smear. The morphological index (MI) is the percentage of solid-stained bacilli, calculated after examining 200 red staining elements, lying singly. It is a useful indicator of progress in patients under treatment and changes more rapidly than the BI. Ideally the MI should become zero in about 6 weeks after starting MDT as more than 99% of bacilli are killed with the first dose. A rise in the MI while a patient is receiving treatment indicates lack of compliance by the patient, incomplete absorption of the drug, or that the bacilli have become resistant to the drug. SSS is no longer recommended by WHO in screening or diagnosis of leprosy because it has the potential risk of transmitting HIV and HBV infections and wide interobserver variations [62].

Skin/Nerve Biopsies

The histopathology of the skin or nerves is an important modality for confirmation of diagnosis and classification of disease and evolution of the disease which may not be evident clinically (downgrading/upgrading). Hematoxylin-eosin staining should always be complemented with Fite-Faraco staining or one of its variations to detect the bacillary load. Sometimes the inflammatory infiltrate of the nerves may be distinct from the ones in the cutaneous lesions, with lower histological grading (toward the lepromatous pole) in the nerves as compared to the cutaneous lesions. Histopathology is also important in differentiating relapse from reversal reactions [63].

Skin Tests Efforts to develop a diagnostic skin test using proteins and peptides from antigens purified from *M. leprae* are underway since a long time, but we still do not have an ideal skin test to diagnose patients with paucibacillary spectrum where the CMI predominantly plays a role in the disease pathogenesis [64]. The lepromin test is not a useful diagnostic tool; it consists of injecting a calibrated number of autoclaved *M. leprae* into the skin; the results are assessed after 3–4 weeks. The test does not measure exposure or infection. A positive test reflects the ability to develop a granuloma following exposure to *M. leprae* antigens; a positive test does not indicate exposure to leprosy [64]. Tuberculin skin tests (TSTs) do not significantly cross-react with *M. leprae* infection; in one study of a population in which tuberculosis was highly endemic, 70% of controls had positive TST, but only 15–50% of leprosy patients had positive TST [65, 66].

Immunohistochemistry

Immunohistochemistry using monoclonal or polyclonal antibodies to detect *M. leprae* antigens may provide higher sensitivity and specificity than conventional methods, especially in the initial stage of illness or in PB cases. The antibodies

against PGL-1, S-100, 35 kDa, 65 kDa, and BCG are used to demonstrate *M. leprae* in the tissues [63].

19.2 Serology and PCR

PCR is helpful in detecting slow-growing or uncultivable microorganisms and, based on the available genetic data, has been used to detect *M. leprae*, since 1989. PCR made it possible to detect, quantify, and determine *M. leprae* viability, showing significantly better results compared to common microscopic examinations. PCR may allow confirming cases of initial, PB, and pure neural leprosy, demonstrating subclinical infection in contacts, monitoring treatment, determining patients' cure or their resistance to MDT drugs, and helping to understand the mechanisms of *M. leprae* transmission [67, 68].

Serologic tests—Serologic tests for *M. leprae* phenolic glycolipid-1 (PGL-1) are described but are not freely available because they are not sufficiently sensitive to provide a reliable measure of infection without other clinical or histologic evidence [69, 70]. Patients with lepromatous disease develop a strong polyclonal antibody response to *M. leprae* and have positive serologic responses to PGL-1. Patients with tuberculoid disease seldom produce antibody to PGL-1, and therefore the test is not helpful for diagnosis in these patients. Many contacts have been found to have antibodies to PGL-1 also, but only a small percentage of them go on to develop the infection. Thus, PGL-1 is not a reliable diagnostic test nor is it satisfactorily predictive of the development of infection. Further development of serologic tests is an area of active investigation [70].

Detection of anti-PGL-1 by immunochromatographic flow test (ML-Flow test) which is a simple dipstick assay using whole blood samples has been shown to be comparable to the ELISA in its sensitivity, being able to detect >90% of MB patients and 40% of PB patients, with background seropositivity in endemic controls at around 10%. It can prove to be a useful tool in high endemic areas for the control of transmission, and it may even obviate the need for SSS [71, 72].

Another protein termed LID-1 (leprosy IDRI diagnostic 1) constructed from two proteins named ML0405 and ML2331 has also shown promising results as diagnostic tool for leprosy. Positive titers of antibodies against LID-1 protein were found in 87–92% MB and 7–48% PB patients in different populations [71, 73]. Some individuals had high titers of anti-LID-1 antibodies 1 year before the appearance of clinical symptoms of leprosy suggesting a role of this protein in the monitoring of contacts. Interestingly, LID-1 can also be used in a cell-based IGRA assay to determine the cell-mediated immune status as in case of the "QuantiFERON" assay for TB. There has been a lot of research on the immunology and biomarkers of leprosy and its reactions in the past few decades; however, it has not translated into clinical practice, and at present there are no good biomarkers for leprosy diagnosis, susceptibility, detection of reactions, or neuritis [71].

19.3 Evaluation of Nerve Damage

Electrophysiology (EPG) of the peripheral nerves especially the nerve conduction studies with or without sympathetic skin response (which measures autonomic dysfunction) is a sensitive tool for the detection of the earliest alterations of sensory fibers or autonomic functions, thereby detecting the neuropathy much before the clinical symptoms appear. However, EPG is an invasive procedure, and it requires expensive equipment and a neurologist for its interpretation, which limits its applications in leprosy. Moreover it is overly sensitive and at times may detect changes which are not of clinical significance and is not preferred by some researchers [36].

Another emerging investigational modality is high-resolution ultrasonography (HRUS) which is a useful noninvasive tool in the evaluation of the nerve involvement of leprosy. HRUS provides useful information about the nerves involved and degree of nerve enlargement, alterations in nerve morphology, echotexture, vascularity, and fascicular pattern that may be helpful in the diagnosis and treatment of peripheral nerve disorders. The increased blood flow and vascularity observed on ultrasound (US) have been associated with the inflammatory process, and therefore, it could be a useful modality for determining the need to initiate corticosteroid therapy to prevent/treat the nerve damage associated with reactions [74].

19.4 Reactions in Leprosy

Leprosy reactions are responsible for the morbidity associated with the disease. Reactions are seen in up to 50% of patients and are the consequence of the dynamic nature of the immune response to *M. leprae***.** Reactions are responsible for much of the permanent nerve damage, leading to disability and deformities. The term reaction is used to describe the appearance of symptoms and signs of acute inflammation in the lesions of leprosy. Clinically redness, swelling, and tenderness of skin lesions are present with associated swelling, pain, and tenderness of nerves often accompanied by nerve function impairment. New skin lesions may appear and new nerve involvement may become apparent. Three types of reactions are seen in leprosy: type 1 (reversal reaction), type 2 (erythema nodosum leprosum), and type 3 (Lucio phenomenon). These reactions can occur before, during, and after treatment. Some patients have recurrent or persistent reactions which are difficult to manage and cause significant morbidity; it is essential to identify the triggering factors for reaction in these patients.

One of these triggering factors is oral infections (periodontitis, gingivitis, poor dental hygiene and caries teeth, etc.) which favor the expression of intracellular cytokines and probably the inflammatory reaction, acting as a stimulatory signal triggering the reactional episodes, and sometimes treatment of these coinfections may prevent recurrent reactional episodes [36, 75, 76].

19.5 Type 1 Reaction (Reversal Reaction)

It is the delayed type of hypersensitivity due to killing of *M. leprae* and can occur in any spectrum of the disease but mostly seen in the unstable borderline (BT, BB, BL) leprosy. Type 1 reactions in leprosy can be both upgrading and downgrading reactions; however, it is difficult to clinically differentiate the two, so the term reversal reaction is used for both. It is associated with rapid increase in the specific CMI which presents as inflammatory reaction in the existing skin and nerve lesions. It is characterized by increase in redness, swelling, tenderness/discomfort, and even ulceration in the existing lesions (Fig. 9). There may be a rapidly progressive neuritis which can lead to muscle paralysis and subsequent deformities. Constitutional symptoms are usually absent in these cases [75, 76].

19.6 Pathogenesis of Type 1 Reaction

It is related to upregulation in CMI and subsequent interaction of the breakdown product of lepra bacilli with T lymphocytes. Some studies have observed that it is not only the killed bacilli or bacillary products but also the metabolically active *M. leprae* in the skin or nerve lesion(s) can also trigger type 1 reaction especially in BT-BL spectrum. Incomplete killing or refractoriness to treatment or persistence of *M. leprae* increases the risk of reaction [77]. There is activation of CD4+ T lymphocytes and macrophages with production of Th1-type cytokines—IFN-γ, IL-2, and IL-12. TNF-α plays a crucial role in induction of type 1 reaction which

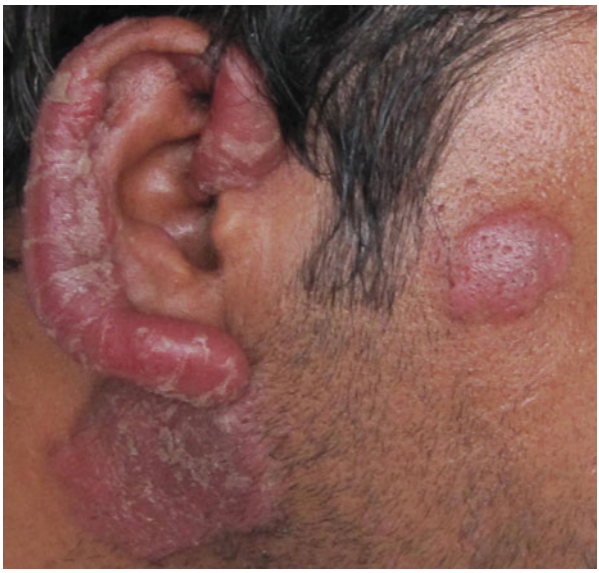


Fig. 9 Intense erythema and edema of a lesion in type 1 reaction

is evident from the rise of TNF-α levels 4–8 weeks before type 1 reaction and reaction-related nerve impairment episodes [75, 77]. However so far there are no routine laboratory tests or biomarkers to assist in the diagnosis. Elevated serum levels of chemokine CXCL10 have been strongly associated with the occurrence of T1R, although CXCL10 levels are not elevated prior to occurrence of reaction and therefore are not predictive [78, 79].

19.7 Type 2 Reaction

It occurs in patients with multibacillary disease and is seen in the LL and BL spectrum. Attacks are often acute in onset but may become chronic or recur over several years. The cutaneous lesions in type 2 reaction or erythema nodosum leprosum (ENL) typically manifest as painful, red evanescent nodules on the face and extensor surfaces of the limbs (Fig. 10). Rarely the lesions may be bullous, pustular, necrotic, or ulcerative (Fig. 11). Systemic symptoms like fever and malaise are often present, and in severe form, uveitis, dactylitis, arthritis, neuritis, lymphadenitis, myositis, and orchitis are also observed. Sometimes type 2 reaction may present without ENL or cutaneous lesions, and the patient may present with fever and arthritis or severe neuritis. Nerve involvement in the form of acute or subacute neuritis with or without nerve function impairment (NFI) is one of the major criteria for distinguishing mild and severe ENL [75, 76].

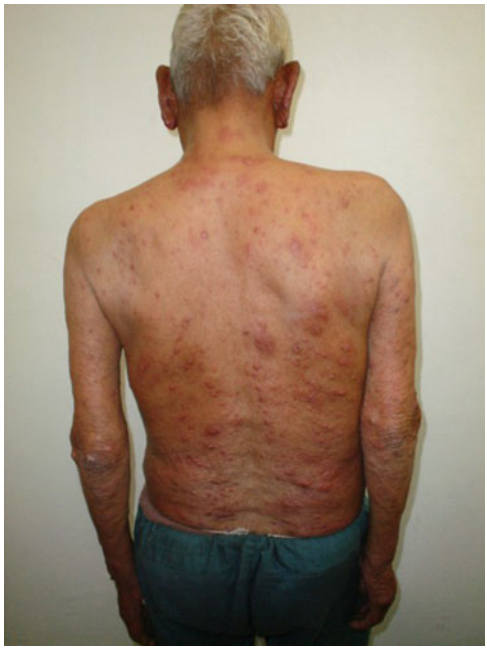


Fig. 10 Nodular lesions of LL and red evanescent nodules on the back in type 2 reaction

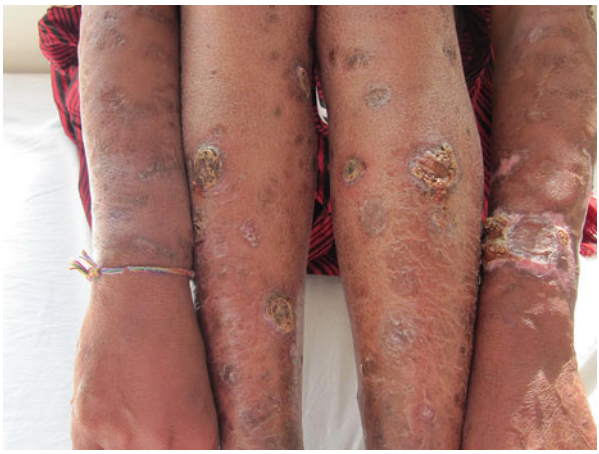


Fig. 11 Ulcero-necrotic lesions in severe type 2 reaction

19.8 Pathogenesis of Type 2 Reaction

Type 2 reaction is due to Th2-mediated type 3 hypersensitivity. Lepromatous leprosy is mainly characterized by the predominance of CD8+ cells. With onset of type 2 reaction, macrophages present the *M. leprae* antigens to the T cells, and there is infiltration of CD4+ cells in the dermis. Increase in IL-4, IL-5, and IL-10 cytokines is indicative of Th2-type response. They stimulate antibody release. Following antibody release, immune complex deposition takes place followed by complement stimulation. There is neutrophilic chemotaxis and levels of TNF-α are increased. This is followed by fever and tissue damage including the nerves [75, 76, 78]. Some studies have also implicated interleukin-6 as a susceptibility gene for leprosy type 2 reaction [80].

20 Systemic Involvement

Leprosy manifestations can resemble many dermatologic and neurological diseases and affect multiple organs, making its recognition challenging. In particular, the neurological and endocrine manifestations caused by leprosy have been long recognized but underestimated, even by specialists. Systemic involvement is usually seen in long-standing disease and predominantly in patients near lepromatous pole due to bacillary dissemination and associated granulomatous infiltration affecting various organs especially the nasal mucosa, eyes, bones, testes, kidneys, lymph nodes, liver, and spleen [81]. Besides the disease, systemic manifestations in the form of constitutional symptoms like fever, malaise, joint pains, and acute inflammation of eyes, joints, and related to reticuloendothelial system, etc. occur mostly as a part of type 2 lepra reaction. Systemic involvement is of significance

because it may serve as a sanctuary of *M. leprae*, which may be responsible for relapse even after adequate therapy [81].

20.1 Treatment

Multidrug therapy (MDT) recommended by WHO in 1982 has proved to be the most effective tool in the treatment of leprosy. It has cured approximately 16 million patients over the last 15 to 20 years. The three main objectives in management of leprosy are to interrupt transmission, cure patients, and prevent development of deformities due to reactions [82].

The concept of giving multiple drugs for treatment of leprosy was based on estimation that an advanced, untreated lepromatous leprosy patient harbors about 11 logs of live bacilli. Out of these, the proportion of naturally occurring drug-resistant mutants is estimated to be 1 in 7 logs for rifampicin and 1 in 6 logs each for dapsone and clofazimine. The organisms resistant to one drug will be susceptible to the other drugs in MDT, because their mechanisms of action are different. With combination therapy, the probability of emergence of mutant resistance to any two drugs reduces to 1 in 13 logs, which is negligible [83].

MDT comprises three drugs: dapsone, rifampicin, and clofazimine.

PB patients are treated with MDT-PB (rifampicin + dapsone) regimen for 6 months, and MB patients are treated with MDT-MB (rifampicin + dapsone + clofazimine) regimen for 12 months [82].

MDT is safe and effective and can be easily administered under field conditions. It is available in convenient monthly calendar blister packs.

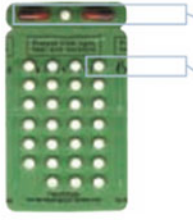
MDT-PB		
Age (in years)	Dapsone: daily unsupervised (mg)	Rifampicin: monthly supervised (mg)
<10	2 mg/kg	10 mg/kg
10–14	50	450
15 and above	100	600
Duration, 6 months		

MDT-MB				
			Clofazimine	
Age group (in years)	Dapsone: daily unsupervised (mg)	Rifampicin: monthly supervised(mg)	Unsupervised: (mg)	Monthly supervised (mg)
<10	2 mg/kg	10 mg/kg	1 mg/kg	6 mg/kg
10–14	50	450	50 on alternate days	150
15 and above	100	600	50 daily	300
Duration, 12 months				

MDT Regimens

Each blister pack contains treatment for 4 weeks.

It is crucial that patients understand which drugs they have to take once a month and which every day.



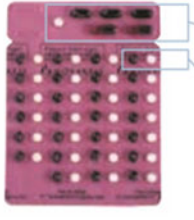
PB adult blister pack

PB adult treatment:

Once a month: Day 1
– 2 capsules of rifampicin (300 mg X 2)
– 1 tablet of dapsone (100 mg)

Once a day: Days 2–28
– 1 tablet of dapsone (100 mg)

Full course: 6 blister packs



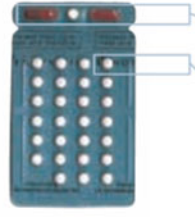
MB adult blister pack

MB adult treatment:

Once a month: Day 1
– 2 capsules of rifampicin (300 mg X 2)
– 3 capsules of clofazimine (100mg X 3)
– 1 tablet of dapsone (100 mg)

Once a day: Days 2–28
– 1 capsule of clofazimine (50 mg)
– 1 tablet of dapsone (100 mg)

Full course: 12 blister packs



PB child blister pack

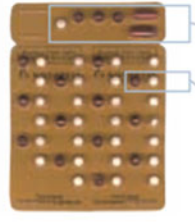
PB child treatment (10–14 years):

Once a month: Day 1
– 2 capsules of rifampicin (300 mg+150 mg)
– 1 tablet of dapsone (50 mg)

Once a day: Days 2–28
– 1 tablet of dapsone (50 mg)

Full course: 6 blister packs

For children younger than 10, the dose must be adjusted according to body weight.



MB child blister pack

MB child treatment (10–14 years):

Once a month: Day 1
– 2 capsules of rifampicin (300 mg+150 mg)
– 3 capsules of clofazimine (50 mg X 3)
– 1 tablet of dapsone (50 mg)

Once a day: Days 2–28
– 1 capsule of clofazimine every other day (50 mg)
– 1 tablet of dapsone (50 mg)

Full course: 12 blister packs

For children younger than 10, the dose must be adjusted according to body weight.

Children must receive the same multidrug therapy regimen, and the doses should, ideally, be calculated based on the weight of the child.

21 Other Regimens for Special Situations

Uniform MDT (U-MDT) Uniform MDT implies same treatment for all patients irrespective of the classification; all leprosy patients (PB and MB) receive 6 months of treatment with rifampicin, clofazimine, and dapsone. WHO in collaboration with the Global Leprosy Programme (GLP) launched a clinical trial for comparative evaluation of U-MDT with conventional WHO-MDT regimens for MB and PB leprosy. In a study from Bangladesh, 1612 patients were enrolled and followed up for over 7 years after diagnosis. During 11,425 person years at risk (PYAR) of follow-up, no relapses were detected, by bacteriological or clinical criteria, in the 918 patients in the 6-month MB-MDT group nor in the 694 patients in the control group. Rate of decline of BI in those who were smear positive was not significantly different between groups. The authors were of the opinion that shortening the duration of treatment from 12 months to 6 months MDT for MB leprosy patients does not lead to increased rates of relapse [84]. However the other view is that although U-MDT for 6 months is well tolerated and may have a marginal beneficial effect in PB leprosy, it is too short a regimen to adequately treat MB leprosy [83].

Accompanied MDT (A-MDT) It was recommended by WHO for people living in hard-to-reach border areas, urban slums, areas of civil strife, and those working as migrant laborers. The patient is provided with a full course of treatment on their first visit to the leprosy clinics after diagnosis. It is no longer a favorite with leprosy specialists because of issues of nonadherence [83].

Special treatment regimens are required for individual patients who cannot take rifampicin or clofazimine due to side effects or intercurrent diseases or are infected with drug-resistant *M. leprae*.

The WHO Expert Committee on Leprosy has recommended alternative regimen for adult patients, comprising of ofloxacin, minocycline, and clarithromycin, given in different dosage schedules and different durations for PB and MB leprosy [37, 83].

Drug Resistance MDT has been the backbone of leprosy elimination so far; hence, drug resistance in leprosy is a matter of concern. The WHO convened a working group of collaborating laboratories to use molecular methods for monitoring of drug resistance globally [85]. There are sporadic reports of drug resistance for dapsone, rifampicin, and ofloxacin, from India and various parts of the world [86]. However, as per the drug resistance surveillance report of 2014:

> drug resistance in leprosy is not a big problem at present but it is a potential future challenge [87]. Longitudinal observation however, should be continued, alongside primary and other secondary leprosy case surveillance. The situation in leprosy control is not the same as in

TB, and vigilance needs to be continued to prevent the occurrence and spread of drug resistance and thus maintain the effectiveness of MDT [87].

Although mouse foot pad assay is considered to be the gold standard for diagnosing drug resistance, it is a very cumbersome and tedious method and has been replaced by mutation detection by sequencing or DNA microarray for the identification of several mutations associated with resistance to individual agents [86]. The drug resistance-determining region (DRDR) of the genome is amplified for mutations in the *rpoB*, *folP*, and *gyrA* genes to look for resistance for rifampicin, dapsone, and ofloxacin, respectively [86].

Response to Treatment and Follow-up The erythema and induration of skin lesions may decrease within a few months of starting MDT. Most lesions heal without scarring; however, it may take a few years for cutaneous lesions to resolve fully, and some lesions may persist, depending on the initial number of lesions and severity of infection [82].

After treatment completion, the dead *M. leprae* are removed from the tissues very slowly; some may persist in the tissues for several years. There is no definitive bacteriological endpoint for treatment since *M. leprae* cannot be grown in culture and its viability cannot be assessed in biopsies. The presence of bacilli in smears or biopsies after treatment does not indicate treatment failure or drug resistance, and there is no evidence that prolonged antimicrobial treatment enhances the removal of dead *M. leprae* from tissues although immunotherapy may be helpful in some of these cases.

Given the lack of a definitive therapeutic endpoint (clinical, bacteriological, or immunological), assessing treatment adherence is extremely important in assessing treatment completion. Laboratory evidence has shown that *M. leprae* are killed rapidly after exposure to rifampicin and the other drugs used in the treatment of leprosy. Experience with WHO-MDT protocols with 1–2 years of treatment has provided good evidence of cure with very few relapses. Therefore, if adherence to these WHO-MDT protocols is good, killing of the bacilli and resolution of the lesions can be expected in majority of patients [88].

During the treatment, the first follow-up visit should be done in 2–4 weeks to evaluate for side effects of medications. After that, routine follow-up visits can be scheduled every 1–3 months. Follow-up visits should include clinical examination, including assessment of the skin, nerves, limbs, and eyes, and laboratory studies to assess drug toxicity wherever facilities are available. Patients should be instructed to report appearance of new skin lesions, nerve function impairment in the form of sensory or motor loss, eye symptoms, reactions, or other complaints. Routine laboratory studies to assess drug toxicity while on treatment include a complete blood count, urinalyses, and renal and liver function tests. Drug toxicity is relatively uncommon after the first year of treatment, and serious toxicity may manifest clinically before it is detected in the laboratory. Asymptomatic liver enzyme elevation of up to three times normal is acceptable.

It is believed that disease progression or worsening during therapy is almost always due to poor adherence to treatment regimes. Therefore, patient education is

an important part of each visit; compliance with a prolonged drug regimen is unlikely unless the patient fully understands its necessity. Family cooperation is also very important in ensuring good adherence and treatment completion [89].

The patients should be taught to evaluate the anesthetic or hypoesthetic areas of their hands and feet regularly for evidence of injury and should seek treatment if they find any evidence of injury. Special protective shoes are also required to avoid injury or ulceration in cases of sensory impairment of feet. Motor loss resulting in deformities and disabilities may require physiotherapy and eventually corrective surgery.

Ocular examination should include assessment of lid closure, cornea, and conjunctiva which should be done at the first visit and repeated on follow-up. Complex problems such as iridocyclitis should be managed by an ophthalmologist. Potentially sight-threatening ocular involvements like corneal anesthesia and lagophthalmos require protective measures and corrective surgery.

After completion of treatment, annual follow-up for 3 more years is warranted for tuberculoid (PB) cases and for at least 9 more years for lepromatous (MB) patients. Patients should be advised to return for evaluation if new lesions or other problems develop. The follow-up visits can be planned every 3–6 months where, besides the clinical, neurological, and ocular examination, SSS and biopsy can be repeated in (MB) cases. Skin biopsies should be done preferably from the same lesion at 1- to 2-year intervals to assess the response to treatment by evaluating the reduction of granulomatous inflammation and the decline of bacilli in the tissues.

The proportion of MB cases has been gradually increasing in the post-elimination era. There are unpublished reports about clinical nonresponsiveness to fixed duration MDT in some patients with high bacillary index, and some leprosy workers/physicians feel that something other than the standard MDT may be required for this subset of patients. Some experts feel that since the standard MDT has only one bactericidal drug, rifampicin, newer bactericidal agents like rifapentine, moxifloxacin, sparfloxacin, ofloxacin, minocycline, clarithromycin, etc. may be used in alternative antileprosy regimes for these patients and may prove to be helpful in patients who have drug resistance or are not responsive to WHO-MDT MBR. However there are no guidelines or recommendations for these alternative drug regimes [89].

21.1 Vaccines in Leprosy

Vaccination in leprosy can be immunoprophylactic or immunotherapeutic. Several studies have found immunotherapy with *MIP* (*Mycobacterium indicus pranii*) *or BCG* to be useful particularly in multibacillary patients with high bacterial load as it enhances bacterial clearance by upregulating the specific immune response [90, 91]. Vaccines are better than drugs for a sustained protection because vaccines provide memory but drugs do not. There is an emerging need in leprosy research to

further evaluate the vaccines BCG, BCG with heat-killed *M. leprae*, MIP, ICRC (Indian Cancer Research Centre) strain, *M. vaccae*, *M. vaccae* with BCG, and *M. habana* for leprosy prevention and immunomodulation. Newer vaccines like a subunit vaccine developed by IDRI (Infectious Disease Research Institute) have been tested in armadillo, wherein it was able to prevent nerve damage and may prove useful in humans [89].

MIP has been found to be effective as an immunotherapeutic modality in few studies from India. It was seen that the MIP vaccine led to a faster reduction in bacillary load along with decrease in the frequency and severity of type 2 reactions without exacerbating type 1 reactions and neuritis. It can be used as an adjunct to MDT in leprosy patients with a high bacillary load [90, 91].

21.2 Management of Lepra Reactions

Lepra reactions should be treated promptly to control and prevent complications and deformities. The principles of treatment of reactions are to control the acute inflammation in skin and nerves, alleviate the pain, stop progression of eye damage, and prevent disease progression or worsening. Standard antileprosy treatment (WHO-MDT) should be started or continued along with treatment of reactions. Clinical evidence of ongoing neuritis or new nerve function impairment (NFI) (nerve tenderness, new anesthesia, and/or motor loss) should be carefully sought, and, if present, corticosteroid treatment should be started immediately. Patients need to be reassured and explained about the need to continue MDT [92].

Mild reversal reactions can be treated with aspirin and nonsteroidal anti-inflammatory drugs (NSAIDS). In severe reversal reactions, the presence of neuritis, or involvement of facial lesions, corticosteroids are the drug of choice. The 7th WHO Expert Committee on Leprosy recommended (WHO, 1998) [37] that most reactions and neuritis can be treated successfully under field conditions by a standard 12-week course of prednisolone (starting dose 40–60 mg daily); however, some clinicians believe that this duration might be short and could lead to recurrences. The dose should be maintained until the inflammatory changes in skin lesions and the neuritic pain have subsided. Then tapering of the drug can begin slowly until a maintenance dose of 10–15 mg (0.3 mg/kg) prednisolone daily is reached, which must be continued for 3–4 months. Resting of the affected limb in case of neuritis to prevent further deterioration and splinting in the functional position with gentle joint movements to prevent contractures are important components of care. Once the acute inflammation has subsided, physiotherapy should be initiated. For the patients who cannot tolerate or become steroid dependent, alternate drugs most commonly used are azathioprine, methotrexate, cyclosporine, mycophenolate mofetil, etc. In cases of persistent severe nerve pain or nerve abscess, surgery (decompression) is necessary.

Mild cases of *type 2 lepra reaction* can be managed symptomatically with analgesics and rest and taking care of the precipitating factors like intercurrent

infections like malaria, filariasis, tuberculosis, and helminthic infections and psychological and physical stress. In cases of severe reaction, thalidomide is the drug of choice, if available and not contraindicated in the given patient. Thalidomide is given in an initial dosage of 300–400 mg/day (6 mg/kg/day), which will control the reaction within 48 h. The dose is then reduced gradually (100 mg/week). Patients should be stabilized on the lowest possible dose of thalidomide to control symptoms (50–100 mg daily) and be maintained on this till the control of the disease. A good alternative if thalidomide is unavailable or contraindicated in the patient is corticosteroids; 60 mg of prednisolone (1–2 mg/kg/day) daily will control most reactions, and tapering can usually be done more quickly compared with type 1 lepra reaction.

In a significant proportion of patients, ENL becomes chronic and the patient will need continuous steroid treatment. In these cases, high-dose clofazimine is useful, given in a dose of 300 mg daily until the steroids are discontinued. The dose of clofazimine can be then tapered gradually to 50 mg daily. Other drugs tried in refractory cases of ENL are azathioprine, cyclosporine, methotrexate, pentoxifylline, colchicine, mycophenolate mofetil, zinc, and infliximab. Newer drugs like leukotriene receptor antagonist (zafirlukast and montelukast), thalidomide derivative (Revlimid and Actimid), and anti-TNF-α antibody like infliximab have also been tried successfully in few cases, but the evidence is lacking [75, 76]. Immunotherapy (*MIP*/*BCG*) and minocycline can also be tried in some patients with high bacillary load as these agents also help to reduce the bacillary load, and minocycline has additional anti-inflammatory properties [93].

21.3 Treatment and Prophylaxis for Nerve Damage

The multidrug therapy used in the treatment in leprosy is mostly targeted at killing the organism but not preventing the nerve damage. Hence interventions to prevent or treat the nerve damage are required. Steroids are the most commonly used drug for this purpose although the recovery may be limited and prevention is not guaranteed. Few studies have been undertaken to see the response to treatment in prevention and recovery of the nerve damage in leprosy.

TRIPOD study is a triad of randomized, controlled trial on prevention of neuropathy which leads to impairment and disability in leprosy.

TRIPOD I In the study done in Nepal and Bangladesh, 20 mg of prednisolone was given to multibacillary patients requiring 12 months of MDT. Prednisolone was started with MDT; full dose was given for the first 3 months and tapered and stopped in the 4th month. There was significant reduction of the events of neuritis/reaction by 75% in the first 4 months, but the effect was not sustained. At the end of 12 months, only 31% reduction in the events occurred as compared to control group which was not significant [94].

TRIPOD II It was designed to investigate whether the leprosy patients with mild sensory impairment have a better prognosis when treated with steroids than similarly impaired patients treated with placebo. The patients were treated with prednisolone starting at 40 mg daily and tapered over 16 weeks. The study showed no significant difference in the outcome at the end of study [95].

TRIPOD III In this study, patients with untreated nerve function impairment for less than 6 months and those with NFI of more than 6 months and less than 24 months were included. The patients with nerve function impairment of more than 6 months are usually not offered any treatment. The patients were started on prednisolone 40 mg OD, and it was tapered by 5 mg every 2 weeks until 16 weeks when the steroids were tapered and stopped. The study showed there was no significant difference between the patients administered with steroids and in the placebo group both with recent or long-standing NFI [96].

Although steroids are effective in acute neuritis and recent onset NFI, evidence from these three randomized controlled trials is insufficient to draw robust conclusions about the long-term effect of corticosteroids for treating nerve damage in leprosy. Two trials, of which one treated long-standing nerve function impairment and the other mild sensory impairment, did not show significantly better outcomes with corticosteroids than placebo for treating nerve damage in leprosy in the long term [97]. However, another study with 5-month duration of steroid therapy reported better results compared to the group which was given steroids for 3 months. May be the dose or duration of therapy was not adequate—in the TRIPOD series [98]. However, it was observed that standard corticosteroid regimens are not significantly more harmful than placebo treatment. Despite known adverse effects of corticosteroids [98], in a recent update on use of corticosteroids in management of leprous neuropathy, it was concluded that more RCTs are required to establish optimal corticosteroid regimens (dose and duration) and to examine the efficacy and safety of adjuvant or new therapies for treating nerve damage in leprosy. Nerve decompression is another alternative in case of acute neuritis or nerve abscess in leprosy which is generally reserved for cases not responding to medical treatment, although some researchers believe that it can be considered in early management of acute neuritis as well. Besides the efficacy and safety, we should also address nonclinical aspects, such as costs and impact on quality of life, which are highly relevant indicators for both policymakers and participants [98].

22 Other Treatment Issues

22.1 Relapse

WHO recommends 1 year of MB-MDT for MB patients (12 pulses in 18 months) and 6 months of PB-MDT (6 pulses in 9 months) for PB patients. At any point of time during therapy, the patient should have ingested two-thirds of the pulses till

that time. For operational purposes, once a patient receives adequate chemotherapy, he is considered "cured" [99]. Histopathological resolution of the lesions and clinical subsidence of the disease may take months to years after MDT is stopped. So it is difficult to define relapse when there are no defined criteria of clinical or bacteriological cure. However for operational purposes, the WHO in 1988 proposed that "A patient who successfully completes an adequate course of MDT, but who subsequently develops new signs and symptoms of the disease, either during the surveillance period (2 years for PB and 5 years for MB leprosy) or thereafter" as relapse [37]. However some of the leprosy researchers are of the opinion that a period of 2 and 5 years is very short when we are defining follow-up for detecting relapse rate and should be extended to 3 and 9 years. The other definitions and criteria like clinical, bacteriological, histopathological, or immunological criteria are used for research. Relapse of leprosy is relatively rare and must be distinguished from immunologic reaction (which is more common). The World Health Organization has reported that among 103 countries reporting relapses, 57 reported zero relapses and 46 reported relapse after treatment, with a total of 3039 cases reported in 2015 [4].

Most relapses occur 5–10 years after completion of treatment. Relapse is more likely to occur in the setting of incomplete treatment or a very high bacterial load at the onset of treatment [100]. Patients who have had recurrent or persistent reactions and have been treated with steroids are also more likely to relapse than patients with lesser reactions during true relapse; the tissue bacterial load generally rises steadily. Relapse can be distinguished from immunologic reaction in that the latter should improve after a short course of prednisone [99].

There is little evidence to guide the approach to retreatment after relapse. In general, treatment consists of reinitiating the same regimen used for initial therapy [99]. Patients who presented initially with tuberculoid (paucibacillary, PB) disease but relapse with lepromatous (multibacillary, MB) leprosy should be retreated with an MB regimen. Drug resistance is extremely unlikely to have developed as long as the original *M. leprae* strain was fully sensitive to the drugs used, although there is no role for baseline testing of drug resistance. When indicated, testing for mutations can be done from paraffin-embedded tissues taken at the time of diagnosis (i.e., before treatment) and at the time of suspected relapse or resistance.

Leprosy and HIV There has been no increase in leprosy in regions where HIV is prevalent. In patients coinfected with *M. leprae* and HIV, initiation of antiretroviral therapy may trigger a type 1 reaction; this is a manifestation of the immune reconstitution inflammatory syndrome [101]. Although worsening of disease and increased incidence of reactions in patients with HIV coinfection have been observed by some, this has not been confirmed in all the studies.

The response to leprosy treatment in HIV-infected individuals appears to be comparable with the response in HIV-uninfected individuals.

Leprosy and Pregnancy Management of leprosy and immunologic reactions in pregnancy is the same as described above for other patients. Leprosy can be exacerbated during pregnancy, and without treatment it can permanently damage

the skin, nerves, limbs, and eyes; therefore WHO-MDT should be taken regularly in all the stages of pregnancy and during lactation as it also protects the child from getting infected. Immunologic reactions appear to occur more frequently in pregnancy and postpartum period. In two small series, such reactions were observed in up to 38 percent of patients [102]. Type 2 reactions were observed more frequently during pregnancy; type 1 reactions were observed more frequently in the postpartum period.

22.2 Prevention

Control measures for leprosy include clinical management of active cases as well as contact management. Household contacts should be evaluated annually for evidence of disease for at least 5 years and should be educated to seek immediate attention if skin or neurologic changes suggestive of leprosy develop.

There are no universally accepted recommendations for prophylaxis in contacts; however, a randomized controlled trial with chemoprophylaxis for contacts of leprosy patients using a single dose of rifampicin (SDR) has shown an overall protective effect of approximately 60%, effective in the first 2 years after the intervention. This protective effect increased to 80% in contacts who had previously received BCG vaccination. It was observed that SDR for contacts of patients with newly diagnosed leprosy was effective for preventing development of clinical disease within 2 years, and the authors observed no difference between the placebo and rifampicin groups after 2 years.

BCG alone or in combination with *Mycobacterium leprae* or related mycobacterial vaccines has been used in vaccine trials for immunoprophylaxis in contacts of leprosy patients, with BCG giving the best results. Meta-analysis shows that the protective effect of BCG is better in observational studies (60%) than in clinical trials, 41%, and it is higher among contacts of leprosy patients than among the general population, 68% versus 53% [103].

BCG is administered at birth in most countries with high rates of leprosy, and vaccination with BCG is partially protective for leprosy; a single dose appears to be 50% protective, and two doses further increase this protection. However vaccination for prevention of leprosy is economically feasible only in areas with an extremely high incidence of the disease and may not be feasible in other areas with low endemicity. There has been a renewed interest in development of an improved BCG vaccine, BCG booster, or alternate vaccine strain that could benefit control of both tuberculosis and leprosy. Skin test antigen studies and the identification of the appropriate protective *M. leprae* genomic DNA sequence could also help in the development of an improved vaccine for leprosy. The future leprosy control strategy should include contact management, consisting of a contact survey, and chemoprophylaxis or immunoprophylaxis. Modeling studies have shown that both interventions will lower the incidence of leprosy in the population by

interrupting the transmission of *M. leprae*. Implementation studies of such contact-based strategy are the need of the hour.

In an attempt to check the transmission of leprosy and further reduce the leprosy burden, the Indian government has started a pilot project in five districts of Bihar and Gujarat where the MIP vaccine will be administered as a preventive measure to people living in close contact with those infected.

22.3 Rehabilitation in Leprosy: Prevention of Impairment and Disabilities and Deformities

Although leprosy is responsive to MDT, disabilities of the eyes, hands, and feet due to neuropathy are often not reversible and may require lifelong care and rehabilitation. Therefore, early diagnosis and complete treatment are necessary to minimize the likelihood of these disabilities. The central goal of rehabilitation is to restore the health and dignity of someone affected by an illness that may have caused physical, mental, or emotional hurt and that may have led to social problems, such as the loss of a job or the disruption of close relationships [82].

Transient or permanent nerve damage is responsible for most of the impairments and disabilities in leprosy. A baseline nerve function assessment should be done at the time of diagnosis, and it should be repeated at regular intervals; after diagnosis the frequency of assessment can be more if patients have neuritis at the time of diagnosis or are undergoing treatment for neuropathy [104]. Motor function can be assessed and monitored by the manual muscle strength tests, and sensory function can be assessed by tests for touch or temperature perception.

Footwear with a hard under sole and soft, cushioned insoles are required for people with sensory impairment of feet to prevent further injuries and deformities. In cases with severe structural deformity of feet due to previous ulcers, fitted shoes may be necessary. Regular physiotherapy or specific exercises should be taught to prevent (or overcome) contractures of paralyzed hands. The leprosy-affected person with paralyzed eyelid muscles needs to develop the habit of attempting regular eye closure (think and blink). They should take safety precautions when cooking, insulating all the cooking utensils and keeping a distance from open fires. They also need to rehydrate dry skin due to loss of autonomic nerve function via soaking and oiling [104].

22.4 Role of Surgery

The residual disability or deformity due to leprosy is a serious long-term condition, which requires ongoing care and attention and eventually surgery. Surgical management is required for deformities and disabilities, like claw hand and

lagophthalmos, by reconstructive and tendon transfer surgeries. Decompression of nerves (neurolysis) was undertaken as a management of acute neuritis, and, although it does provide pain relief in chronic painful nerves and can be used to evacuate nerve abscesses, the evidence to recommend it for all cases of neuritis is lacking [104].

22.5 Stigma and Socioeconomic Rehabilitation with Empowerment of Leprosy Patients

Leprosy stigma is still a global phenomenon in both endemic and non-endemic countries. In the past, the stigma was due to the fact that leprosy was considered incurable, but now when we have a robust MDT which can cure leprosy, the stigma continues in the mind of community due to deformities associated with leprosy. The social and emotional consequences of leprosy are often far more deep-seated and disruptive and last a lifetime. Rehabilitation in the field of leprosy is an immense and wide-ranging challenge due to all these factors. Community education and changes in legislation are still needed in leprosy-endemic countries to eradicate the stigma associated with leprosy and ensure equal rights and opportunities for people affected by leprosy.

The rehabilitation interventions and the organizations associated with it have changed dramatically over years. In the past, the focus was on the professional, biomedical remedies organized by hospitals and similar institutions; however, this approach was costly and underutilized resources were available in the community. Initiatives by individuals and groups in the community gradually led to community-based rehabilitation (CBR) [3]. CBR has grown in scope over the years and now has strong links with community development and poverty alleviation as poverty and disability generally go hand in hand [105].

In order to be truly restored to their lives of dignity, those affected need to feel that they have more control over key areas of their lives. A certain level of empowerment is needed to provide the motivation for change, leading to normalization. This has led to self-care and self-help movements in the leprosy-endemic areas The leprosy patients need to be actively involved in leprosy control and rehabilitation programs [105].

22.6 Multimorbidity in Leprosy

Leprosy patients are also at risk of incurring numerous other comorbidities or chronic conditions (like hypertension tuberculosis, diabetes, etc.) which can further adversely affect their quality of life. Integration of leprosy into basic healthcare services (with leprosy-related treatment being offered by the same staff at the same

place and time as treatment for other medical conditions) is one solution to tackle this problem and is being done in most endemic countries, although such full integration is not yet achieved everywhere [106].

Another potential approach is the concept of "reverse integration," which means bringing other healthcare services into existing leprosy services, which is difficult but is being done in some endemic countries. Advantages include accessibility to specialized services under one place, affordability, and better integration of patients into rehabilitation programs [107].

22.7 Leprosy Control

The overall declining trend in the prevalence or new case detection rates should not lead to complacency and stopping further active surveillance or contact tracing. There are millions of cases that are not detected by the current practice of self-reporting as about half of the undiagnosed cases are symptomatic, which clearly indicates the failure of practice to identify all cases. According to a mathematical model to predict the number of cases at a given time, a study estimated that more than 4 million cases will be missed worldwide between 2000 and 2020 [108]. This clearly indicates the necessity of additional strategies such as chemoprophylaxis to household contacts and preferably a wider range of contacts, as well as active case finding in high-risk populations [109].

22.8 Prognosis

Long-term prognosis in leprosy patients treated with the recommended dose and duration of therapy is good, as evidenced by the low relapse rates. Mortality in leprosy is very low; however, it causes significant physical and psychological comorbidity due to its sequelae-like deformities and disfigurement. Despite high cure rates after introduction of MDT, nerve involvement, neuritis, and nerve function impairment frequently lead to deformities which are common in all types of leprosy especially where diagnosis is delayed and in those with manual occupations. This leads to social stigmatization which remains one of the most unfortunate aspects of the disease.

22.9 Research Priorities in Post-elimination Era

Leprosy has been eliminated (as a public health problem) but it has not been eradicated. Leprosy transmission continues unabated in the endemic countries as the new case detection rate has not reduced significantly. MDT alone may not be

sufficient to eradicate leprosy. There is the need for continued efforts that promote educational programs for health professionals, sustain control activities in the developing world, and promote investment in primary care systems. We will have to alleviate poverty, illiteracy, and ignorance if we want to eradicate leprosy. Leprosy research should continue on all fronts and the priorities in the next few years will be:

- To develop better and sensitive laboratory tools for early diagnosis of leprosy
- To work on identification of predictors for reactions and their treatment
- To develop tools for predicting neuritis and its prevention and development of neuroprotective agents for treatment of neuritis
- To develop vaccine for prophylaxis
- To improve the understanding of transmission dynamics
- To monitor and evaluate treatment failures and delayed and altered clinical responses
- To train and reorient workers with latest technologies and developments
- To monitor drug resistance
- To eradicate the stigma by creating awareness about leprosy in the community and empower people affected by leprosy

References

1. Robbins G, Tripathy VM, Misra VN, Mohanty RK, Shinde VS, Gray KM, Schug MD. Ancient skeletal evidence for leprosy in India (2000 B.C.). PLoS One. 2009;4(5):e5669.
2. Eichelmann K, González González SE, Salas-Alanis JC, Ocampo-Candiani J. Leprosy. An update: definition, pathogenesis, classification, diagnosis, and treatment. Actas Dermosifiliogr. 2013;104:554–63.
3. Suzuki K, Akama T, Kawashima A, Yoshihara A, Yotsu RR, Ishii N. Current status of leprosy: epidemiology, basic science and clinical perspectives. J Dermatol. 2012;39:121–9.
4. Global Leprosy Update, 2016: Accelerating Reduction Of Disease Burden. Wkly Epidemiol Rec. 2017;92:501–20.
5. WHO. Global leprosy strategy 2016–2010. Accelerating towards a leprosy-free world World Health Organization (2015).
6. Franco-Paredes C, Rodriguez-Morales AJ. Unsolved matters in leprosy: a descriptive review and call for further research. Ann Clin Microbiol Antimicrob. 2016;15(1):33.
7. NLEP – Annual Report for the year 2016–17. Central Leprosy Division, DGHS, Govt of India. 2017.
8. NLEP. Programme Implementation Plan (PIP) for 12th Plan Period (2012–13 to 2016–17). Available from nlep.nic.in/pdf/Final%20PIP,on%203%20May%202013.pdf.
9. NLEP – Progress Report for the year 2015–16 Central Leprosy Division, DGHS, Govt of India. 2016.
10. Scollard D, Stryjewska B. Epidemiology, microbiology, clinical manifestations, and diagnosis of leprosy. http://www.uptodate.com/patients/content/topic.do. Accessed 13 Oct 2016.
11. Sekar B. Bacteriological aspects. In: Kumar B, Kar HK, editors. IAL textbook of leprosy. New Delhi: Jaypee; 2016. p. 90–104.
12. Gupta UD, Katoch VM. Animal models in leprosy. Indian J Med Microbiol. 1995;13:57–64.

13. Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, et al. Massive gene decay in the leprosy bacillus. Nature. 2001;409:1007.
14. Eiglmeier K, Parkhill J, Honoré N, Garnier T, Tekaia F, Telenti A, Klatser P, James KD, Thomson NR, Wheeler PR, Churcher C, Harris D, Mungall K, Barrell BG, Cole ST. The decaying genome of Mycobacterium leprae. Lepr Rev. 2001;72(4):387.
15. Monot M, Honoré N, Garnier T, Zidane N, Sherafi D, Paniz-Mondolfi A. Comparative genomic and phylogeographic analysis of Mycobacterium leprae. Nat Genet. 2009;41:1282.
16. Han XY, Quintanilla M. Diffuse lepromatous leprosy caused by mycobacterium Lepromatosis in Quintana Roo, Mexico. J Clin Microbiol. 2015;53:3695–8.
17. Singh P, Benjak A, Schuenemann VJ. Insight into the evolution and origin of leprosy bacilli from the genome sequence of Mycobacterium lepromatosis. Proc Natl Acad Sci USA. 2015;112:4459–64.
18. Han XY, Seo Y-H, Sizer KC, et al. A new Mycobacterium species causing diffuse lepromatous leprosy. Am J Clin Pathol. 2008;130:856–64.
19. Han XY, Sizer KC, Thompson EJ, et al. Comparative sequence analysis of Mycobacterium leprae and the new leprosy-causing Mycobacterium lepromatosis. J Bacteriol. 2009;191:6067–74.
20. Goulart LR, Goulart IM. Leprosy pathogenetic background: a review and lessons from other mycobacterial diseases. Arch Dermatol Res. 2009;301(2):123–37.
21. Gaschignard J, Grant AV, Thuc NV, Orlova M, Cobat A, Huong NT, Ba NN, Thai VH, Abel L, Schurr E, Alcaïs A. Pauci- and multibacillary leprosy: two distinct, genetically neglected diseases. PLoS Negl Trop Dis. 2016;10(5):e0004345.
22. Siddiqui MR, Meisner S, Tosh K, Balakrishnan K, Ghei S, Fisher SE, et al. A major susceptibility locus for leprosy in India maps to chromosome 10p13. Nat Genet. 2001;27:439–41.
23. Mira MT, Alcaïs A, Van Thuc N, Thai VH, Huong NT, Ba NN, et al. Chromosome 6q25 is linked to susceptibility to leprosy in a Vietnamese population. Nat Genet. 2003;33:412–415. doi:10.1038/ng1096. PMID:12577057.
24. Tosh K, Meisner S, Siddiqui MR, Balakrishnan K, Ghei S, Golding M, et al. A region of chromosome 20 is linked to leprosy susceptibility in a South Indian population. J Infect Dis. 2002;186:1190–3.
25. Bhat RM, Prakash C. Leprosy: an overview of pathophysiology. Interdiscip Perspect Infect Dis. 2012;2012:181089.
26. Wilder-Smith EP, Van Brakel WH. Nerve damage in leprosy and its management. Nat Clin Pract Neurol. 2008;4:656–63.
27. Turankar RP, Lavania M, Singh M, Siva Sai KSR, Jadhav RS. Dynamics of Mycobacterium leprae transmission in environmental context: deciphering the role of environment as potential reservoir. Infect Genet Evol. 2012;12:121–6.
28. Wheat WH, Casali AL, Thomas V, Spencer JS, Lahiri R, Williams DL, McDonnell GE, Gonzalez Juarrero M, Brennan PJ, Jackson M. Long-term survival and virulence of Mycobacterium leprae amoebal cysts. PLoS Neglec Trop Dis. 2014;8(12):e3405.
29. Truman R, Fine PE. 'Environmental' sources of Mycobacterium issues and evidence. Lepr Rev. 2010;81:89–95.
30. Sharma R, Singh P, Loughry WJ, Lockhart JM, Inman WB, Duthie MS, Pena MT, Marcos LA, Scollard DM, Cole ST, Truman RW. Zoonotic leprosy in the Southeastern United States. Emerg Infect Dis. 2015;21:2127–34.
31. Bratschi MW, Steinmann P, Wickenden A, Gillis TP. Current knowledge on Mycobacterium leprae transmission: a systematic literature review. Lepr Rev. 2015;86:142–55.
32. Polycarpou A, Walker SL, Lockwood DN. New findings in the pathogenesis of leprosy and implications for the management of leprosy. Curr Opin Infect Dis. 2013;26:413–9.
33. Scollard DM, Truman RW, Ebenezer GJ. Mechanisms of nerve injury in leprosy. Clin Dermatol. 2015;33:46–54.

34. Shetty VP. Pathomechanisms of nerve damage. In: Kumar B, Kar HK, editors. IAL textbook of leprosy. New Delhi: Jaypee; 2016. p. 170–81.
35. Pinheiro RO, De Souza Salles J, Sarno EN, Sampaio EP. Mycobacterium leprae host-cell interactions and genetic determinants in leprosy: an overview. Future Microbiol. 2011;6:217–30.
36. Foss NT, Motta AC. Leprosy, a neglected disease that causes a wide variety of clinical conditions in tropical countries. Mem Inst Oswaldo Cruz. 2012;107(Suppl 1):28–33.
37. WHO Expert Committee on Leprosy. WHO Expert Committee on Leprosy: Eighth report Report. WHO Tech Rep Ser. 968; Geneva, Switzerland 2012.
38. Lockwood DNJ, Sarno E, Smith WC. Classifying leprosy patients – searching for the perfect solution? Lepr Rev. 2007;78:317–20.
39. Moschella SL. An update on the diagnosis and treatment of leprosy. J Am Acad Dermatol. 2004;51(3):417–26.
40. Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis. 1966;34:255–73.
41. Kumar B, Dogra S. Case definition and clinical types of Leprosy. In: Kumar B, Kar HK, editors. IAL textbook of leprosy. New Delhi: Jaypee; 2016. p. 236–53.
42. Vargas-Ocampo F. Diffuse leprosy of Lucio and Latapí: a histologic study. Lepr Rev. 2007;78(3):248–60.
43. Wade HW. The histoid variety of lepromatous leprosy. Int J Lepr. 1963;31:129–42.
44. Kaur I, Dogra S, De D, et al. Histoid leprosy: a retrospective study of 40 cases from India. Br J Dermatol. 2009;160:305–10.
45. Kumar B, Kaur I, Dogra S, Kumaran MS. Pure neuritic leprosy in India: an appraisal. Int J Lepr Other Mycobact Dis. 2004;72:284–90.
46. Rao PN, Suneetha S. Pure neuritic leprosy: Current status and relevance. Indian J Dermatol Venereol Leprol. 2016;82(3):252–61.
47. Nascimento OJ. Leprosy neuropathy: clinical presentations. Arq Neuropsiquiatr. 2013;71:661–6.
48. Van Brakel WH, Khawas IB. Silent neuropathy in leprosy: an epidemiological description. Lepr Rev. 1994;65:350–60.
49. van Brakel WH, Nicholls PG, Wilder-Smith EP, Das L, Barkataki P, Lockwood DN, INFIR Study Group. Early diagnosis of neuropathy in leprosy – comparing diagnostic tests in a large prospective study (the INFIR cohort study). PLoS Negl Trop Dis. 2008;2(4):e212.
50. Garbino JA, Heise CO, Marques W Jr. Assessing nerves in leprosy. Clin Dermatol. 2016;34:51–8.
51. Raicher I, Stump PR, Baccarelli R, Marciano LH, Ura S, Virmond MC, Teixeira MJ, Ciampi de Andrade D. Neuropathic pain in leprosy. Clin Dermatol. 2016;34:59–65.
52. Gitte SV, Sabat RN, Kamble KM. Childhood leprosy in an endemic area of Central India. Indian Pediatr. 2016;53:221–4.
53. Dogra S, Narang T, Khullar G, Kumar R, Saikia UN. Childhood leprosy through the post-leprosy-elimination era: a retrospective analysis of epidemiological and clinical characteristics of disease over eleven years from a tertiary care hospital in North India. Lepr Rev. 2014;85:296–310.
54. Oliviera MB, Diniz LM. Leprosy among children under 15 years of age: literature review. An Bras Dermatol. 2016;91:196–203.
55. WHO Global Leprosy Strategy agreed for 2011–2015. www.searo.who.int/EN/Section980/Section2572/Section2578_14961.htm
56. Grzybowski A, Nita M, Virmond M. Ocular leprosy. Clin Dermatol. 2015;33:79–89.
57. Malik AN, Morris RW, Ffytche TJ. The prevalence of ocular complications in leprosy patients seen in the United Kingdom over a period of 21 years. Eye (Lond). 2011;25:740–5.
58. Chauhan S, Wakhlu A, Agarwal V. Arthritis in leprosy. Rheumatology (Oxford). 2010;49:2237–42.

59. Prasad S, Misra R, Aggarwal A, Lawrence A, Haroon N, Wakhlu A, Krishnani N, Agrawal V, Paliwal VK, Jha S, Agarwal V. Leprosy revealed in a rheumatology clinic: a case series. Int J Rheum Dis. 2013;16:129–33.
60. Mahajan V, Sharma NL. Differential diagnosis of dermatological conditions. In: Kumar B, Kar HK, editors. IAL textbook of leprosy. New Delhi: Jaypee; 2016. p. 298–315.
61. Shah BJ. Differential diagnosis of neurological and other disorders in relation to leprosy. In: Kumar B, Kar HK, editors. IAL textbook of leprosy. New Delhi: Jaypee; 2016. p. 316–30.
62. Mahajan VK. Slit-skin smear in leprosy: lest we forget it! Indian J Lepr. 2013;85:177–83.
63. Lastória JC, de Abreu MA. Leprosy: a review of laboratory and therapeutic aspects – part 2. An Bras Dermatol. 2014;89:389–401.
64. Brennan PJ. Skin test development in leprosy: progress with first-generation skin test antigens, and an approach to the second generation. Lepr Rev. 2000;71 Suppl:S50.
65. Mitra DK, Joshi B, Dinda AK, Rai AK, Girdhar BK, Katoch K, Bindra MS, Sengupta U. Induction of lepromin reactivity in cured lepromatous leprosy patients: impaired chemokine response dissociates protective immunity from delayed type hypersensitivity. Microbes Infect. 2009;11(14–15):1122.
66. Kaplan G, Laal S, Sheftel G, Nusrat A, Nath I, Mathur NK, Mishra RS, Cohn ZA. The nature and kinetics of a delayed immune response to purified protein derivative of tuberculin in the skin of lepromatous leprosy patients. J Exp Med. 1988;168:1811.
67. Job CK, Jayakumar J, Williams DL, Gillis TP. Role of polymerase chain reaction in the diagnosis of early leprosy. Int J Lepr Other Mycobact Dis. 1997;65:461–4.
68. Banerjee S, Biswas N, Kanti Das N, Sil A, Ghosh P, Hasanoor Raja AH, Dasgupta S, et al. Diagnosing leprosy: revisiting the role of the slit-skin smear with critical analysis of the applicability of polymerase chain reaction in diagnosis. Int J Dermatol. 2011;50:1522–7.
69. Butlin CR, Soares D, Neupane KD, Failbus SS, Roche PW. IgM anti-phenolic glycolipid-I antibody measurements from skin-smear sites: correlation with venous antibody levels and the bacterial index. Int J Lepr Other Mycobact Dis. 1997;65:465.
70. Oskam L, Slim E, Bührer-Sékula S. Serology: recent developments, strengths, limitations and prospects: a state of the art overview. Lepr Rev. 2003;74:196.
71. De Souza VN, Iyer AM, Lammas DA, Naafs B, Das PK. Advances in leprosy immunology and the field application: a gap to bridge. Clin Dermatol. 2016;34(1):82–95.
72. Aseffa A, Brennan P, Dockrell H, Gillis T, Hussain R, Oskam L, Richardus JH, Ideal Consortium. Report on the first meeting of the IDEAL (Initiative for Diagnostic and Epidemiological Assays for Leprosy) consortium held at Armauer Hansen Research Institute, ALERT, Addis Ababa, Ethiopia on 24–27 October 2004. Lepr Rev. 2005;76:147.
73. Fabri Ada C, Carvalho AP, Vieira NF, Bueno Ide C, Rodrigues RN, Monteiro TB, Correa-Oliveira R, Duthie MS, Lana FC. Integrative literature review of the reported uses of serological tests in leprosy management. Rev Soc Bras Med Trop. 2016;49:158–64.
74. Jain S, Visser LH, Suneetha S. Imaging techniques in leprosy clinics. Clin Dermatol. 2016;34:70–8.
75. Kar HK, Chauhan A. Leprosy reactions: pathogenesis and clinical features. In: Kumar B, Kar HK, editors. IAL textbook of leprosy. New Delhi: Jaypee; 2016. p. 416–40.
76. Kamath S, Vaccaro SA, Rea TH, Ochoa MT. Recognizing and managing the immunologic reactions in leprosy. J Am Acad Dermatol. 2014:1268–7.
77. Save MP, Dighe AR, Natrajan M, Shetty VP. Association of viable Mycobacterium leprae with Type 1 reaction in leprosy. Lepr Rev. 2016;87:78–92.
78. Stefani MM, Guerra JG, Sousa AL, Costa MB, Oliveira ML, Martelli CT, Scollard DM. Potential plasma markers of Type 1 and Type 2 leprosy reactions: a preliminary report. BMC Infect Dis. 2009;9:75.
79. Scollard DM, Chaduvula MV, Martinez A, Fowlkes N, Nath I, Stryjewska BM, Kearney MT, Williams DL. Increased CXC ligand 10 levels and gene expression in type 1 leprosy reactions. Clin Vaccine Immunol. 2011;18:947.

80. Sousa AL, Fava VM, Sampaio LH, Martelli CM, Costa MB, Mira MT, Stefani MM. Genetic and immunological evidence implicates interleukin 6 as a susceptibility gene for leprosy type 2 reaction. J Infect Dis. 2012;205:1417–24.
81. Ramesh V, Kataria JK. Systemic involvement in leprosy. In: Kumar B, Kar HK, editors. IAL textbook of leprosy. New Delhi: Jaypee; 2016. p. 331–43.
82. Scollard D, Stryjewska B. Treatment and prevention of leprosy. https://www.uptodate.com/contents/treatment-and-prevention-of-leprosy? Accessed 30 Sep 2016.
83. Kar HK, Gupta R. Treatment of leprosy. Clin Dermatol. 2015;33:55–65.
84. Butlin RC, Pahan D, Maug AKJ, Withington S, Nicholls P, Alam K, Salim AH. Outcome of 6 months MBMDT in MB patients in Bangladesh-preliminary results. Lepr Rev. 2016;87:171–82.
85. Sentinel surveillance for drug resistance in leprosy. Report of the WHO Global Leprosy Programme Meeting Cotonou, Benin, 12–13 November 2012.
86. Moraes MO. Drug-resistance in leprosy: moving toward understanding the scope of the problem and how to tackle it. Clin Infect Dis. 2016 Aug 23. pii: ciw574. No abstract available.
87. World Health Organisation. Drug resistance surveillance in leprosy: report of the WHO-ILEP joint meeting 4–6 February 2014, Cebu City, Philippines. 1–31. SEA−GLP−2014.1.
88. Kaur I, Dogra S, Kumar B, Radotra BD. Combined 12-month WHO/MDT MB regimen and Mycobacterium w. vaccine in multibacillary leprosy: a follow-up of 136 patients. Int J Lepr Other Mycobact Dis. 2002;70:174–81.
89. Singh I, Lavania M, Nigam A, Turankar RP, Ahuja M, John AS, Sengupta U. Symposium on emerging needs in leprosy research in the post elimination era: The Leprosy Mission Trust India. Lepr Rev. 2016;87:132–43.
90. Katoch K, Katoch VM, Natrajan M, et al. Treatment of bacilliferous BL/LL cases with combined chemotherapy and immunotherapy. Int J Lepr. 1995;63:202–12.
91. Narang T, Kaur I, Kumar B, et al. Comparative evaluation of immunotherapeutic efficacy of BCG and Mw vaccines in patients of borderline lepromatous and lepromatous leprosy. Int J Lepr. 2005;73:105–14.
92. Sehgal VN, Sardana K, Dogra S. Management of complications following leprosy: an evolving scenario. J Dermatolog Treat. 2007;18:366–74.
93. Narang T, Sawatkar GU, Kumaran MS, Dogra S. Minocycline for recurrent and/or chronic erythema nodosum leprosum. JAMA Dermatol. 2015;151:1026–8.
94. Smith WC, Anderson AM, Withington SG, van Brakel WH, Croft RP, Nicholls PG, Richardus JH. Steroid prophylaxis for prevention of nerve function impairment in leprosy: randomised placebo controlled trial (TRIPOD 1). BMJ. 2004;328:1459.
95. Van Brakel WH, Anderson AM, Withington SG, Croft RP, Nicholls PG, Richardus JH, Smith WC. The prognostic importance of detecting mild sensory impairment in leprosy: a randomized controlled trial (TRIPOD 2). Lepr Rev. 2003;74:300–10.
96. Richardus JH, Withington SG, Anderson AM, Croft RP, Nicholls PG, Van Brakel WH, Smith WC. Treatment with corticosteroids of long-standing nerve function impairment in leprosy: a randomized controlled trial (TRIPOD 3). Lepr Rev. 2003;74:311–8.
97. Van Veen NH, Nicholls PG, Smith WC, Richardus JH. Corticosteroids for treating nerve damage in leprosy. A Cochrane review. Lepr Rev. 2008;79:361–7.
98. Van Veen NH, Nicholls PG, Smith WC, Richardus JH. Corticosteroids for treating nerve damage in leprosy. Cochrane Database Syst Rev. 2016;23(5):CD005491. https://doi.org/10.1002/14651858.CD005491.pub3.
99. Kaimal S, Thappa DM. Relapse in leprosy. Indian J Dermatol Venereol Leprol. 2009;75:126–35.
100. WHO Expert Committee on Leprosy. WHO Expert Committee on Leprosy: Seventh Report. WHO Tech Rep Ser. 874; Geneva, 1998.
101. Lockwood DN, Lambert SM. Human immunodeficiency virus and leprosy: an update. Dermatol Clin. 2011;29:125.

102. Lockwood DN, Sinha HH. Pregnancy and leprosy: a comprehensive literature review. Int J Lepr Other Mycobact Dis. 1999;67:6.
103. Richardus JH, Oskam L. Protecting people against leprosy: chemoprophylaxis and immunoprophylaxis. Clin Dermatol. 2015;33:19–25.
104. Brandsma JW. Rehabilitation of Leprosy-affected people: an overview. Clin Dermatol. 2016;34:66–9.
105. Saunderson P. Rehabilitation & social aspects: introductory overview. International textbook of leprosy. www.internationaltextbookofleprosy.org. Last Accessed 05 Oct 2016.
106. Butlin CR. The challenge of multi-morbidity in the context of leprosy. Lepr Rev. 2016;87:151–7.
107. Iyer FT. Reverse integration in leprosy: lessons from Mker, Nigeria. Asia Pacific Disabil Rehab J. 2006;17:35–41.
108. Smith WC, van Brakel W, Gillis T, Saunderson P, Richardus JH. The missing millions: a threat to the elimination of leprosy. PLoS Negl Trop Dis. 2015;9:e0003658.
109. de Matos HJ, Blok DJ, de Vlas SJ, Richardus JH. Leprosy new case detection trends and the future effect of preventive interventions in Para State, Brazil: a modelling study. PLoS Negl Trop Dis. 2016;10:e0004507.

Trachoma

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Contents

1 Introduction

Trachoma is the outcome of ocular infection by *Chlamydia trachomatis*. The clinical spectrum of the disease ranges from minor conjunctivitis, congestion and follicular inflammation to advanced long-term conjunctival scarring leading to trichiasis and corneal opacity. These long-term corneal opacities often lie in the visual axis and interfere with the pathway leading to visual impairments and corneal blindness. Once the leading cause of blindness worldwide, trachoma has since largely disappeared from the developed countries. It still remains the leading infectious cause of blindness. It is recognized among the priority neglected tropical diseases (NTDs), disproportionately affecting poor communities and contributing

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to the vicious cycle of poverty and ill health among them. Trachoma is estimated to cause a loss of US$5.3 billion annually (in 2003) when measuring trachoma blindness associated with productivity losses. The burden is believed to be much higher if trachoma-associated visual impairments were also considered [1]. There are still nearly 200 million people globally at risk of trachoma, and focused efforts are needed to achieve elimination of this disease.

2 Historical Aspects

Trachoma disease is an ancient scourge causing blindness over the millennia. Among skeletons in Australia dating as far back as 8000 BC, lesions consistent with trachoma have been identified. Egyptians knew of the disease 3500 years back. It is believed that Central Asia is one of the areas from where the disease originated and spread. First usage of the term *trachoma* has been attributed to Dioscorides (40–90 AD). Syrians knew the disease to be a contagious condition as early as thirteenth century. Over history, the disease is believed to have been limited to specific segments of communities, with spread occurring across communities during times of war, due to the associated large-scale population displacements. Trachoma is recognized as one of the causes of *military ophthalmia* that blinded a large number of soldiers during the Egyptian wars of the eighteenth century. After the wars, the return of various armies (Napoleon, British and others) to Europe is believed to have contributed to the spread of trachoma in Europe. In 1891, trachoma was the first ever disease that was classified as a 'loathsome and contagious' condition by the US congress, and immigrants suffering from the disease were excluded from entering the country. In the USA, trachoma was common in eastern states, and the area came to be known as the 'trachoma belt'. By the end of the nineteenth century, with increasing travel, the disease was widespread all over the world across all continents [2]. The causative organism of trachoma was still unknown. Intracellular cytoplasmic inclusion bodies were detected among samples from apes and humans suffering from trachoma in 1907 by Halberstaedter and Prowazek who considered the organism to be a protozoan. Inclusion bodies were accepted as the cause of the disease, and their detection became the pathognomonic sign of trachoma. Debates continued however as to the exact nature of the organism. Based on their successful culture, ability of cultured organisms to infect and cause disease and filtration experiments, T'ang et al. believed it to be a virus, a view which gained much traction. In 1966, Moulder conclusively proved these to be bacteria since the organism possesses both DNA and RNA and was susceptible to antibiotics.

3 The Causative Agent

Trachoma is caused by an obligate intracellular Gram-negative organism *Chlamydia trachomatis*. The genus *Chlamydia* had three species, *Chlamydia trachomatis* (a human pathogen), *Chlamydia suis* (affects only swine) and *Chlamydia muridarum* (affects only mice and hamsters). *C. trachomatis* has three human biovars or biotype groups of strains distinguishable from others of the same species on the basis of physiological characteristics:

1. Serotypes A, B, Ba or C—cause trachoma
2. Serotypes D–K—cause genital infections, neonatal pneumonia and neonatal conjunctivitis
3. Serotypes L1, L2 and L3—lymphogranuloma venereum

Chlamydia cannot synthesize their own ATP molecules and are obligate intracellular organisms, requiring energy-rich metabolic intermediates from host cells to complete their replication cycle. *C. trachomatis* has a biphasic development cycle distinguished by two forms, elementary body (EB) and reticulate body (RB) (Fig. 1). Elementary bodies are the small, spherical or, rarely, pear shaped and 0.2–0.3 μm in diameter, metabolically inert infectious particles which attach to and enter the susceptible host cells. They appear to bind to susceptible host cells via heparin bridges. Successful attachment of the EB is followed by its entry into the host cell. Once inside a host cell, over the next 2 hours, the elementary body transforms into the larger (0.5–1.0 μm diameter) metabolically active RB within a membrane-bound vacuole known as an inclusion body. The RB is non-infectious and metabolically active. It grows and replicates by binary fission, remaining within the inclusion membrane for the entire duration of the organism's intracellular phase. The inclusion body can occupy as much as 90% of the cytoplasmic space of the host cell. After an 18–30 h of exponential growth, progeny RBs differentiate back into EBs. These infective EBs as well as active RBs subsequently get released into the extracellular space from the host through cell lysis, nearly 40–48 h after the initial infection. This leads to the spread of infection to more cells. Sometimes, the RBs may experience a prolonged life cycle resulting in persistent infection of the host cell.

3.1 Immunity

The major outer membrane protein (MOMP, also called *omp1*) is the immunodominant surface antigen that differentiates all *C. trachomatis* isolates into serogroups and serotypes. The mucosal response to *C. trachomatis* infection involves several components of the immune system. The acquisition of repeated infections with *C. trachomatis* in trachoma-endemic areas suggests the absence of any long-lasting protective immunity. Neutralizing antibodies against the major outer

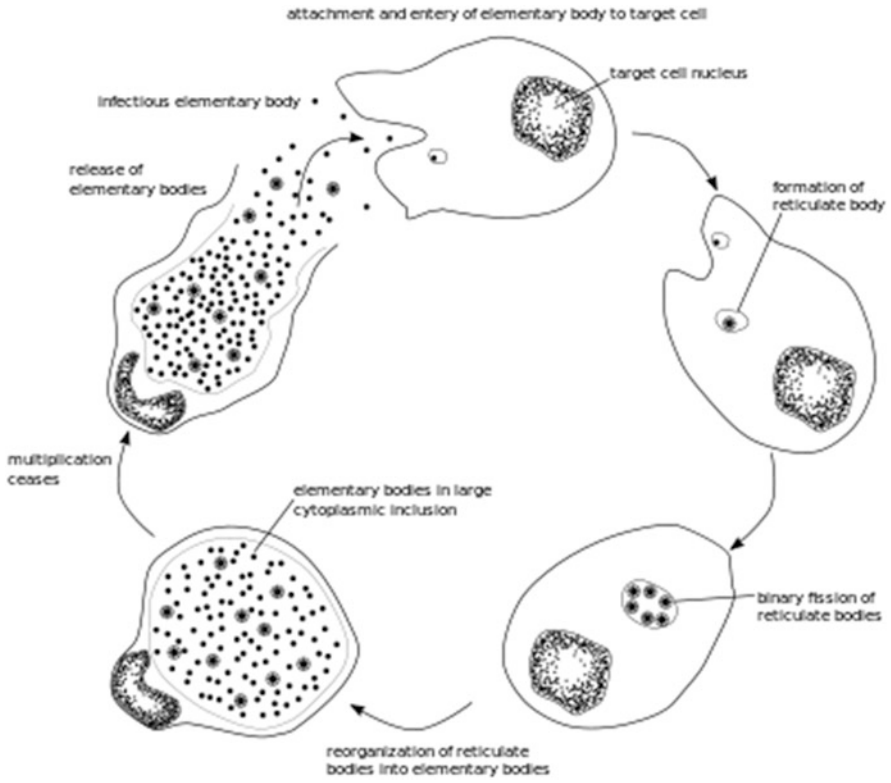

Fig. 1 Developmental cycle of the *Chlamydia trachomatis*

membrane protein (MOMP) have been shown to protect against infection in the laboratory, but the extent of natural immune response in producing some protection is still unclear. The infection triggers an innate immune response, possibly driven from the epithelium through pattern recognition receptors. Infection of human epithelial cells in vitro provokes a pronounced pro-inflammatory response, with the production of chemokines and cytokines. In fact, the immune response may well be responsible for the serious clinical manifestations of trachoma. Severe inflammation may be the result of a delayed hypersensitivity response in ocular tissues elicited by the 57-kDa chlamydial heat shock protein.

The resolution of chlamydial infection is thought to be dependent on interferon γ, mediated through nitric oxide free radicals and the depletion of intracellular tryptophan and iron. Infected children have increased expression of the genes *IFN*γ and *IL12p40*, which suggests a type-1 CD4-expressing T-helper lymphocyte (Th1) cell-mediated immune response. Other sources of interferon γ could include natural killer cells and CD8-expressing T cells.

The understanding of host defence mechanisms that promote the persistence or eliminate the growth of *C. trachomatis* is essential for the design of an effective

vaccine. Specifically, research should focus on clarifying the role of Thl-like responses in clearance of infection as well as the role of Th-2 responses in producing more severe disease. In animal models, immunity was shown to be short lived and serotype specific. Reinfection with a different serovar tends to raise antibody responses to the previous serovar. There is no evidence that tear antibodies confer protection against chlamydial infection. Byrne and Krueger have shown that gamma interferon inhibits chlamydial growth and viability in cell cultures and probably serves as one host defence mechanism.

3.2 Pathophysiology

The first sign of infection is a nonspecific vasodilatation of conjunctival blood vessels, characterized by a diffuse inflammatory cell infiltrate of the conjunctiva punctuated by lymphoid follicles. Specific changes may be noted after infection of several weeks duration, with the development of follicles subjacent to the conjunctivae of the fornices, the tarsal plates and the limbus. When inflammation is severe, an intense papillary reaction on the tarsal conjunctiva is associated with a diffuse thickening of the conjunctiva, obscuration of the deep tarsal vessels and, sometimes, eyelid oedema. The presence of follicles and papillae is a feature of active trachoma. Most children with active trachoma do not develop scarring and trichiasis.

Resolution of active trachoma may be accompanied by scarring of the sub-epithelial conjunctiva. This scarring is the result of a complex interaction between chlamydial infection and the T-cell-mediated immune response occurring over many years. Trachomatous conjunctival scarring shows disruption of loose, regular type 1 stromal collagen and dense sheets and bundles of compact type V collagen. The epithelial cells are thinned and goblet cells reduced. Scar deposition is most prominent in the upper tarsal plate, although the conjunctival fornices, the bulbar conjunctiva and the upper part of the cornea may also be involved. In areas where trachoma is endemic, upper tarsal plate scars derived from repeated episodes of infection can eventually accumulate to such an extent that they become visible macroscopically after eversion of the upper lid, appearing as white bands against the erythematous background of the conjunctiva. Conjunctival scarring precedes the development of trichiasis. If sufficient tarso-conjunctival scarring accumulates, contraction of scars over the years will cause the upper eyelid to turn inward so that the eyelashes rub against the globe. This is known as trichiasis. When the whole lid margin is turned in, the condition is known as entropion. Scars around the bases of hair follicles can pull individual eyelashes into contact with the cornea, even without entropion. Trichiasis is intensely irritating and painful. Sufferers may use homemade forceps to remove their own lashes or attempt to keep their lids elevated with strips of cloth tied around their heads. Besides being painful, trichiasis injures

the cornea. In addition to the direct abrasive effect of the in-turned lashes on the cornea, secondary bacterial and fungal infections of the cornea and corneal drying due to scarring of forniceal mucous, lacrimal and Meibomian glands accelerate epithelial damage of cornea. As a part of healing process, the collagenous scar is laid down on the cornea. Because scars are opaque, vision can be affected by scarring that involves the central part of the cornea.

3.3 Transmission

The main source of trachoma infection is infected eye secretions in persons with trachoma. The active infective stages of trachoma are usually found in children. There is no animal reservoir for trachoma. The following are the routes of transmission for trachoma:

- Direct spread due to close physical contact (e.g. while playing or sharing a bed, mothers of affected children)
- Indirect spread through sharing towels, fomites, pillows, etc.
- Flies particularly the eye-seeking fly *Musca sorbens*
- Coughing and sneezing

A combination of these and other transmission mechanisms probably operates in most environments, although their relative importance may vary.

4 Diagnosis

The diagnosis of trachoma is made on clinical grounds. The available assays include microscopy of conjunctival scrapings, isolation in cell culture, direct fluorescent antibody, enzyme immunoassay, serology, nucleic acid hybridization probes and nucleic acid amplification tests.

Microscopy This is the oldest and most widely used method for detection of active chlamydial infection. This method is based on detection of mature inclusion bodies, also known as Halberstaedter-Prowazek body. With Giemsa stain, mature inclusions appear as dark purple masses in the cytoplasm of epithelial cells. This method is time-consuming and has probably the lowest sensitivity [3]. Acridine orange and iodine can also be used for detection of these chlamydial inclusion bodies.

Cell Culture This test is highly specific, hence is considered the gold standard for chlamydial detection. Chlamydiae are highly fastidious organisms, and hence, efficient isolation, transport and plating are necessary. Successful culture relies on

the use of enriched sucrose phosphate transport medium and strict maintenance of the cold chain during transport. In the laboratory, clinical specimens are inoculated onto McCoy cells, HeLa 229 cells or L434 mouse fibroblasts. Culture takes approximately 3–6 days.

Immunofluorescence A direct fluorescent antibody test, the 'Syva MicroTrak' was the first diagnostic reagent that used a monoclonal antibody against *C. trachomatis* and began the move away from culture to techniques that do not rely on chlamydial viability. The MicroTrak uses labelled antibody to detect a species-specific epitope in MOMP (major outer membrane protein), which is then visualized under a fluorescent microscope. Usually five to ten elementary bodies are taken as threshold for positive sample.

Enzyme-Linked Immunosorbent Assays (ELISA) It refers to an antigen detection test, with antibody used to detect chlamydial antigen contained in the specimen. Conjunctival scrapings with chlamydial liposaccharide as antigen are bound with antibodies, to give a coloured substance after addition of an enzyme.

Serology A complement fixation test that detects serum antibodies against the polysaccharide antigens of lipopolysaccharide. Because these epitopes are common to all chlamydial species, the specificity of the test is low. Additionally, it has low sensitivity for ocular infections**.** Moreover, a large percentage of the population in endemic areas may demonstrate high titres, so the test is not very specific.

Polymerase Chain Reaction PCR is a technique for amplifying DNA, and assays based on it are part of the group of nucleic acid amplification tests. A number of different nucleic acid sequences have been used as targets in PCRs for the detection of *C. trachomatis*. These include the chlamydial cryptic plasmid (pCT), omp1, coding for MOMP, the gene coding for 16S rRNA, and omp2, coding for omcB. With the exception of pCT, all of these targets are sequences found on the *C. trachomatis* chromosome, which includes two complete rRNA operons and single copies of omp1 and omp2.

The specimen for PCR is collected with the help of Dacron swabs that can be stored at 2–25 degrees for up to 7 days. The presence of a luminescent DNA-RNA hybrid is measured in luminometer, and the whole process takes about 2–3 h. The sensitivity and specificity of these procedures are close to nearly 90% [4].

Tests results may often contradict with clinical findings. Tests detect infection and therefore positive tests may be observed before the development of clinical symptoms. Further, sometimes tests may be positive after the resolution of clinical symptoms. Currently, no tests exist which can be used easily in a field setting. Therefore, identifying cases in the community is based primarily on clinical features.

5 Clinical Features

Clinically, trachoma can be divided into its acute (active) and chronic (cicatricial) late-stage manifestations. Acute and chronic signs can occur at the same time in the same individual. Many infections due to *Chlamydia trachomatis* are asymptomatic. In some children, following an incubation period of 5–10 days, conjunctival infection produces an irritated, red eye and scanty mucopurulent discharge. The common symptoms of active infection are irritation of the eyes, mucoid or mucopurulent discharge and often a swelling of upper eyelids, though children may present without any symptoms. Specific changes may be observed on clinical examination after infection of several weeks duration, with the development of follicles subjacent to the conjunctivae of the fornices, the tarsal plates and the limbus. Follicles are lymphoid germinal centres and are found immediately below the epithelial cell layer. They are grey or creamy masses measuring approximately 0.2–3.0 mm in diameter. As the superficial layer of the conjunctival stroma lacks lymphoid tissue until about 3 months after birth, newborns are unable to mount a follicular response to ocular chlamydial infection. Papillae may also be noted at this stage; in mild cases, a few isolated, small red dots can be seen with the naked eye. With the aid of a slit lamp, papillae appear as small swellings of the conjunctiva, each with a central vascular core. When inflammation is severe, an intense papillary reaction on the tarsal conjunctiva is associated with a diffuse thickening of the conjunctiva, obscuration of the deep tarsal vessels and, sometimes, eyelid oedema. Vascular corneal pannus or superficial infiltrates occur in some cases, usually at the superior corneal limbus.

The chronic stage of trachoma results in cicatrizing sequelae. Resolution of follicles may be accompanied by scarring of the sub-epithelial conjunctiva. Scar deposition is most prominent in the upper tarsal plate, although the conjunctival fornices, the bulbar conjunctiva and the upper part of the cornea may also be involved. At the limbus, replacement of the active follicles by scar tissue results in Herbert's pits. Scarring at the upper tarsal conjunctiva is initially seen as stellate scars, which later forms a thick band of scar tissue near the lid margin known as the Arlt's line. Scarring may lead to obscuring of the underlying vasculature. If sufficient tarso-conjunctival scarring occurs, tylosis or thickening of the upper eyelid margin, Meibomian gland atrophy and dry eyes are evident. Contraction of the scar tissue eventually causes the upper eyelid to turn inwards so that the lashes rub against the globe, known as trichiasis. Sometimes the whole lid margin turns inwards known as entropion. Constant rubbing of the eyelashes on the corneal surface leads to formation of corneal ulcers, corneal scarring and eventually corneal opacities that cause visual impairment and irreversible corneal blindness. Presence of trichiasis is associated with intense irritation in the eyes.

5.1 Disease Classification

Dioscorides (40–90 AD) described roughness of everted eyelids as *trachoma*, while Galen (129–200 AD) introduced the term 'trichiasis'. Galen also introduced four stages of trachoma, namely, psorophthalmia (itch), choma (trachoma, roughness), sycosis (scar) and tylosis (trichiasis), a system which was used till the seminal works of MacCallan in the twentieth century [3]. Multiple grading methods for assessing severity of disease in a standardized manner have been developed over the years with over ten systems documented [4]. The better known of these include the systems devised by MacCallan in 1931 and the FPC grading system by Dawson, Jones and Tarrizo in 1981 (also called modified World Health Organization [WHO] system).

The 1981 classification comprised of assessment of follicles and papillae in grades F0–F3 and P0–P3, respectively, based on upper tarsal conjunctival examination and combinations. Conjunctival scarring, trichiasis/entropion and corneal scarring were classified as C0–C3, T/E0–T/E3 and CC0–CC3, respectively. Further, trachomatous inflammation was classified as trivial, mild, moderate or severe based on combinations of follicular and papillary scoring [5]. However, such a detailed classification system was deemed too complex for use by non-specialist health personnel. Therefore, the FPC evolved into the WHO simplified trachoma grading system in 1987, specifically designed for use in epidemiologic field surveys for assessment of trachoma by non-specialist personnel [6]. The WHO simplified trachoma grading system includes only five stages: trachomatous inflammation follicular (TF), trachomatous inflammation intense (TI), trachomatous conjunctival scarring (TS), trachomatous trichiasis (TT) and trachomatous corneal opacity (CO), also often abbreviated as the FISTO classification (Table 1). Since its introduction, the WHO simplified trachoma grading scheme has been used widely in various research studies and surveys and for developing indicators for epidemiologic monitoring endemicity and elimination status of trachoma.

Table 1 The WHO simplified trachoma grading system

Grade	Description
TF = trachomatous inflammation (follicles)	Five or more follicles, at least 0.5 mm in size, on the 'flat' surface of the upper tarsal conjunctiva
TI = trachomatous inflammation (intense)	Inflammatory thickening of the upper tarsal conjunctiva with more than half of the normal deep tarsal vessels obscured
TS = trachomatous scarring	Scarring of the tarsal conjunctiva (fibrosis)
TT = trachomatous trichiasis	At least one eyelash rubbing on the eyeball or evidence of eyelash removal. This should be in conjunction with evidence of trachomatous scarring
CO = corneal opacity	At least part of the pupil is blurred or obscured

6 Epidemiology

6.1 Prevalence and Burden

6.1.1 Global Burden

Trachoma was once the most important cause of blindness. The US Public Health Services (USPHS) in its surveys conducted during 1912–1915 identified 0.03–12.6% prevalence of disease in school children and 12.7–68.7% prevalence among Native American Indians. Trachoma ceased to be a problem in the affected regions of the USA by the early 1950s with less than 800 cases being reported in 1954 [7]. It has been reported in 1949 that trachoma was a worldwide distributed disease with population prevalence of Egypt 98%; India 75%; Siam 65%; Malay and Poland 50%; Italy, Greece, Argentina, Brazil and Central America 30%; and Mexico 25% [8]. However, these numbers were commented to be too high, and prevalence quoted were Poland 1.5%, Italy 3–6%, Greece 4.5% and Argentina 2.3% [9]. In any case, the burden of trachoma was considered high enough to warrant its recognition as a dangerous disease upon the inception of the WHO. The lack of adequate information but at the same time the perceived severity of the problem of trachoma at a global level can be assessed if one reads the following section from a report by the WHO:

> Owing to its world-wide distribution for no continent is exempt from its ravages trachoma is one of the most important of all the communicable diseases. In the questionnaire replies it is mentioned by 33 countries, although only 5 gave it any measure of priority. Yet the total of the cases which are notified is formidable. In 1960 over 230,000 cases of trachoma were notified to the WHO by India and more than 180,000 in Morocco. Iran and Japan recorded totals of 57,000 and 45,000 respectively. Even in Europe it is regularly reported, notably in the countries of the Balkans. It remains a great social problem because of the blindness it causes. Nevertheless, its control by antibiotics is now feasible. Above all it is one of the diseases in which health education from the school onwards and insistence on elementary hygiene can yield most gratifying results [10].

Over time, the importance of trachoma has decreased. In the year 2000, it was estimated that the disease was endemic in 66 countries worldwide. Among these 66 countries, 7 are currently claiming elimination of trachoma, while one has been validated as having eliminated trachoma. As can be observed in Table 2, and Fig. 2, North America and Europe are nonendemic for trachoma, while Africa, Southern America, Southeast Asia and West Pacific Region countries continue to have a high burden. China and India, the two most populous countries of the world comprising nearly 25% of global population, are considered endemic. It is estimated that nearly 192 million people lived in endemic areas in 2016 and are at risk of trachoma. Considering that in 2007, nearly 1260 million were at risk for trachoma, it is clear that much progress has been made over the last decade in controlling this disease. Three countries (Mexico, Morocco and Oman) have been validated as having eliminated trachoma in recent years, and another seven countries (Cambodia, China, Gambia, Ghana, Islamic Republic of Iran, Lao Peoples Democratic Republic and Myanmar) are pending validation. The Southeast Asia region too reported low levels of active trachoma in 2016 [11].

Table 2 Status of elimination of trachoma as a public health problem in various countries and population living in endemic areas, by WHO Region, 2016

Status of trachoma	Africa	Americas	Eastern Mediterranean	Europe	Southeast Asia	Western Pacific	Global
Nonendemic countries	14	29	9	53	7	16	128
Endemic countries	33	6	12	0	4	11	66
Claim to have eliminated	2	1	2	–	1	1	7
Known to require interventions	26	3	4	–	1	8	42
Status uncertain	3	2	4	–	2	1	12
Thought to not require interventions	2	–	1	–	–	1	4
Validated as having eliminated	–	–	1	–	–	–	1
Population living in endemic areas, 2015 (millions)	173.9 (90.5%)	4.7 (2.4%)	10.7 (5.6%)		0.2 (0.1%)	2.6 (1.4%)	192.1 (100%)

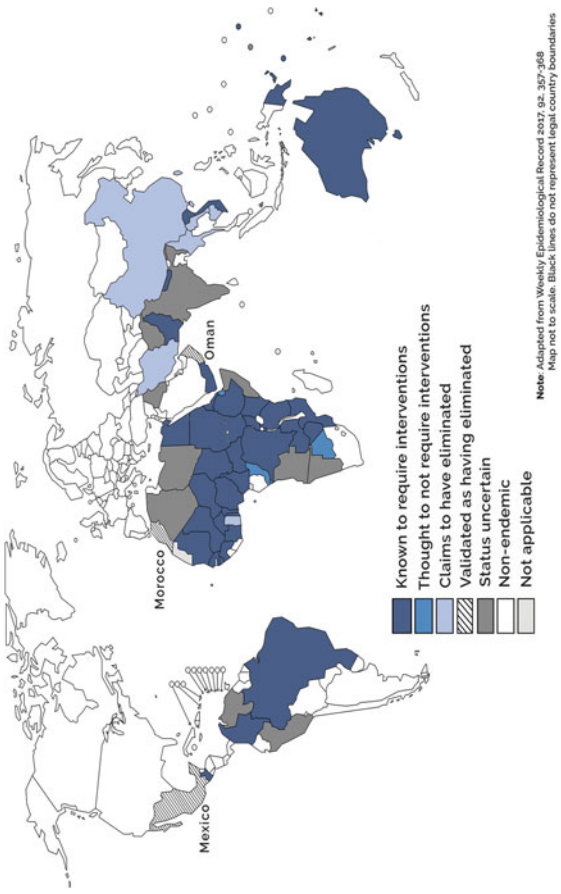

Fig. 2 Distribution of trachoma worldwide, 2017 [11]

6.1.2 Burden in SEAR

The South East Asia Region as defined by WHO includes three currently endemic countries: Nepal, Myanmar and India. In addition, Pakistan and Afghanistan too are high-burden South Asian countries. In 1949, the limited information on burden of trachoma in SEAR which was available suggested a sizeable problem in Afghanistan, less problem in Burma (Myanmar) and uncommon in Ceylon (Sri Lanka), high prevalence in northern India, as well as, a serious problem in Indonesia and Thailand. No information was available for Nepal. Lack of knowledge of local epidemiology of trachoma prompted pilot projects in the region with the support of the WHO to assess the prevalence, factors of disease transmission and effectiveness of antibiotics and other methods of control. Severe problem was identified in two provinces of Afghanistan, three in Thailand and in selected areas in Malaysia. In Indonesia, the prevalence was estimated as 52% [12]. Trachoma was the most frequently notified communicable disease from India in 1960 with 231,867 cases being reported. It was one of the most important health problems in school children in Thailand with the whole country being endemic and higher prevalence in northeastern parts during the year 1959 [10]. Data on trachoma from Pakistan again is limited. Recently, under the Global Trachoma Mapping Project, mapping for baseline prevalence has been completed in many districts in Pakistan (excluding insecure districts). Over years, the burden in the regions has decreased substantially (Table 3).

Table 3 Burden of trachoma in South Asian countries

	Status of elimination of trachoma as a public health problem (2016)	Population in areas that warrant treatment with antibiotics, facial cleanliness and environmental improvement for elimination of trachoma as a public health problem (2015)	Number of people operated for trachomatous trichiasis (2015)	Number of people who received treatment with antibiotics for trachoma (2015)
India	Status uncertain	No data	No data	No data
Myanmar	Claims to have eliminated	No data	No data	No data
Nepal	Known to require interventions	249,157	1325	0
Timor-Leste	Status uncertain	No data	No data	No data
Afghanistan	Status uncertain	No data	0	0
Pakistan	Known to require interventions	3,074,598	987	0

Note: Bangladesh, Bhutan, Democratic People's Republic of Korea, Indonesia, Maldives, Sri Lanka and Thailand are nonendemic for trachoma

India

In India, studies done in 1958–1964 showed trachoma prevalence ranged from 0.5% to 79.1% across various states (Table 4). In the high endemic regions, even 90% prevalence was observed in some villages, while the moderate endemic states too had pockets of high prevalence. The studies also showed that the prevalence of active trachoma was high among children under 10 years of age in areas of high overall trachoma prevalence and among children under 15 years of age in moderate prevalence areas. The high endemic areas had dry and dusty climates; overcrowding, poor personal and environmental hygiene; and exposure to smoke. Sex of the child was not a predisposing factor till 6 years of age, but beyond that, females had a higher risk of severe disease [13]. These hyperendemic states had dry and dusty climates and were the focus on initial control efforts. During the WHO-NPCB Survey on Blindness in India (1986–1989), it was observed that the prevalence of active trachoma infection (TI/TF) in all the previously known hyperendemic states had reduced to under 10% among children under ten years of age, and no state was hyperendemic. Active trachoma was observed among 8.5% school-aged children (5–15 years) in two districts of the present Uttarakhand state in 1995. Trachoma rapid assessments conducted among the ten districts of previously hyperendemic states in 2007–2009 in purposively identified high-risk areas reported TI/TF prevalence of 0.6–15.2% among children and TT prevalence of 0.03–0.52%, while a very high prevalence was reported from Andaman Islands [14, 15]. National-level surveys in the country also estimated the contribution of trachoma to blindness. The 1971–1974 survey of blindness conducted across seven centres in India showed that the corneal involvement was the cause of blindness in 7.31% of blind eyes, and the burden was much lower than lens pathologies (74.65%). Trachoma being a major cause of corneal blindness, these results indicated a downward trend in the burden of trachoma. The subsequent nationwide WHO-NPCB blindness survey of 1986–1989 showed that trachoma was responsible for blindness only among 0.39% of all blind individuals in the survey across all ages [16]. The survey observed that the prevalence of active infection had significantly reduced in all the previously known hyperendemic states in the country. The prevalence of active infection among 0–14-year age group had decreased to 21.79% in Punjab, 17.11% in Rajasthan, 11.73% in Uttar Pradesh, 10.24% in Gujarat and 15.0% in Haryana, while the corresponding prevalence among 0–10 year age group were 6.94%, 4.19%, 9.49%, 4.76% and 3.83%, respectively. No state remained in the hyperendemic zone in 1986 with a prevalence of under 10% among population

Table 4 Prevalence of trachoma in India, Trachoma Control Pilot Project, India, 1956–1963 [13]

High Endemic	Gujarat (56.0%), Rajasthan (74.2%), Punjab (79.9%), Uttar Pradesh (72.7%)
Moderate Endemic	Madhya Pradesh (43.3%), Bihar (35.1%), Assam (25.2%), Mysore (22.6%)
Low Endemic	West Bengal (0.5%), Orissa (2.72%), Maharashtra (11.3%), Madras (4.6%), Andhra Pradesh (5.3%), Kerala (8.7%), Jammu and Kashmir (16.8%)

aged <10 years. Trachoma cause was attributed to 0.89% blindness (presenting visual acuity <6/60) in the 1999–2001 blindness survey conducted among 50+ year aged populations [17]. In the last national blindness survey done in 2007, 0.3% of blindness (presenting visual acuity <6/60) was attributed to trachoma [18]. Since 2006, numerous trachoma rapid assessments and trachoma prevalence surveys have also been conducted in the country. The 2006–2007 rapid assessment surveys were conducted in ten selected districts in six previously hyperendemic states (including new states of Uttaranchal which was split from Uttar Pradesh). A rapid assessment was conducted in Car Nicobar Island in 2010 followed by a prevalence survey in all districts of Andaman and Nicobar Islands in 2013 (Table 5). Currently, a national survey is underway in the country which would yield the most updated results of the burden once its results are declared.

Table 5 Prevalence of trachoma in India, Trachoma Rapid Assessments, India, 2006–2013

Districts and states surveyed	Type of survey	Year	Children (1–10 years) with active trachoma TI/TF %	TT among people aged ≥ 10 years (%)
Kutch (Gujarat)	Rapid assessment	2006–2007	0.03	0.57
Banaskantha (Gujarat)	Rapid assessment	2006–2007	0.04	1.14
Dholpur (Rajasthan)	Rapid assessment	2006–2007	0.31	6.35
Tonk (Rajasthan)	Rapid assessment	2006–2007	0.03	5.05
Bikaner (Rajasthan)	Rapid assessment	2006–2007	0.06	11.38
Bulandshahr (Uttar Pradesh)	Rapid assessment	2006–2007	0.27	5.88
Pauri Garhwal (Uttaranchal)	Rapid assessment	2006–2007	0.05	15.15
Mahendragarh (Haryana)	Rapid assessment	2006–2007	0.06	2.20
Mewat (Haryana)	Rapid assessment	2006–2007	0.52	5.81
Hoshiarpur (Punjab)	Rapid assessment	2006–2007	0.22	5.66
Car Nicobar Island	Rapid assessment	2010	1.00	50.78
Car Nicobar (Andaman and Nicobar Islands)	Prevalence survey	2013	6.80	3.91

Myanmar

Myanmar (erstwhile Burma) was also a high-burden country for Trachoma. Trachoma was hyperendemic in the central region of Myanmar, a region which has low rainfall and is usually dry. A survey conducted in the Meiktila, Magwe, Myingyan and Yemethin districts of the region revealed the prevalence of disease to be directly associated with poverty, dry environmental conditions, overcrowding and the general and health education. Among 82 townships surveyed during 1965–1975 in Myanmar, 37 had active trachoma rate above 30%, 27 had 15–30% and rest had rates of less than 15%. An average active trachoma rate of 43.05% was reported with gross visual loss 1.8% and severe conjunctival scarring and/or entropion 7.5%. Trachoma was the most important cause of blindness in Myanmar responsible for 57% of blindness in the country. The prevalence of active trachoma had decreased to 18.7% in 1975, 11.3% in 1985, 6.2% in 1995 and 4.8% by 1998. Further, the prevalence of TT was 0.75% among males and 1.9% among females, and CO was 0.2% and 0.4%, respectively, in the 1997 survey. The contribution of trachoma to blindness had also decreased to 0.53% in dry zones of the country, 0.41% in hilly zones, 0.03% in delta zones and 0.08% in coastal zones [19]. The 2005 blindness survey among adults 40+ years of age revealed population prevalence of TT as 1.4% and CO as 1.3% and overall CO + TT prevalence of 2.7% in the Meiktila province. Proportion of blindness due to trachoma was 3.5% and the disease accounted for 0.91% of low vision [20]. Currently, Myanmar claims to have eliminated trachoma and is pending validation.

Nepal

Extremely limited information was available from Nepal till 1985 when the Nepal Blindness Survey results (done in 1980–1981) revealed trachoma to be the most important potentially blinding disorder of all ocular morbidities in the country with 6.5% of the population affected by the disease and 36.3% of all ocular morbidities were identified as trachoma. Trachoma was responsible for 2.4% of all blindness (best visual acuity $<$3/60 in better eye) [21]. The disease was more common in the Terai region (lowlands) as compared to the hilly regions. In 1989–1990, the prevalence of TI/TF trachoma was 23.6% among children 2–6 years of age with much higher prevalence in certain sampled areas in Sarlahi district of Nepal [22]. Similarly, high TI/TF prevalences (16%, range 4–39%) were also observed among children in Geta and Sripur areas in Kailali district in 1998 which subsequently decreased to 4% 12 months after two rounds of antibiotic treatment programmes [23, 24]. In the Lumbini zone of the country, 3.4% of blindness among adults (9/241) was due to trachoma in 1998 [25]. The 2006 Lumbini survey showed that 3.8% of blindness was due to corneal causes while not explicitly mentioning trachoma and a similar result (3.1%) was observed in Gandaki zone [26, 27]. Currently, the disease has been eliminated in many parts of the country including the Kailali, Kanchanpur, Chitawan, Nawalparasi, Surkhet, Banke,

Bardiya, Dang, Kapilvastu, Bara, Parsa, Doti, Rolpa, Dailekh, Sarlahi, Rukum, Rautahat, Rasuwa, Achham and Baitadi districts [28–30].

6.2 Risk Factors

Trachoma is an infectious disease, and as such it passes through the stages of transmission, latent infection, symptomatic phase and, finally, the recovery. In certain cases, the disease progresses and patient develops complications leading to long-term sequelae. Accordingly, the risk factors can be discussed as those for the active disease and those for chronic disease. The information about risk factors has accumulated through observations about the disease occurrence over the years. The understanding of its epidemiology has significantly increased over the last century through scientifically designed systematic studies. Longitudinal studies conducted in Africa have generated information about natural history of disease and the factors responsible for the development of chronic scarring disease. Presence of active trachoma disease can be studied either on the basis of clinical signs (TI/TF as grade) and through microbiological and, more recently, serological methods. Chronic trachoma has mostly been studied on the basis of the presence of clinical signs. Understanding and addressing the epidemiologic risk factors for trachoma was a key to the disappearance of the disease from many countries even before the isolation of the bacteria.

6.2.1 Risk Factors for Active Disease

Trachoma exhibits geographical clustering. The cases are clustered in households with villages as well as across specific villages in a district. The initial infection often occurs early in infancy, and most of the initial infections happen in children under 5 years of age, and almost all the affected children are infected by the time they are 10 years of age. Repeated reinfections are also common. The signs of active infection are common in children and decrease with increasing age. Risk for active infection is similar across males and females, but in some communities, higher rates have been observed among females. Presence of unclean faces has been identified as an important risk factor for active disease, most likely through a concomitant increased risk of acquiring infection. Cleanliness of faces is directly linked to availability of water, and poor access to a water source is also a risk factor for active infection. Risk of infection is higher in households where overcrowding is present since it is associated with closer physical contact as well as sharing of linen, beds and other fomites enabling transmission of infection. Houseflies especially the eye-seeking 'bazaar fly' *Musca sorbens* in Asia and Africa, the 'bush fly' *M. vetustissima* in Australia and eye gnats *Hippelates* spp. and *Liohippelates* spp. in Latin America have been proposed as the vectors for transmission. *C. trachomatis* have been isolated from houseflies, but these have not

been confirmed as vectors. Presence of a functional latrine has also been found to be associated with decreased risk of infection. The mechanism of action is unclear though it could be through decreased fly breeding or it could be due to a simultaneous improved hygienic status of the family [31]. In a recent meta-analysis, environmental factors associated with lower risk of trachoma measured by the presence of trachomatous inflammation TF/TI included access to sanitation (Odds Ratio [OR] 0.85), having a clean face (OR 0.42), lack of ocular discharge (OR 0.42) and lack of nasal discharge (OR 0.62), face washing at least once daily (OR 0.76), face washing at least twice daily (OR 0.85), soap use (0.76), towel use (OR 0.65) and daily bathing (OR 0.76). Factors associated with lower risk of trachoma measured by the presence of *C. trachomatis* infection include access to sanitation (OR 0.67), lack of ocular discharge (OR 0.40) and lack of nasal discharge (OR 0.56) [32].

6.2.2 Risk Factors for Blinding Disease

Not all infections lead to development of scarring and blinding disease. The incidence of trachomatous scarring has been observed to be varying between 0.5% and 5% per year across various longitudinal studies. Ramadhani and colleagues in their recent systematic review concluded that the various studies were too diverse to enable a meta-analysis. Severe conjunctival inflammation is the most consistent finding leading to the development and progression of scarring [33]. There are two major hypotheses for development of scarring disease. The first is classically held view of the occurrence of repeated trachoma infections leading to conjunctival inflammation and scarring. Thus, all the risk factors for active disease also become relevant to development of chronic blinding disease. Females are more likely to develop blinding trachoma presumably due to prolonged duration of contact with children. Over the last 20 years, genetic studies have supported a role of persistence of infection to later years scarring. About 8–10% of the infected children in hyperendemic areas may not clear the initial infection and have persistent inflammation leading to scarring. The incidence of conjunctival scarring is almost five times higher in these children than in children with active trachoma but without severe inflammation. This seems to be the course of disease only in a minority of those affected by scarring disease [34]. Risk of scarring has also been proposed to be associated with the presence of other ocular pathogens which may lead to non-chlamydial conjunctival inflammation. It has also been theorized that the presence of chronic inflammation due to any reason is associated with dryness of the eye and changes in ocular surface which may favour reinfections. Recent studies have also suggested a role of environmental factors such as lower precipitation, higher land surface temperature, higher mean annual temperature and rural classification with a greater risk of TT/CO [35].

7 Prevention and Elimination of Trachoma

Given the natural history of the disease, the prevention programmes need to address the issues related to disease transmission and acute infections seen among children and, at the other end, the complications of TT and CO that lead to visual impairments and blindness. Breaking the cycle of transmission will control acute infections and future blinding trachoma, but at the same time, there will be adults in the community who suffered infections in their childhood and are now having TT/CO. The cycle of transmission can be broken by improving hygiene and treating acute infections. Treatment of TT requires lid surgery while that of CO requires keratoplasty procedures. It is obvious that TT/CO treatments require more skilled eye care professionals, while the prevention and management of acute infections requires a wider coverage by health personnel probably with not that high skill levels. Challenges are more while implementing surgical interventions in resource-constrained settings that are typical of the locations where trachoma is endemic.

7.1 Historical Aspects in Control of Trachoma

Given the ancient history of the disease, a wide variety of treatments were recommended in various cultures over the centuries. These include pulling eyelashes, application of products to the eyes (such as mixture of myrrh, animal blood, dung, red ochre, malachite, etc.), removing infected tissue by scraping or cutting and physically fixing eyelashes away from the eyeball. The rapid spread of trachoma in the nineteenth century led to an intensification of efforts to identify methods to prevent transmission of trachoma. Practices such as isolation of afflicted patients, ensuring sanitation by frequent washing of affected persons and their linen, avoiding sharing of linen and towels and avoiding overcrowding in schools and military camps were initiated in England after an expert team of professionals in 1810 studied trachoma cases and gave its recommendations for control. The effectiveness of sanitary practices was recognized as a key to control trachoma. Efforts to control trachoma also resulted in setting up of ophthalmic schools and ophthalmic hospitals. In the USA, the US Public Health Service (USPHS) studied the communities where trachoma was endemic and identified overcrowding, sharing of dirty bedsheets and towels and unsanitary conditions around the house as associated factors. Trachoma was often referred to as ‘family disease’ due to the strong clustering of cases observed within families. Topical treatments for trachoma in vogues in the early nineteenth century included the use of copper sulphate and silver nitrate to debride the conjunctival surface and antiseptic eye washes. Surgical treatments included *grattage* (scraping of follicles followed by mercury bichloride and argyrol application) and tarsectomy. Given the disastrous results, tarsectomy was stopped in 1927 in the USA, while the practice continued to be

practiced in Africa [7, 36]. In 1937, oral sulphanilamide was found to be effective in control of trachoma, and this revolutionized the treatment of disease [37].

7.2 Current Efforts for Control of Trachoma

Control of trachoma was achieved in developed countries before the World War II. After the World War II, trachoma control was taken up on a priority by the WHO, and several countries launched pilot projects and standalone programmes for control of the disease. The vertical programmes tended to be successful but very resource intensive. With the move towards primary health care, the standalone vertical programmes gave way to horizontally integrated control efforts, mostly with other blindness control programmes. These integrated programmes tend to be more sustainable in the long term. Based on accumulated epidemiological evidence, the control efforts for trachoma elimination have been crystallized into the SAFE strategy: Surgery, Antibiotics, Facial cleanliness and Environmental hygiene. The SAFE strategy was adopted by the WHO in 1993 and has become the cornerstone of trachoma elimination in all affected countries. In 1998, the Fifty-First World Health Assembly adopted resolution WHA51.11 on the global elimination of trachoma at a public health problem. In 1997, Alliance for the Global Elimination of Trachoma by the year 2020 (GET2020) was launched. Total cost of eliminating trachoma, excluding drug and latrine construction costs, has been estimated at about $430 million for confirmed burden. This includes $14 million for surveillance and surveys, $182 million for surgery, $94 million for drug distribution, $28 million for face washing and $112 million for environmental hygiene efforts. This estimate is expected to increase to $748 million if half of suspected districts are confirmed to be endemic. It has been estimated that each $20 spent in trachoma control results in one additional person spending one additional year without severe vision loss or blindness [38].

7.2.1 Surgery

Corrective lid surgery to correct TT is an essential component of the SAFE strategy. The purpose of surgery is to evert the in-turned lid margin which stops the eyelashes from rubbing against the eyeball. The WHO recommends two procedures: (1) the bilamellar tarsal rotation (BLTR) and (2) the posterior lamellar tarsal rotation or Trabut procedure for upper eyelid TT [39]. There is still a chance of recurrence of trichiasis after surgery; incidence may be as high at 20–40% after 1 year and 60% after 3 years. Quality of training of surgeons is critical to achieve good outcomes. Lower rates of recurrence have recently been observed in a clinical trial where patients received oral azithromycin or topical tetracycline. Newer evidence is also emerging as to the superiority of the Trabut over the BLTR procedure. Patients who refuse surgery should be offered high-quality epilation forceps as well as counselled

about the proper use of forceps and the need for regular follow-up. Refusals can also be minimized by ensuring strong community-level support and mobilization to overcome the issues of fear, lack of awareness, costs of surgery and other social barriers [40]. It was estimated that 8.2 million required TT surgery in 2007 and 7.3 million required TT surgery in 2012, and the best estimate of 2015 global trichiasis backlog is 3.6 million people. To maximize the uptake of surgical services, it is recommended to hold regular surgical sessions held at fixed sites, such as district hospitals, and periodic outreach sessions held in trachoma-endemic communities. The cost of surgery has been estimated to be $27–$50 (average $40) depending on the setting. A TT prevalence of 10/1000 in the population aged ≥15 years constitutes a public health problem which is equivalent to 5 cases/1000 in all ages, and programmes should achieve an 80% relative reduction below the minimum level at which TT constitutes a public health problem. Elimination of trachoma as a public health problem includes a target of prevalence of TT 'unknown to the health system' of <1 case per 1000 total population. TT 'unknown to the system' excludes TT in individuals with postsurgical recurrence, TT in individuals who have refused surgery and TT in individuals who are listed for surgery but have not yet received an operation but for whom a surgical date has been set [41].

7.2.2 Antibiotics

Antibiotics treatment is the cornerstone of management of acute infection. Azithromycin 20 mg/kg (maximum 1 g) given as a single dose is the drug of choice for trachoma prevention programmes. Where azithromycin is not available and for children under 6 months of age, topical 1% tetracycline administered to both eyes twice daily for 6 weeks is the recommended choice. Clinical signs can persist even after infection has been cleared by antibiotics. Annual mass antibiotic treatment of all residents for 3 years is recommended if the baseline district prevalence of TF in 1–9 year-old children is 10% or greater. Targeted distribution in high-burden communities is recommended in case TF prevalence is 5–9%. WHO recommends complete coverage in MDA (mass drug administration), with a minimum coverage of 90% of population. At least 6 months after the last planned round of antibiotic mass drug administration, an impact survey should be undertaken in endemic districts. If the TF prevalence <5%, surveillance is recommended for 2 years without any antibiotic mass drug administration, which should be followed by a pre-validation survey. Elimination criteria for trachoma include TF prevalence <5% in children aged 1–9 years in previously endemic districts. At prevalences >30%, seven or more annual MDAs may be required to achieve the elimination target [42]. In Car Nicobar Islands, in India, after three rounds of MDA, the prevalence of TF was reduced to 6.8% from 50.9% at baseline [14]. Concerns have been raised about the development of resistance in *Streptococcus pneumoniae* after MDA with azithromycin for trachoma. A recent review observed that in communities where baseline resistance to azithromycin in pneumococcus is low, MDA

increased resistance only transiently, with the proportion of resistant cases gradually reducing over time (due to fitness cost associated with maintaining resistance); while in communities with high initial resistance and antibiotic usage, the MDAs were associated with an increased subsequent resistance [43]. Resistance to azithromycin in *Chlamydia trachomatis* has not been reported. The 'A' component of the GET2020 campaign has internationally received support from Pfizer in the form of large donations of azithromycin. It is recommended that wherever appropriate, azithromycin MDA programmes should be linked up with programmes for other NTDs such as yaws. MDA for trachoma has also been reported to have beneficial effects in decreasing child mortality and reducing acute respiratory infections.

7.2.3 Facial Cleanliness and Environmental Factors (F and E)

Addressing personal hygiene is fundamental to ensuring long-term success in trachoma control and preventing resurgence once burden of trachoma has been reduced by azithromycin MDA programmes. Environmental risk factors that need to be addressed include supply of water, provision of toilets, disposal of wastes, animal pens within households and fly density. There is strong evidence from historical observational studies about association between face washing with trachoma infection. A community trial has concluded that face washing promotion combined with topical tetracycline may be effective in reducing severe active trachoma and in increasing the prevalence of clean faces at 1-year follow-up. Evidence base for environmental measures and trachoma control is weak, but given the biological plausibility, these have been made an integral component of SAFE strategy [44, 45]. The F and E components should be targeted specifically if TF prevalence >5% among 1–9 year population. For optimal penetration, the F and E interventions need to be integrated with the water, sanitation and hygiene (WASH) interventions in the communities. Therefore, quite often these interventions do not fall directly under the preview of health ministries which makes coordination an operational challenge. In districts where trachoma is endemic, it is vital that these challenges be surmounted which can be done by establishing close interdepartmental ties as well as by engaging local self-governance organizations in trachoma control. The Sustainable Development Goals (SDGs) also have a goal of providing access to safe drinking water and sanitation which ties in with the goal for control of eliminating trachoma (among the bouquet of NTDs).

7.2.4 Planning for Trachoma Control

Trachoma elimination efforts need to be planned and implemented in a systematic manner that is based on local context where the interventions need to be implemented. It is estimated that a district takes 4–6 years from start to elimination and the time can often be more. A major first step towards elimination is an

epidemiological assessment of true burden on trachoma in all the previously hyper-endemic districts of the region. Endemicity of trachoma is determined based on the prevalence of TF among children in the age group of 1–9 years, usually through a survey. If the TF prevalence is 10% or above, implementation of AFE interventions is required, while if TF prevalence is between 5 and 9.9%, 1 year of MDA with F&E is recommended followed by impact assessments. If TF prevalence is under 5% and the area was previously not known to be endemic, no action is required. Similarly if the district was previously endemic, and a TF prevalence of <5% is observed for 2½ years after the last round of MDA, no interventions are required. The second major consideration is the prevalence of TT. A district may have TF prevalence of under 5% but a TT prevalence of >1 per 1000 population in which case surgical interventions (S component of SAFE strategy) for trichiasis are needed, but the AFE components do not need to be addressed specifically. Specific guidelines are available for calculation of district level ultimate intervention goals for TT surgeries and distribution of azithromycin during the MDA.

While the prevalence of TF is measured only in 1–9 year population, the AFE interventions must be planned for the entire population. The planning of SAFE interventions in a district requires additional data about the population size, proportion of population aged 15+ years, number of people receiving TT management since the last survey, previous MDA coverage and data on face washing and environmental improvement (if available), in addition to the TT and TF prevalence data.

The planning exercise also must involve all concerned district health officials, water and sanitation officials, supervisors, community representatives, medical stores representatives, NGOs and all the other relevant stakeholders. Implementation of A and S interventions is usually the domain of the health sector, and that of F&E interventions requires close coordination with the WASH sector. Once a plan has been prepared, the SAFE interventions are implemented in the district. The timeline of interventions is often dependent on the duration of scaling up, on the number of rounds of MDA that are required and on the surgical capacity and productivity. Achieving community support in trachoma programmes can help overcome barriers of ignorance, providing access to safe water and sanitation, and generate demand for interventions among the affected. It also keeps the programme on its toes.

7.3 Elimination of Trachoma

Elimination of trachoma as a public health problem is defined as (1) a prevalence of trachomatous trichiasis (TT) 'unknown to the health system' of <1 case per 1000 total population and (2) a prevalence of trachomatous inflammation follicular (TF) in children aged 1–9 years of <5%, in each formerly endemic district. Districts are defined as the normal administrative unit for health care management, which for the purposes of clarification consist of a population unit between 100,000 and

250,000 persons. In addition, there must be evidence that the health system can continue to identify and manage incident cases of TT. Once a country is ready for validation, they submit a dossier documenting the achievement of elimination targets to the World Health Organization. India was among the first countries to launch a blindness control programme under which trachoma control activities are being conducted. The Nepal Netra Jyoti Sangh, an NGO, is actively pursuing the agenda in that country. Myanmar has already reported elimination of trachoma through concerted efforts of the country's government and is undergoing validation. In India, the recent launch of *Swachh Bharat* (Clean India) programme has the potential to giving a big boost to F and E interventions in previous hyperendemic districts and further contribute to NTD and trachoma elimination. With only 3 years to 2020, it is vital that properly managed SAFE interventions be implemented in all affected districts in the region, while the districts where the disease is believed to be eliminated should plan for certification of the same through post-elimination surveillance.

References

1. Frick KD, Hanson CL, Jacobson GA. Global burden of trachoma and economics of the disease. Am J Trop Med Hyg. 2003;69(5 Suppl):1–10.
2. Katherine Schlosser. Trachoma-Through-History-2.pdf [Internet]. [cited 2016 Dec 9]. Available from: https://www.k4health.org/sites/default/files/Trachoma-Through-History-2.pdf
3. Taylor HR. Doyne Lecture: trachoma, is it history? Eye. 2009;23(11):2007–22.
4. Solomon AW, Peeling RW, Foster A, Mabey DCW. Diagnosis and assessment of trachoma. Clin Microbiol Rev. 2004;17(4):982–1011.
5. Dawson CR, Jones BR, Tarizzo ML, Organization WH. Guide to trachoma control in programmes for the prevention of blindness. Guide pour la lutte contre le trachome dans les programmes de prévention de la cécité [Internet] 1981 [cited 2016 Nov 1]. Available from: http://www.who.int/iris/handle/10665/38939
6. Thylefors B, Dawson CR, Jones BR, West SK, Taylor HR. A simple system for the assessment of trachoma and its complications. Bull World Health Organ. 1987;65(4):477–83.
7. Allen SK, Semba RD. The trachoma "Menace" in the United States, 1897–1960. Surv Ophthalmol. 2002;47(5):500–9.
8. Murray WB. A review of trachoma. Can Med Assoc J. 1949;60(6):574–80.
9. Stern JJ. Trachoma. Can Med Assoc J. 1949;61(5):539–40.
10. World Health Organization. Second report on the world health situation, 1957–1960 [Internet]. World Health Organization; 1963 [cited 2016 Dec 13]. Available from: http://www.who.int/iris/handle/10665/85752
11. Mariotti SP, Pascolini D, Rose-Nussbaumer J. Trachoma: global magnitude of a preventable cause of blindness. Br J Ophthalmol. 2009;93(5):563–8.
12. World Health Organization. WHO alliance for the global elimination of trachoma by 2020: progress report on elimination of trachoma, 2014–2016. Wkly Epidemiol Rec. 2017;92(26):357–68.
13. Gupta UC, Preobragenski VV. Trachoma in India – Endemicity and epidemiological study. Indian J Ophthalmol. 1964;12(2):39.
14. Malhotra S, Vashist P, Gupta N, Kalaivani M, Satpathy G, Shah A, et al. Prevalence of trachoma in Car-Nicobar Island, India after three annual rounds of mass drug administration with azithromycin. PLoS One. 2016;11(7):e0158625.

15. Vashist P, Gupta N, Rathore AS, Shah A, Singh S. Rapid assessment of trachoma in underserved population of Car-Nicobar Island, India. PLoS One. 2013;8(6):e65918.
16. Damodar Bachani, Rachel Jose. Blindness in India – trends in prevalence and magnitude. New Delhi, India: Ophthalmology Division, Ministry of Health & Family Welfare, Government of India; 1996.
17. Murthy GVS, Gupta SK, Bachani D, Jose R, John N. Current estimates of blindness in India. Br J Ophthalmol. 2005;89(3):257–60.
18. John N, Jose R, Vashist P, GVS M, RAAB India Study Group. Rapid assessment of avoidable blindness in India. PLoS One. 2008;3(8):e2867.
19. Ko Ko U, Lwin K, Thanung U, editors. Conquest of scourges in Myanmar. Yangon, Myanmar: Myanmar Academy of Medical Science; 2002.
20. Durkin SR, Casson RJ, Newland HS, Aung TH, Shein WK, Muecke JS, et al. Prevalence of trachoma-related trichiasis and corneal opacity in rural Myanmar: the Meiktila Eye Study. Ophthalmology. 2007;114(5):e7–11.
21. Brilliant LB, Pokhrel RP, Grasset NC, Lepkowski JM, Kolstad A, Hawks W, et al. Epidemiology of blindness in Nepal. Bull World Health Organ. 1985;63(2):375–86.
22. Katz J, West KP, Khatry SK, LeClerq SC, Pradhan EK, Thapa MD, et al. Prevalence and risk factors for trachoma in Sarlahi district, Nepal. Br J Ophthalmol. 1996;80(12):1037–41.
23. Holm SO, Jha HC, Bhatta RC, Chaudhary JS, Thapa BB, Davis D, et al. Comparison of two azithromycin distribution strategies for controlling trachoma in Nepal. Bull World Health Organ. 2001;79(3):194–200.
24. Jha H, Chaudary JSP, Bhatta R, Miao Y, Osaki-Holm S, Gaynor B, et al. Disappearance of trachoma from Western Nepal. Clin Infect Dis Off Publ Infect Dis Soc Am. 2002;35(6):765–8.
25. Pokharel G, Regmi G, Shrestha S, Negrel A, Ellwein L. Prevalence of blindness and cataract surgery in Nepal. Br J Ophthalmol. 1998;82(6):600–5.
26. Sapkota YD, Pokharel GP, Nirmalan PK, Dulal S, Maharjan IM, Prakash K. Prevalence of blindness and cataract surgery in Gandaki Zone, Nepal. Br J Ophthalmol. 2006;90(4):411–6.
27. Sherchan A, Kandel RP, Sharma MK, Sapkota YD, Aghajanian J, Bassett KL. Blindness prevalence and cataract surgical coverage in Lumbini Zone and Chetwan District of Nepal. Br J Ophthalmol. 2010;94(2):161–6.
28. Nepal Netra Jyoti Sangh. National Trachoma Program Annual Programme Review 2015 [Internet]. 2016 [cited 2016 Dec 22]. Available from: http://nnjs.org.np/uploads/2015%20%20Annual%20Report-Nepal(TrachoaJune2016).pdf
29. Pant BP, Bhatta RC, Chaudhary JSP, Awasthi S, Mishra S, Sharma S, et al. Control of trachoma from Achham district, Nepal: a cross-sectional study from the Nepal National Trachoma Program. PLoS Negl Trop Dis. 2016;10(2):e0004462.
30. Yee A. Nepal sees end in sight for trachoma. Lancet Lond Engl. 2012;379(9834):2329–30.
31. Taylor HR, Burton MJ, Haddad D, West S, Wright H. Trachoma. Lancet. 2014;384(9960):2142–52.
32. Stocks ME, Ogden S, Haddad D, Addiss DG, McGuire C, Freeman MC. Effect of water, sanitation, and hygiene on the prevention of trachoma: a systematic review and meta-analysis. PLoS Med. 2014;11(2):e1001605.
33. Ramadhani AM, Derrick T, Holland MJ, Burton MJ. Blinding trachoma: Systematic review of rates and risk factors for progressive disease. PLoS Negl Trop Dis [Internet] 2016 [cited 2016 Nov 28];10(8). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4970760/
34. West SK, Courtright P. Trachoma control: 14 years later. Ophthalmic Epidemiol. 2015;22(3):145–7.
35. Smith JL, Sivasubramaniam S, Rabiu MM, Kyari F, Solomon AW, Gilbert C. Multilevel analysis of trachomatous trichiasis and corneal opacity in Nigeria: the role of environmental and climatic risk factors on the distribution of disease. PLoS Negl Trop Dis. 2015;9(7):e0003826.
36. Boase AJ. Tarsectomy. Br J Ophthalmol. 1952;36(11):645–8.
37. Loe F. Sulfanilamide treatment of trachoma: preliminary report. J Am Med Assoc. 1938;111(15):1371–2.

38. INSight: The end in sight. International Coalition for Trachoma Control; 2011.
39. Anthony W. Solomon. Trichiasis surgery for trachoma [Internet]. 2nd ed. World Health Organization; 2015 [cited 2016 Dec 28]. Available from: http://www.who.int/trachoma/resources/9789241549011/en/
40. Anthony W. Solomon, editor. Second global scientific meeting on trachomatous trichiasis, Cape Town, 4–6 November 2015 [Internet]. WHO Department of control of neglected tropical diseases; 2016 [cited 2016 Dec 28]. Available from: http://www.who.int/trachoma/resources/who_htm_ntd_2016.5/en/
41. Anthony W. Solomon. Validation of elimination of trachoma as a public health problem. World Health Organization; 2016.
42. Jimenez V, Gelderblom HC, Mann Flueckiger R, Emerson PM, Haddad D. Mass drug administration for trachoma: how long is not long enough? PLoS Negl Trop Dis. 2015;9(3):e0003610.
43. Ho DK-H, Sawicki C, Grassly N. Antibiotic resistance in Streptococcus pneumoniae after azithromycin distribution for trachoma. J Trop Med [Internet] 2015 [cited 2016 Dec 28]; 2015. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4628654/
44. Ejere HOD, Alhassan MB, Rabiu M. Face washing promotion for preventing active trachoma. Cochrane Database Syst Rev. 2015;2:CD003659.
45. Rabiu M, Alhassan MB, Ejere HOD, Evans JR. Environmental sanitary interventions for preventing active trachoma. Cochrane Database Syst Rev. 2012;2:CD004003.

Overview of Leptospirosis

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1 Introduction

Leptospirosis has re-emerged as a serious public health problem that is no longer limited to those who work and live in a rural setting [1]. The migration of poverty-stricken individuals to urban settings, together with climate change, has resulted in dramatic increases in the incidence of leptospirosis worldwide [2], particularly in tropical and subtropical developing countries [3, 4]. Leptospirosis is caused by pathogenic *Leptospira* spp. that have been characterized into >300 serovars [5]. Urban leptospirosis is mainly spread by rats that have proved difficult if not impossible to control. Rural leptospirosis is equally difficult to control due to the plethora of animal reservoirs and transmission between wild and domestic animal hosts. To further complicate matters, clinical diagnosis of leptospirosis is difficult as the symptoms are similar to febrile illnesses caused by dengue, Zika, chikungunya, hantavirus, as well as viral hepatitis and malaria. Laboratory diagnosis is carried out using the WHO-recommended microagglutination test (MAT), yet this is not widely available and is usually limited to reference laboratories [6]. The MAT requires two serum samples taken during the acute and convalescent phase of the disease so it has limited application to patient management. Alternative, commercially available, diagnostic tests are typically based on whole-cell antigens (inactivated leptospires), and sensitivity and specificity during the acute phase are highly variable (28–71%) [7, 8]. Therefore, there is an urgent need for new diagnostic tests that are faster, able to detect the disease at an early stage and that may be performed in areas having limited laboratory capacity, allowing for the rapid initiation of treatment. Inactivated vaccines, known as bacterins, are highly effective against the target serovar, but they are poorly cross-protective against other serovars and can cause severe side effects, and few countries around the world have approved their use as human vaccines [9]. Severe leptospirosis is life-threatening and can manifest as acute kidney injury (AKI), the classic manifestation of Weil's disease, and more recently as leptospirosis pulmonary haemorrhage syndrome (LPHS) [10]. The aim of this chapter is to provide an overview of human leptospirosis and its causative agent, the pathogenic *Leptospira* spp.

2 Epidemiology of Leptospirosis in South Asia

The latest report by the WHO on the annual worldwide incidence of leptospirosis was >1 million cases, ca. 59,000 fatalities, and 73% of cases are in countries located within the Tropics of Cancer and Capricorn [3]. Leptospirosis is severely underreported in the region, and this has hindered attempts to determine the actual incidence and burden of the disease. As a region, South Asia was estimated to have an annual incidence of 18/100,000 population and a mortality of 1/100,000 [3]. Towards combating global epidemics and zoonoses in the region, the One

Health Network South Asia (http://www.onehealthnetwork.asia) was established in 2010 and includes all countries in the region. The principal objective of the network is to train public health specialists and veterinarians in combating priority zoonoses through formal training programmes in epidemiology and biosecurity.

The Islamic Republic of Afghanistan Due to ongoing wars in the region, there is very little published literature available. There are reports of ca. 200 cases in 2008, equivalent to an annual incidence of 0.6/100,000 population and 0.3/100,000 in 2009; however, no data is available after 2009 [11]. The WHO estimated the annual incidence and mortality of leptospirosis as 3 and 0.4/100,000, respectively [3]. The burden of the disease was estimated as 6686 disability-adjusted life years (DALYs) or 23/100,000 [4].

The People's Republic of Bangladesh From 2008 to 2009, two cases were diagnosed, equivalent to 0.3% of febrile cases studied [12], compared to 18% during an outbreak of dengue fever in 2001 [13]. Lack of a confirmatory diagnosis resulted in most febrile cases being classified as malaria; however, a community survey from 2007 to 2010 found that 25–40% of febrile cases were seropositive for leptospirosis [14]. The WHO estimated the annual incidence and mortality as 19 and 1/100,000, respectively [3]. The burden of the disease was estimated as 84,270 DALYs or 51/100,000 [4].

The Kingdom of Bhutan There was one reported case in 2011, equivalent to an incidence of 0.14/100,000 [11, 15]. The WHO estimated the annual incidence and mortality as 11 and 0.6/100,000, respectively [3]. The burden of the disease was estimated as 231 DALYs or 33/100,000 [4].

The Republic of India A multicentric study in India showed that leptospirosis accounts for ca. 13% of cases of acute febrile illness in hospitals. Regular leptospirosis epidemics occur, and most are located in the coastal regions and the islands, reviewed in [16]. India does not have a national database for leptospirosis even though it is responsible for 12% of acute febrile illness [17]. Outbreaks are associated with the monsoon season (July–October), and seropositivity ranges from 13 to 35%, depending on the laboratory diagnostic test employed; the MAT is not generally available [18–24]. Leptospirosis is highly endemic in the Andaman Islands, and there are reports of outbreaks among the convict population in 1892 [25]. Leptospirosis re-emerged in the 1980s and is now recognized as a serious public health problem, and outbreaks regularly occur in the post-monsoon period. A report from the Andaman Islands found that 10% of water samples collected from urban areas were positive for pathogenic *Leptospira* spp., compared to 7% of rural water samples [26]. Recent studies reported a seroprevalence of >55% in the general population [27]. Agricultural workers were most at risk, 63% of those tested were seropositive, followed by 39% of sewage workers and 38% of animal handlers. *L. interrogans* serogroup Grippotyphosa was most common among seropositive samples; other serogroups included Australis and Canicola. Risk factors for leptospirosis include planting and harvesting of crops, handling animals, heavy rain, exposure to contaminated water, poor living conditions and lack of protective

footwear [21]. The WHO estimated incidence and mortality of leptospirosis as 20 and 1/100,000, respectively [3]. The burden of the disease was estimated as 684,369 DALYs or 56/100,000 [4].

The Republic of Maldives The first reported case was in 2000, although in 2003 the incidence had increased to ca. 4/100,000 [11, 15]. The WHO estimated the incidence and mortality of leptospirosis as 274 and 11/100,000, respectively [3]. The burden of the disease was estimated as 1791 DALYs or 570/100,000 [4].

The Federal Democratic Republic of Nepal In a study of suspected leptospirosis in 2011, 28% of the cases were ELISA positive [28]. Nepal introduced a One Health Network study in 2013, and 295 febrile patients were recruited; however, there are no MAT results available yet [29]. In a study during 2007–2008, seropositivity was 37–43% [30]. There are reports of an unidentified febrile illness from 2009 to 2010; 50% of the cases were seropositive for leptospirosis. The WHO estimated the incidence and mortality of leptospirosis as 16 and 0.9/100,000, respectively [3]. The burden of the disease was estimated as 1086 DALYs or 6/100,000 [4].

The Islamic Republic of Pakistan There are few published reports of leptospirosis in Pakistan [31], and the earliest available reported a prevalence of 25% among patients in Karachi hospital [32]. They also found 100% seropositivity in rats captured in urban or rural settings, and the most common serovars were Grippotyphosa and Icterohaemorrhagiae. In a study of patient's resident in Lahore, seropositivity as high as 44% was reported during 2010–2011 [33]. Leptospirosis prevalence peaked (50–67%) during March–September, overlapping with the rainy season. As part of the One Health Network South Asia, Pakistan recognized the need for increased surveillance of zoonotic diseases. The WHO estimated incidence and mortality of leptospirosis as 8 and 0.5/100,000, respectively [3]. The burden of the disease was estimated as 47,432 DALYs or 26/100,000 [4].

The Democratic Socialist Republic of Sri Lanka Prior to 1991, there were <200 reported cases and incidence was ca. 1/100,000. However, when leptospirosis became a notifiable disease in 1991, this rose to 1000–2000 annual cases and an incidence of ca. 11/100,000 up to 2007. Then in 2008, more than >7000 suspected cases were reported, equivalent to an incidence 36/100,000 and a case fatality rate of ca. 3% [34]. In 2013, there were >4000 reported cases, and the incidence was 21/100,000 [35]. Leptospirosis outbreaks were concentrated in nine districts, the so-called wet zones, where leptospirosis is endemic and the annual incidence varies from 31 to 164/100,000 [36]. While *L. interrogans* was responsible for most cases in these districts, an outbreak in 2010 in a nonendemic, dry zone reported that *L. kirschneri* was responsible [36]. It remains unclear what caused the dramatic increase in cases; however, as there is a lack of laboratory diagnosis in Sri Lanka, most cases were based on clinical suspicion which is often inaccurate due to its similarity with other febrile illnesses. In a study to confirm the clinical diagnosis of

leptospirosis, only 38% of the cases were confirmed by laboratory diagnosis [37]. In addition, there is a lack of public awareness of leptospirosis and the risk factors associated with its transmission [34, 38]. Sri Lanka has a large agricultural economy and heavy rainfall, which makes the transmission of leptospirosis an occupational hazard [35]. As part of the One Health Network, Sri Lanka introduced a project that includes a hospital-based case-control study to identify risk factors and the reservoir species involved in the transmission of leptospirosis [39]. The WHO estimated incidence and mortality of leptospirosis as 301 and 18/100,000, respectively [3]. The burden of the disease was estimated as 164,368 DALYs or 805/100,000 [4].

3 Pathogenic *Leptospira* spp.: The Causative Agent of Leptospirosis

Currently, 22 species of *Leptospira* have been identified (Table 1); 7 species are saprophytic and cannot establish an infection; the remaining 15 species are divided in 2 taxonomic clades, the pathogenic and intermediate *Leptospira* spp. The pathogenic species are the causative agents of leptospirosis, while the five

Table 1 *Leptospira* spp. and their distribution among pathogenic, intermediate and saprophytic species groups

Taxonomic clade	Species	Reference
Pathogenic species	*L. alexanderi*	[40]
	L. alstonii	[41]
	L. borgpetersenii	[42]
	L. interrogans	[43]
	L. kirschneri	[44]
	L. kmetyi	[45]
	L. mayottensis	[46]
	L. noguchii	[42]
	L. santarosai	[42]
	L. weilii	[42]
Intermediate species	*L. broomii*	[47]
	L. fainei	[48]
	L. inadai	[42]
	L. licerasiae	[49]
	L. wolffii	[50]
Saprophyte species	*L. biflexa*	[43]
	L. idonii	[51]
	L. meyeri	[42]
	L. terpstrae	[41]
	L. vanthielii	[41]
	L. wolbachii	[42]
	L. yanagawae	[41]

intermediate species cause only a mild disease in humans and animals [52]. The taxonomic classification of *Leptospira* spp. is defined by genome to genome distance, currently performed mainly by in silico approaches after whole genome sequencing. Leptospires are also serologically classified into >300 serovars that are grouped in to 24 serogroups [53]. The structural heterogeneity of the O antigen of leptospiral lipopolysaccharide (LPS) is responsible for the vast serological variability observed for *Leptospira*.

All members of the *Leptospira* genus share common morphological features, such as the distinctive cork-shaped, right-handed helical cell, usually with hooked ends. Leptospires are highly motile bacteria due to the presence of two periplasmic flagella, one attached at each extremity. The leptospiral cell is thin, 0.1–0.2 μm in diameter with 10–20 μm length, making it difficult to visualize by light microscopy [53]. The routine observation of live leptospires is performed by dark-field microscopy (Fig. 1). Therefore, classical staining techniques such as gram staining are not applicable to *Leptospira* spp. Like gram-negative bacteria, leptospires are diderms, with an inner and outer membrane (OM) and a periplasmic space in between. The inner membrane is thinner than the OM and is closely associated with a peptidoglycan layer [1]. The outer leaflet of the OM is composed of LPS on the bacterial surface. Several proteins are also associated with leptospiral membranes; transmembrane α-helix proteins span the inner membrane, while β-barrel transmembrane proteins are located in the OM [54, 55]. Many lipoproteins are associated with both membranes, including surface-exposed lipoproteins [55]. Leptospires have complete type I secretion systems and a partial type II secretion system [56]. Although leptospiral secreted proteins have been described, the experimental functionality of these secretion systems remains to be explored [57, 58]. Other OM proteins (OMPs) include transporters such as TonB-dependent transporters, porins and OM assembly machinery [54, 55]. Indeed, many of these surface-exposed proteins have been evaluated as vaccine candidates [9].

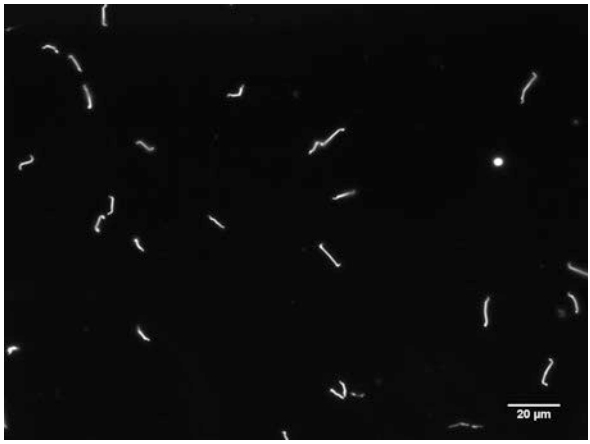

Fig. 1 *Leptospira interrogans* under dark-field microscopy

Leptospires are fastidious aerophilic bacteria that grow in vitro in enriched media supplemented with rabbit serum and bovine serum albumin. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium is the most commonly used culture media for *Leptospira* spp. [53]. Long-chain fatty acids are used by leptospires as the carbon source; they cannot use glucose as an energy source due to the lack of an uptake system. Additional nutritional supplements include calcium, iron, phosphate, thiamine, copper, manganese and sulphate [59]. Vitamin B12 is also routinely added to culture medium, even though some pathogenic species can synthesize cobalamin [60]. *Leptospira* spp. are usually cultured in vitro at 28–30 °C, although the saprophytic leptospires can grow at 5–10 °C, compared to a minimum of 13–15 °C for the pathogens [61]. The pathogenic leptospires grow at physiologic temperatures, i.e. 37 °C. The doubling time in vitro varies from 8 to 31 h depending on the species, growth medium, temperature and starting inoculum, among other factors. Pathogenic *Leptospira* spp. lose virulence after a high number of in vitro passages, although this can be restored by recovery from infected animal models [62]. The mechanism and the number of passages required for loss of virulence have not been not properly determined.

The genomes of *Leptospira* spp. are ca. 4.2 Mbp and includes two chromosomes; the larger one is >3.6 Mbp, and the smaller one ranges from 278 to 300 kbp [60]. Each genome includes approximately 4100 CDS, many of them encoding proteins with unknown function. The core and pan genomes of 20 *Leptospira* spp. (*L. mayottensis* and *L. idonii* were not included) have been studied and are composed of 1764 and 17,477 genes (1592 and 13,822 when the paralogs are collapsed), respectively [60]. Leptospiral genomes include multiple insertion sequences, several phages and CRISPR/Cas systems [60, 63]. Only a small number of plasmids have been identified, and this has severely hampered the development of tools for genetic manipulation [1, 64]. Some 750 genes are arranged in operons within the *L. interrogans* genome, and the majority are composed of one or two genes [65].

There is evidence that *Leptospira* spp. regulate gene expression in response to environmental cues [66–69]. Leptospires are readily adaptable; they can infect a range of animal hosts and, unlike other spirochaetes, can survive outside the host in harsh conditions such as wet soil and water [52]. Gene expression is regulated by housekeeping and alternative sigma factors, two component systems and other bacterial regulation factors. Among the host signals are physiologic temperature, serum, osmotic pressure, phagocytes and innate immune response. Gene promoters are not easily identified, and a surprisingly large number of mRNAs (~440) are leaderless [65]. Several putative regulatory small RNAs (sRNA) are expressed by *Leptospira* spp., as demonstrated by total RNA sequencing (RNA-Seq) and Northern blotting experiments [65, 66]. As expected, there is little similarity between mRNA and protein levels in *L. interrogans* grown under the same conditions [70], probably due to post translational regulation of gene expression, most likely by sRNAs [65].

Leptospires differentially express proteins under different environmental conditions [71–73]. Some proteins are not expressed under standard in vitro culture conditions, e.g. the leptospiral immunoglobulin-like (Lig) proteins. The expression

of the Lig proteins was demonstrated to be induced by osmolarity [74], and an RNA thermometer was suggested to additionally regulate expression [75]. The lipoproteins LipL32, LipL41, LipL21, LipL36 and Loa22 are among the most highly expressed proteins in *L. interrogans* [71]. Post-translational modifications in leptospiral proteins have been reported and most likely play a role in protein function and interactions with the environment and their hosts. Methylation [76], acetylation [77] and addition of sialic acid have been described for leptospiral proteins [78].

4 Transmission and Pathogenesis of Leptospirosis

Leptospires are maintained in nature by asymptomatic animals, such as dogs, cattle, several wild animals and especially rodents (Fig. 2). Leptospires are excreted in their hosts' urine and can survive outside the animal reservoir for months [79, 80]. Transmission of leptospires to humans or other animals occurs mainly through direct contact with infected animals or indirect contact with soil or water contaminated with leptospires [81, 82]. Entry into the host is usually via the mucous membranes or abraded skin [1]. Once through the skin barrier, leptospires can attach to host molecules, invade blood vessels and rapidly spread throughout the

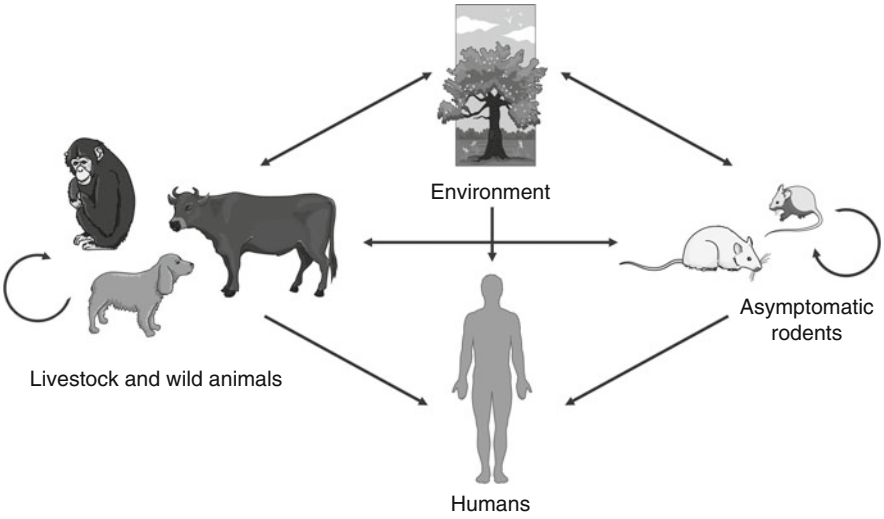

Fig. 2 The transmission cycle of pathogenic *Leptospira* spp. Rodents are the main asymptomatic carriers of leptospires in either urban or rural settings. Domestic and wild animals can be asymptomatic or susceptible depending on the infecting serogroup and the health status of the host. Leptospires are excreted in urine resulting in contamination of the environment where they can survive for months in damp conditions until they infect another host

host [1]. In the first days of infection, leptospires can be found in the bloodstream at high density ($>10^4$ leptospires/ml of blood is associated with poor prognosis) [83, 84]. The activation of the immune response (see below) usually clears leptospires from the bloodstream of the maintenance hosts. In asymptomatic carriers, leptospires colonize the proximal renal tubules from where they are excreted back into the environment. In susceptible animals that develop leptospirosis, the spirochaetes can be found in other organs, such as the liver and the lungs [82].

The understanding of the molecular basis for host-pathogen interaction and virulence has advanced in recent years. Several leptospiral proteins have been described as adhesins by in vitro experiments with recombinant proteins, reviewed in [85]. These proteins can bind a diverse range of extracellular matrix components, complement proteins, cadherin, fibronectin, thrombin and plasminogen. There is some evidence that leptospires can secrete proteins that could potentially interact with host cells, contributing to pathogenesis [57, 58]. *Leptospira* spp. genomes encode several sphingomyelinases, phospholipases and proteases whose functions and roles in pathogenesis are still under evaluation [1].

The pathogenic *Leptospira* spp. are not readily amenable to genetic manipulation, although several mutants have been generated, mostly by random transposon mutagenesis [86, 87], or targeted mutagenesis [88–91]. The evaluation of mutant virulence in animal models, especially for acute disease, has enabled the identification of several virulence factors essential for colonization and disease. The first leptospiral virulence gene to fulfil molecular Koch's postulates was Loa22, which contains an OmpA-like domain [92]. The *L. interrogans loa22* knockout mutant was attenuated for virulence in both hamster and guinea pig models of lethal leptospirosis. The genetic complementation of Loa22 restored the virulent phenotype. However, the function and subcellular localization of Loa22 remain unclear. Other virulence factors identified by mutagenesis and subsequent loss of virulence in animal models include LPS [93], motility [91, 94–96], haem oxygenase (HemO) [97], the chaperons ClpB [98] and HtpG [99], the lipoprotein LruA [100] and the catalase KatE [101], a protein related to oxidative stress. Mutants to the abundant OM lipoproteins LipL32 [87], LipL41 [102] and LigB [88] were not attenuated in virulence, although this is likely due to redundancy in the leptospiral genome [103].

5 Host Response and Immunity

Macrophages are important cell types in the immune response against leptospirosis. *Leptospira* spp. stimulate macrophages to produce pro-inflammatory chemokines including IL-1β, IL-6, IL-8 and TNF-α [104]. However, pathogenic leptospires can survive within macrophages; several proteins have demonstrated a role in leptospiral adhesion and uptake by these cells [89, 105]. Catalase activity by pathogenic *Leptospira* spp. confers resistance to the reactive oxygen species induced by the host innate immune cells [101]. However, *Leptospira* spp. are susceptible to

neutrophil extracellular traps (NETs), and while neutrophil-depleted mice have a significantly higher bacterial burden in their kidneys during infection, this did not alter the course of the disease [106].

Due to an atypical lipid A composition, which has a unique methylated phosphate residue not found in other Gram-negative bacteria, leptospiral LPS only activates the Toll-like receptor (TLR) 2 in human innate immune cells, compared to the activation of both TLR2 and TLR4 in murine and other resistant hosts. The activation of TLR4 is required for early production of immunoglobulins and the subsequent clearance of leptospires from the blood [107]. A study showed that TLR4 knockout mice were susceptible to acute leptospirosis, with an increased burden of leptospires in the liver, lungs and kidneys [108].

Activation of the complement system is another important immune mechanism for the early elimination of leptospires from the host. The saprophyte *L. biflexa* is killed in the presence of normal human serum, while pathogenic *Leptospira* spp. are able to evade complement by binding several of the complement regulatory components, including Factor H (fH) and C4b-binding protein (C4BP), or by secreting proteases that directly cleave the complement molecules [109, 110].

Host immune responses to infection are directly related to severe outcomes, with some hospitalized patients showing symptoms of a cytokine storm; however, a correlation between cytokine levels and clinical features remains unclear [82]. As well as a role in the inflammatory response, high levels of circulating TNF-α may be a predictor of poor prognosis; this cytokine has been associated with pulmonary haemorrhaging and death [111]. Furthermore, high levels of IL-10 have been associated with a fatal outcome, possibly due to the inhibition of a protective Th1 immune response [111, 112]. However, IL-10 anti-inflammatory activity is important for controlling disease progression. Since there is a correlation between a low IL-10/TNF-α ratio and severe cases, this ratio should be evaluated frequently [113].

The humoral response to a leptospiral infection plays a leading role in immune protection, with patients producing substantial amounts of antibody. B-cell-depleted mice were susceptible to lethal leptospirosis induced by pathogenic *Leptospira* spp. in contrast to wild-type mice [114]. Anti-LPS antibodies corresponded to the majority of the antibodies produced in a T-cell-independent manner [115]. Mice lacking T cells produced a protective immune response and survived in different studies [107, 116]. Passive immunization with monoclonal antibodies against leptospiral LPS successfully protected guinea pigs and hamsters against challenge [117, 118]. Despite the importance of LPS antibodies for host survival, the humoral response pattern against leptospires is responsible for the serovar-specific protection conferred by commercial vaccines [119]. This observation is endorsed with the cross-protective immunity elicited by an attenuated LPS mutant [120].

6 Clinical Features of Leptospirosis

The clinical symptoms in leptospirosis patients are highly variable, and the majority (90%) present with an asymptomatic infection or flu-like symptoms. However, the disease can progress to severe forms leading to multiple organ failure and death. Leptospirosis has a biphasic disease course. The early acute phase is characterized by non-specific symptoms including fever, headache, and myalgia, which allow a misdiagnosis with other diseases, such as dengue and malaria. This phase lasts ca. a week, is called the leptospiremic phase, and the bacteria are found in the bloodstream or in the cerebrospinal fluid. With the appearance of IgM antibodies and the concomitant clearance of leptospires from the blood, the immune phase is initiated. The disease may resolve in this phase, with only a small fraction (5–15%) of the cases evolving to more severe forms of the disease [3]. In these cases, leptospires are present in several tissues and organs including the kidneys, liver, lungs and brain and are excreted in the urine. Organ colonization by leptospires causes extensive clinical manifestations, the classic Weil's disease, which is characterized by jaundice, haemorrhage and acute kidney injury and more recently, LPHS. Acute renal and lung injuries are reported in 36 and 17% of leptospirosis hospitalized patients, respectively [121].

6.1 Renal Manifestations of Leptospirosis

The kidneys are a major target organ for leptospires following elevation of immunoglobulin levels in the blood, although *Leptospira* spp. do not demonstrate tropism [82]. Renal involvement varies from mild proteinuria and urinary sediment presenting leukocytes and erythrocytes to severe renal failure. Tubulointerstitial nephritis is the major renal change in leptospirosis, in part due to the recognition of leptospiral OMPs by TLR2 expressed in renal tubule cells, mediating an early inflammation response [122]. Interstitial oedema; cellular infiltration, mainly by mononuclear cells; and epithelium necrosis are the most frequent pathological processes involved in acute kidney injury (AKI) [123]. AKI can occur in 60% of the cases of patients with severe disease. Leptospirosis-induced AKI is typically non-oliguric, although the limited number of oliguric cases have a higher mortality rate [124]. The duration of renal failure can vary from days to several weeks, and patients usually recover within 6 months [125].

6.2 Pulmonary Manifestations of Leptospirosis

Pulmonary manifestation is an important cause of mortality in leptospirosis. Symptoms range from chest pain, cough, dyspnoea and haemoptysis to acute respiratory syndrome, and intra-alveolar haemorrhaging has been detected in the majority of these cases [126]. Pulmonary involvement occurs in 20–70% of severe leptospirosis

cases with high mortality rates [127, 128]. Leptospirosis enhanced lung epithelial and endothelial permeability due to a noninflammatory injury, which in association with an impaired alveolar fluid clearance can promote respiratory failure and death [129]. Patients may also present haemoptysis, tachypnoea and cyanosis, although jaundice can be absent in LPHS [127].

6.3 Other Manifestations of Severe Leptospirosis

Leptospirosis can involve the nervous system, and aseptic meningitis is the commonest manifestation. Patients with leptospiral meningitis usually show severe headache and cerebrospinal fluid pleocytosis [130]. Encephalitis, cerebellitis, myelitis and ataxia are other presentations of neuroleptospirosis already reported [130, 131]. Several studies demonstrated the occurrence of myocarditis in leptospirosis, contributing to arrhythmias, heart failure and potentially lethal outcomes [132]. Uveitis is another well-known late complication presented as a unilateral or bilateral acute non-granulomatous panuveitis [133].

Leptospirosis patients show several changes in haematological parameters. Decrease in haemoglobin levels, probably due to iron sequestration by leptospires, and thrombocytopenia are frequently observed complications during the course of infection [134]. This has been associated with poor prognoses, especially in association with haemorrhagic events. Elevated serum bilirubin levels due to disruption of intercellular junctions between hepatocytes resulting in destruction of bile canaliculi are typical of icteric forms of leptospirosis [135]. Dysregulation of sodium transporters promotes kaliuresis, and consequently hypokalemia presented in leptospirosis AKI can impair lung oedema clearance, resulting in increased lung injury [136].

7 Laboratory Diagnosis of Leptospirosis

Culture and the MAT are the gold standard diagnostic tests for leptospirosis even though the numerous shortcomings of the MAT have been discussed in the literature; see, e.g., [7, 8]. Isolation of pathogenic *Leptospira* spp. remains a challenge as they are fastidious and slow-growing (4–6 months), and cultures are susceptible to contamination. There are many commercially available serological tests; however, sensitivity and specificity and predictive values are highly variable in the acute phase of the disease. The majority use the saprophyte *L. biflexa* as the antigen to detect anti-*Leptospira* antibodies in serum samples. As these antibodies are known to persist for years after the original infection, this can impact on their specificity in endemic regions. There are few multicentre evaluations of the readily available diagnostic tests and even fewer that follow the STARD guidelines (http://www.stard-statement.org), making comparisons difficult. The only way to determine the performance parameters of a diagnostic test is to evaluate it using samples collected from the

target population. While much emphasis has been put on rapid diagnostic tests (RDTs), these are serological tests and rely on the detection of antibodies in serum samples; therefore they will not detect leptospirosis during the first 7–10 days of symptoms [137]. During this leptospiremic phase, the most sensitive test is based on detecting leptospires in blood samples. PCR using whole blood samples and EDTA as the anticoagulant (heparin inhibits the PCR) is recommended for the amplification of leptospiral DNA [138]. Alternatively, the transport of blood samples is easily carried out using Whatman filter paper and represents a simple, inexpensive solution when resources are limited [139]. The use of commercial kits for the extraction of DNA for PCR-based diagnostic tests is recommended [140].

7.1 Direct Examination of the Blood

The cut-off or limit of detection (LOD) is around 10^4 leptospires/ml of blood and requires a dark-field microscope, extensive training and is not recommended by the WHO as false positives can be caused by serum proteins and debris in the blood samples [6]. The LOD usually excludes the use of urine samples as they are too dilute for dark-field microscopy.

7.2 Polymerase Chain Reaction

Several conventional and real-time PCR methods are readily available and have been extensively described in the literature; see, e.g., [138, 141–144]. The LOD is around 100 leptospires/ml of blood or urine, and it is simple to differentiate pathogenic *Leptospira* spp. using primer pairs designed to gene targets that are absent in the saprophytes, e.g. *lipL32*, *ligA*, *ligB*, etc. The identification of pathogenic versus saprophytic and the genotyping of *Leptospira* spp. has been proposed by several groups and could further extend the utility of PCR diagnosis [145–147].

7.3 Isothermal Amplification

Several techniques are available, although loop-mediated isothermal amplification (LAMP) is the most commonly used. The amplification results in production of 10^9 copies of the target and requires a water bath or heating block at 60–65 °C and 1h incubation [148, 149]. The amplified DNA can be detected visually or by gel electrophoresis, making the technique ideal for application in a wide range of settings. The LAMP assays usually target the *lipL32* or *lipL41* genes for pathogenic *Leptospira* spp., although an assay based on the 16S *rrs* genes has been developed

that can discriminate between the pathogenic and intermediate clades [150]. Further studies are needed to determine the performance of LAMP in real-world settings.

7.4 ELISA

There are many commercial and in-house ELISA being used for the diagnosis of leptospirosis [151–154], and while the overall sensitivity of an IgM ELISA in the first week of symptoms is low, it tends to be better than the MAT [7, 8]. However, the ELISA is an invaluable diagnostic tool during the second week of symptoms when IgM levels rise considerably.

7.5 Lateral Flow RDTs

RDTs are ideally suited to clinical health centres that do not have ready access to central laboratory facilities. Early administration of antibiotic therapy is known to aid recovery and can be a major factor in patient prognosis [7, 155]. The use of RDTs to perform a differential diagnosis of leptospirosis in point-of-care settings is highly desirable, although the sensitivity and specificity of these tests are variable [7, 8, 156].

8 Treatment of Leptospirosis

Leptospirosis is treated with antibiotics, most frequently penicillin, ampicillin, ceftriaxone or cefotaxime. Doxycycline and azithromycin can also be used, although they are usually used in chemoprophylaxis rather than as a treatment (see below). However, antibiotic treatment can cause a Jarisch-Herxheimer reaction (JHR) that is characterized by the release of endotoxins from dying microorganisms and production of TNF-α, IL-6 and IL-8 [157]. Cases of JHR following administration of antibiotics against leptospirosis have been previously reported [158]; and although rare, the prevalence of JHR in cases of leptospirosis is unknown. As a JHR can be fatal [159], anti-inflammatory, including anti-TNF-α, inhibitors should be co-administered with the antibiotics, and patients should be monitored for adverse reactions following treatment [160]. Furthermore, a severe immune response is suspected to play a damaging role in cases of advanced leptospirosis, in which the administration of anti-inflammatory drugs is also advised [161]. In severe cases of leptospirosis, where hospitalization is required, symptomatic treatment may also be necessary. Dialysis and/or administration of IV fluids may help combat renal injuries [162–164]; mechanical ventilation with low tidal volume could be beneficial for patients with lung damage and difficulties breathing [165].

9 Prevention and Control of Leptospirosis

9.1 Infrastructure and Sanitation Intervention

The control of leptospirosis relies on understanding its epidemiology and how the disease is transmitted. Routine surveillance to identify and interrupt sources of infection is key in preventing leptospirosis; identifying the circulating serovars is essential towards identifying and controlling the maintenance hosts [2, 52]. In addition, the efficacy of inactivated vaccines or bacterins depends on knowledge of the serovars that are endemic to the target region.

Rats are the main source of human leptospirosis infections in urban settings [166], although domestic animals (e.g. pigs, cattle, dogs, etc.) also play a significant role in the transmission of leptospirosis, especially in rural areas. Occupational exposure where there is water accumulation (rice paddies, mud or water puddles where livestock lives, etc.) is also a potential source of transmission for both humans and non-maintenance animals [167]. The ever-increasing population of slum-dwellers caused by the ongoing migration from rural to urban areas has provided the rat population with ecological conditions for explosive expansion, resulting in increased transmission of leptospirosis in these conditions [3, 166, 168–170]. Eradicating the rat population, or any other type of wildlife reservoir, is virtually impossible, and rodenticides can pose health and environmental problems [171]. Likewise, the use of chemicals to kill bacteria in the environment is ineffective as daily application is required and recontamination will occur unless the source is eliminated [172]. Therefore, local government bodies should invest in sanitation and associated infrastructure interventions to control rodent spread, and floodwater should be properly drained during periods of heavy rainfall. Furthermore, new housing projects should include measures to prevent rodents and floodwater from entering. Livestock housing should be constructed such that effluent can be properly decontaminated before being discarded [172].

High-risk groups including veterinarians, abattoir workers, farmers, agricultural workers, sewage workers, etc. should pay close attention to personal protective measures such as appropriate footwear, gloves and goggles to avoid contact with contaminated soil or water, or the tissue and urine of infected animals [52, 172]. These measures are not always possible to implement from either personal or economic perspectives, but they are essential towards reducing the transmission of leptospirosis. Avoiding wet conditions or places contaminated with the urine of potential reservoirs should also be considered; however, this is often unavoidable [172]. Alternatively, vaccinating livestock and pets is probably the most cost-effective intervention towards preventing transmission of leptospirosis, and this is a legal requirement in several countries in Europe. Finally, people engaging in water-related activities or sports or events that take place in tropical regions should also pay close attention to personal protective measures and should also consider vaccination (if available) or chemoprophylaxis (see next section).

9.2 Chemoprophylaxis

Chemoprophylaxis is the use of antibiotics to prevent an infection or attenuate its symptoms. It is not feasible in animals and not recommended for long-term use in humans [172]. However, prompt treatment is encouraged once leptospirosis or potential infection has been detected (e.g. laboratory accident). Furthermore, chemoprophylaxis often only mitigates clinical signs, but does not prevent infection [173]. Chemoprophylaxis is rarely used for humans, unless temporary exposure is foreseen or known to have happened, e.g. military personnel in the jungle [174], after floods [175]. Doxycycline (200 mg per week) is the drug of choice for human chemoprophylaxis, followed by azithromycin or amoxicillin for photosensitive or infant subjects. Streptomycin may also be used for chemoprophylaxis in livestock. The use of antibiotics is not indicated as a long-term preventive measure [172, 176].

10 Vaccines Against Leptospirosis

10.1 Conventional Vaccines

The first report of a vaccine against leptospirosis dates back to 1916 [177]. Although leptospirosis vaccines have been around for over a century, few countries have approved their use as human vaccines (e.g. China [178], Cuba [179], Japan [180], France [181, 182] and Russia [183]). Such vaccines have also been described in other Asian countries [15]. These vaccines are whole-cell killed *Leptospira* spp., known as bacterins, or OM envelope vaccines with efficacy rates of ca. 75–78% [179, 184]. A French study on sewer workers in Paris showed there were several side effects following vaccination, despite protecting against leptospirosis [185]. Bacterin vaccines have been used in China to protect those at risk of exposure during heavy rainfall and flooding [178] and with mine workers Japan [186]. Bacterin vaccines confer serovar-specific, short-term protection and are associated with several undesirable side effects; annual boosters are necessary to maintain immunity [172]. Bacterin immunity is generated primarily against leptospiral LPS, which is a thymus-independent antigen, this may explain the short-term immunity and lack of cross-protection [9]. Constant epidemiological and serological surveys are necessary to identify the circulating serovars that should be included in the bacterin preparations [9] and to identify the emergence of new serovars in a given region post-vaccination [187]. Human vaccination is only indicated for high-risk groups when other preventive measures (see previous section) are not feasible or have been unsuccessful.

The side effects of bacterins are believed to be caused by contaminating proteins being carried over from the leptospiral culture medium. Protein-free media have

been used in attempts to reduce the side effects; however, they have not been widely adopted [188]. Of note, an LPS-mutant strain was developed towards conferring cross-protective immunity against unrelated serovars by focusing the immune response on surface-exposed proteins rather than on LPS [120]. This LPS-mutant strain protected 100% of vaccinated animals against both homologous and heterologous challenges in the hamster model of acute leptospirosis.

10.2 Recombinant Vaccines Against Leptospirosis

Recombinant vaccines are an alternative to bacterins, and research in this area began almost two decades ago; the first report of a recombinant leptospirosis vaccine was published in 1999, even though only one out of three experiments showed protection [189]. In this study, leptospiral proteins OmpL1 and LipL41 expressed in the OM of *Escherichia coli* conferred significant protection against a homologous *L. kirschneri* serovar Grippotyphosa challenge in hamsters (71% survival). Since then, a myriad of recombinant OMPs and lipoproteins have been evaluated as vaccine candidates with varying degrees of success, reviewed in [176, 190, 191].

The highly conserved protein, LipL32, is expressed during infection and is the immunodominant protein of pathogenic *Leptospira* spp.; comprising up to 75% of the OM [66, 71, 192, 193]. LipL32 has been evaluated in DNA [194], subunit [195–197] and recombinant BCG and viral vector vaccine preparations [198, 199]. Yet, it seldom confers immunity when appropriate controls and rigorous statistical analysis are used, reviewed in [176, 190]. Also, it is not clear whether it is a surface-exposed lipoprotein or is located on the inner leaflet of the OM; the evidence remains contradictory [200, 201]. Furthermore, an *L. interrogans* LipL32 knockout remained virulent [87]. It is surprising that such an abundant protein is neither essential for virulence nor the structural integrity of the OM; in addition, the function of LipL32 remains unclear. However, there is a strong degree of functional redundancy in the surfaceome of pathogenic *Leptospira* spp., which might account for these extraordinary observations [202].

The preeminent recombinant vaccine candidates to date are the LigA and LigB proteins; they are specific to, and are highly conserved in pathogenic *Leptospira* spp. [203–205]. The expression of these virulence factors is upregulated in vivo, and they play a role in adhesion, invasion and evasion of the host immune system [74, 109, 206–209]. However, similar to LipL32, an *L. interrogans* LigB knockout remained virulent [88]. These proteins have been reported to confer up to 100% protection on vaccinated hamsters [210–215]. Despite conferring significant protection, LigA is present in only three pathogenic *Leptospira* spp., while LigB is present in all pathogenic *Leptospira* spp. to date [60]. Although LigA is highly immunogenic, LigB is less so and induced low antibody titres, even though it was protective. Furthermore, we recently demonstrated that a fragment of LigB could induce sterilizing immunity in the hamster model of lethal leptospirosis [213]. LigC has not been evaluated as a vaccine candidate as it is a pseudogene in many strains

and unlikely to be virulence-related [205]. The virulence determinant Loa22 was evaluated as a vaccine candidate, and while immunogenic, it did not induce a significantly protective immune response [216, 217].

Although recombinant vaccines diminish the side effects associated with bacterins, their immunogenicity is drastically reduced; therefore the use of adjuvants is essential. The most used adjuvants for vaccines are aluminium salts [218]. These adjuvants induce a Th2 response, in contrast to bacterin vaccination, which induces a Th1 response [213]. Both Th1 and Th2 responses are capable of affording protection against lethal challenge in hamsters. Notably, vaccination with LigBrep (LigB131-645) using aluminium hydroxide as adjuvant induced sterilizing immunity in hamsters, while a bacterin did not. Oil-based adjuvants, such as emulsions and liposomes, should be considered when working with hydrophobic OMPs as they should help the recombinant protein adopt its correct conformation. Liposomes [219–221], carbon nanostructures [222], CpGs and xanthan gum [223] and flagellin [224] have also been evaluated as alternative adjuvants.

10.3 Reverse and Structural Vaccinology

The term reverse vaccinology was first used in the early 1990s to describe the process of identifying all of the potential vaccine candidates in the genome of a pathogen, rather than trying to discover proteins by screening of in vitro grown microorganisms [225]. This approach became viable as the costs associated with high-throughput sequencing fell, together with the development of bioinformatics tools for the in silico identification of, e.g., signal peptides, subcellular location and lipoprotein motifs. This has led to the availability of hundreds of genome sequences from strains of the same species [60, 187, 226]. Structural vaccinology focuses on predicting the structure of likely exposed antigens (e.g. transmembrane β-barrels, lipoproteins) and identifying their function, as well as any potential role in pathogenesis, to better select proteins or their fragments as vaccine candidates [227]. Furthermore, B- and T-cell epitopes within these vaccine candidates can be predicted in silico [228]. Reverse vaccinology has been applied to *Leptospira* spp. with mixed success to date, reviewed in [9]. The structural vaccinology process was recently applied to pathogenic *Leptospira* spp., and several hundred potential vaccine candidates were identified [54, 229]. Our group is currently evaluating these vaccine candidates in the hamster model of lethal leptospirosis.

10.4 Universal Vaccine Against Leptospirosis

There are many challenges remaining for the successful development of a universal leptospirosis vaccine [191]. There is a lack of basic knowledge on how pathogenic leptospires and their hosts interact, on leptospiral metabolism and on host immunity,

making it difficult to develop a universal vaccine. To date, we know that a protective immune response against leptospirosis requires a humoral immune response; however, there is no correlation between antibody titres and protection [213], and correlates of immunity remain to be discovered [191]. Furthermore, protection in vaccinated cattle is dependent on a cellular immune response rather than on antibodies [230–232]. The development of new tools for genetic manipulation will also be fundamental. Reverse and structural vaccinology will likely play a fundamental role in helping understand these issues as well as in discovering potential targets for a universal vaccine [191].

11 Concluding Remarks

Increasing poverty leading to expanding slum communities and climate change resulting in flooding and population displacement will likely result in leptospirosis having an ever-greater impact on global public health. Basic knowledge of leptospiral microbiology has improved over the last decade, but further studies are needed, and this will require significant investment. Laboratory diagnosis remains a challenge; multicentre studies should be encouraged towards identifying improved techniques for the early diagnosis of leptospirosis and replacement of the MAT. Recombinant vaccines are showing promising results, but there is a lack of investment to bring the current vaccine candidates to clinical trials. Perhaps it is time to reconsider the use of bacterins; modern technology could reduce the side effects, towards improving the bacterin preparations for human use.

References

1. Picardeau M. Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nat Rev Microbiol. 2017;15(5):297–307.
2. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003;3(12):757–71.
3. Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global morbidity and mortality of leptospirosis: a systematic review. PLoS Negl Trop Dis. 2015;9(9):e0003898.
4. Torgerson PR, Hagan JE, Costa F, Calcagno J, Kane M, Martinez-Silveira MS, et al. Global burden of leptospirosis: estimated in terms of disability adjusted life years. PLoS Negl Trop Dis. 2015;9(10):e0004122.
5. Levett PN. Systematics of leptospiraceae. Curr Top Microbiol Immunol. 2015;387:11–20.
6. WHO, ILS. Human leptospirosis: guidance for diagnosis, surveillance and control. Malta: World Health Organization; 2003.
7. McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. Curr Opin Infect Dis. 2005;18(5):376–86.
8. Picardeau M, Bertherat E, Jancloes M, Skouloudis AN, Durski K, Hartskeerl RA. Rapid tests for diagnosis of leptospirosis: current tools and emerging technologies. Diagn Microbiol Infect Dis. 2014;78(1):1–8.

9. Dellagostin OA, Grassmann AA, Rizzi C, Schuch RA, Jorge S, Oliveira TL, et al. Reverse vaccinology: an approach for identifying leptospiral vaccine candidates. Int J Mol Sci. 2017;18(1)
10. Croda J, Neto AN, Brasil RA, Pagliari C, Nicodemo AC, Duarte MI. Leptospirosis pulmonary haemorrhage syndrome is associated with linear deposition of immunoglobulin and complement on the alveolar surface. Clin Microbiol Infect. 2010;16(6):593–9.
11. Gideon - Global Infectious Disease and Epidemiology Online Network [Internet]. GIDEON Informatics, Inc. 2017 [cited 14/07/2017]. Available from: www.gideononline.com.
12. Faruque LI, Zaman RU, Gurley ES, Massung RF, Alamgir AS, Galloway RL, et al. Prevalence and clinical presentation of Rickettsia, Coxiella, Leptospira, Bartonella and Chikungunya virus infections among hospital-based febrile patients from December 2008 to November 2009 in Bangladesh. BMC Infect Dis. 2017;17(1):141.
13. LaRocque RC, Breiman RF, Ari MD, Morey RE, Janan FA, Hayes JM, et al. Leptospirosis during dengue outbreak, Bangladesh. Emerg Infect Dis. 2005;11(5):766–9.
14. Swoboda P, Fuehrer HP, Ley B, Starzengruber P, Ley-Thriemer K, Jung M, et al. Evidence of a major reservoir of non-malarial febrile diseases in malaria-endemic regions of Bangladesh. Am J Trop Med Hyg. 2014;90(2):377–82.
15. Victoriano AF, Smythe LD, Gloriani-Barzaga N, Cavinta LL, Kasai T, Limpakarnjanarat K, et al. Leptospirosis in the Asia Pacific region. BMC Infect Dis. 2009;9:147.
16. Pappas G, Papadimitriou P, Siozopoulou V, Christou L, Akritidis N. The globalization of leptospirosis: worldwide incidence trends. Int J Infect Dis. 2008;12(4):351–7.
17. Kamath R, Swain S, Pattanshetty S, Nair NS. Studying risk factors associated with human leptospirosis. J Glob Infect Dis. 2014;6(1):3–9.
18. Patil DY, Dahake RV, Chowdhary AS, Deshmukh RA. Clinico-epidemiological observations of human leptospirosis from Mumbai, India. J Infect Public Health. 2017;10(2):247–8.
19. Maskey M, Shastri JS, Saraswathi K, Surpam R, Vaidya N. Leptospirosis in Mumbai: post-deluge outbreak 2005. Indian J Med Microbiol. 2006;24(4):337–8.
20. DebMandal M, Mandal S, Pal NK. Serologic evidence of human leptospirosis in and around Kolkata, India: a clinico-epidemiological study. Asian Pac J Trop Med. 2011;4(12):1001–6.
21. Sethi S, Sharma N, Kakkar N, Taneja J, Chatterjee SS, Banga SS, et al. Increasing trends of leptospirosis in northern India: a clinico-epidemiological study. PLoS Negl Trop Dis. 2010;4(1):e579.
22. Shivakumar S, Shareek PS. Diagnosis of leptospirosis utilizing modified Faine's criteria. J Assoc Phys India. 2004;52:678–9.
23. Kumar SS. Indian guidelines for the diagnosis and management of human leptospirosis. In: Muruganathan M, editor. Medicine Update. 23. Tamil Nadu, India; 2013.
24. Angnani R, Pathak AA, Mishra M. Prevalence of leptospirosis in various risk groups. Indian J Med Microbiol. 2003;21(4):271–3.
25. Vijayachari P, Sugunan AP, Singh SS, Mathur PP. Leptospirosis among the self-supporting convicts of Andaman Island during the 1920s--the first report on pulmonary haemorrhage in leptospirosis? Indian J Med Res. 2015;142(1):11–22.
26. Lall C, Kumar KV, Raj RV, Vedhagiri K, Vijayachari P. Prevalence and diversity of leptospires in different ecological niches of urban and rural areas of south Andaman Island. Microbes Environ JSME. 2016;31(1):79–82.
27. Sharma S, Vijayachari P, Sugunan AP, Natarajaseenivasan K, Sehgal SC. Seroprevalence of leptospirosis among high-risk population of Andaman Islands, India. Am J Trop Med Hyg. 2006;74(2):278–83.
28. Dahal KP, Sharma S, Sherchand JB, Upadhyay BP, Bhatta DR. Detection of anti-Leptospira IgM antibody in serum samples of suspected patients visiting National Public Health Laboratory, Teku, Kathmandu. Int J Microbiol. 2016;2016:7286918.
29. Gautam M, Adhikary RP, Koirala P. Investigating the zoonotic risk of human leptospirosis among febrile patients in Nepal. Bhutan: South Asia Regional One Health Symposium; 2013.

30. Kandel N, Thakur GD, Andjaparidze A. Leptospirosis in Nepal. J Nepal Med Assoc. 2012;52 (187):151–3.
31. Verma AK, Kumar A, Dhama K, Deb R, Rahal A. Mahima, et al. Leptospirosis-persistence of a dilemma: an overview with particular emphasis on trends and recent advances in vaccines and vaccination strategies. Pak J Biol Sci. 2012;15(20):954–63.
32. Ahmed IP. Serological studies on leptospirosis in Pakistan. J Pak Med Assoc. 1987;37 (9):233–6.
33. Saleem MH, Khan MS, Durrani AZ, Hassan A, Ijaz M, Ali MM. Leptospirosis: an emerging zoonosis in Pakistan. Pak J Zool. 2013;45(4):909–12.
34. Agampodi S, Peacock SJ, Thevanesam V. The potential emergence of leptospirosis in Sri Lanka. Lancet Infect Dis. 2009;9(9):524–6.
35. Bandara KK, Weerasekera M, Gunasekara CP, Ranasinghe N, Marasinghe C, Fernando N. Molecular characterisation and disease severity of leptospirosis in Sri Lanka. Mem Inst Oswaldo Cruz. 2015;110(4):485–91.
36. Agampodi SB, Dahanayaka NJ, Bandaranayaka AK, Perera M, Priyankara S, Weerawansa P, et al. Regional differences of leptospirosis in Sri Lanka: observations from a flood-associated outbreak in 2011. PLoS Negl Trop Dis. 2014;8(1):e2626.
37. Agampodi SB, Peacock SJ, Thevanesam V, Nugegoda DB, Smythe L, Thaipadungpanit J, et al. Leptospirosis outbreak in Sri Lanka in 2008: lessons for assessing the global burden of disease. Am J Trop Med Hyg. 2011;85(3):471–8.
38. Denipitiya DT, Chandrasekharan NV, Abeyewickreme W, Hartskeerl RA, Hapugoda MD. Identification of cattle, buffaloes and rodents as reservoir animals of Leptospira in the District of Gampaha, Sri Lanka. BMC Res Notes. 2017;10(1):134.
39. Amarasekera J, Agampodi S, Kodituwakku M. Risk factors and reservoir species for leptospirosis in Sri Lanka. South Asia Regional One Health Symposium, Bhutan; 2013.
40. Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for Leptospira alexanderi sp. nov. and four new Leptospira genomospecies. Int J Syst Bacteriol. 1999;49(Pt 2):839–58.
41. Smythe L, Adler B, Hartskeerl RA, Galloway RL, Turenne CY, Levett PN, et al. Classification of Leptospira genomospecies 1, 3, 4 and 5 as *Leptospira alstonii* sp. nov., *Leptospira vanthielii* sp. nov., *Leptospira terpstrae* sp. nov. and *Leptospira yanagawae* sp. nov., respectively. Int J Syst Evol Microbiol. 2013;63(Pt 5):1859–62.
42. Yasuda PH, Steigerwalt AG, Sulzer KR, Kaufmann AF, Rogers F, Brenner DJ. Deoxyribonucleic acid relatedness between serogroups and serovars in the family Leptospiraceae with proposals for seven new Leptospira species. Int J Syst Evol Microbiol. 1987;37(4):407–15.
43. Faine S, Stallman ND. Amended descriptions of the genus *Leptospira* Noguchi 1917 and the species *L. interrogans* (Stimson 1907) Wenyon 1926 and *L. biflexa* (Wolbach and Binger 1914) Noguchi 1918. Int J Syst Evol Microbiol. 1982;32(4):461–3.
44. Ramadass P, Jarvis BD, Corner RJ, Penny D, Marshall RB. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. Int J Syst Bacteriol. 1992;42(2):215–9.
45. Slack AT, Khairani-Bejo S, Symonds ML, Dohnt MF, Galloway RL, Steigerwalt AG, et al. *Leptospira kmetyi* sp. nov., isolated from an environmental source in Malaysia. Int J Syst Evol Microbiol. 2009;59(Pt 4):705–8.
46. Bourhy P, Collet L, Brisse S, Picardeau M. *Leptospira mayottensis* sp. nov., a pathogenic species of the genus Leptospira isolated from humans. Int J Syst Evol Microbiol. 2014;64 (Pt 12):4061–7.
47. Levett PN, Morey RE, Galloway RL, Steigerwalt AG. *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. Int J Syst Evol Microbiol. 2006;56(Pt 3):671–3.
48. Perolat P, Chappel RJ, Adler B, Baranton G, Bulach DM, Billinghurst ML, et al. *Leptospira fainei* sp. nov., isolated from pigs in Australia. Int J Syst Bacteriol. 1998;48(Pt 3):851–8.

49. Matthias MA, Ricaldi JN, Cespedes M, Diaz MM, Galloway RL, Saito M, et al. Human leptospirosis caused by a new, antigenically unique leptospira associated with a rattus species reservoir in the peruvian Amazon. PLoS Negl Trop Dis. 2008;2(4):e213.
50. Slack AT, Kalambaheti T, Symonds ML, Dohnt MF, Galloway RL, Steigerwalt AG, et al. *Leptospira wolffii* sp. nov., isolated from a human with suspected leptospirosis in Thailand. Int J Syst Evol Microbiol. 2008;58(Pt 10):2305–8.
51. Saito M, Villanueva SY, Kawamura Y, Iida K, Tomida J, Kanemaru T, et al. *Leptospira idonii* sp. nov., isolated from environmental water. Int J Syst Evol Microbiol. 2013;63 (Pt 7):2457–62.
52. Adler B. Leptospira and Leptospirosis. In: Adler B, editor. Berlin: Springer; 2015.
53. Cameron CE. Leptospiral structure, physiology, and metabolism. Curr Top Microbiol Immunol. 2015;387:21–41.
54. Grassmann AA, Kremer FS, Dos Santos JC, Souza JD, Pinto LDS, McBride AJA. Discovery of novel Leptospirosis vaccine candidates using reverse and structural vaccinology. Front Immunol. 2017;8:463.
55. Haake DA, Zuckert WR. The leptospiral outer membrane. Curr Top Microbiol Immunol. 2015;387:187–221.
56. Zeng L, Zhang Y, Zhu Y, Yin H, Zhuang X, Zhu W, et al. Extracellular proteome analysis of *Leptospira interrogans* serovar Lai. Omics. 2013;17(10):527–35.
57. Amamura TA, Fraga TR, Vasconcellos SA, Barbosa AS, Isaac L. Pathogenic Leptospira secreted proteases target the membrane attack complex: a potential role for thermolysin in complement inhibition. Front Microbiol. 2017;8:958.
58. Eshghi A, Pappalardo E, Hester S, Thomas B, Pretre G, Picardeau M. Pathogenic *Leptospira interrogans* exoproteins are primarily involved in heterotrophic processes. Infect Immun. 2015;83(8):3061–73.
59. Zuerner RL. Laboratory maintenance of pathogenic Leptospira. Curr Protoc Microbiol. 2005; Chap 12:Unit 12E.1.
60. Fouts DE, Matthias MA, Adhikarla H, Adler B, Amorim-Santos L, Berg DE, et al. What makes a bacterial species pathogenic?: comparative genomic analysis of the genus Leptospira. PLoS Negl Trop Dis. 2016;10(2):e0004403.
61. Johnson RC, Harris VG. Differentiation of pathogenic and saprophytic letospires. I. Growth at low temperatures. J Bacteriol. 1967;94(1):27–31.
62. Haake DA. Hamster model of leptospirosis. Curr Protoc Microbiol. 2006;Chap 12:Unit 12E 2.
63. Nascimento AL, Ko AI, Martins EA, Monteiro-Vitorello CB, Ho PL, Haake DA, et al. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J Bacteriol. 2004;186(7):2164–72.
64. Wang Y, Zhuang X, Zhong Y, Zhang C, Zhang Y, Zeng L, et al. Distribution of plasmids in distinct Leptospira pathogenic species. PLoS Negl Trop Dis. 2015;9(11):e0004220.
65. Zhukova A, Fernandes LG, Hugon P, Pappas CJ, Sismeiro O, Coppee JY, et al. Genome-wide transcriptional start site mapping and sRNA identification in the pathogen *Leptospira interrogans*. Front Cell Infect Microbiol. 2017;7:10.
66. Caimano MJ, Sivasankaran SK, Allard A, Hurley D, Hokamp K, Grassmann AA, et al. A model system for studying the transcriptomic and physiological changes associated with mammalian host-adaptation by *Leptospira interrogans* serovar Copenhageni. PLoS Pathog. 2014;10(3):e1004004.
67. Lo M, Bulach DM, Powell DR, Haake DA, Matsunaga J, Paustian ML, et al. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. Infect Immun. 2006;74(10):5848–59.
68. Xue F, Dong H, Wu J, Wu Z, Hu W, Sun A, et al. Transcriptional responses of *Leptospira interrogans* to host innate immunity: significant changes in metabolism, oxygen tolerance, and outer membrane. PLoS Negl Trop Dis. 2010;4(10):e857.

69. Qin JH, Sheng YY, Zhang ZM, Shi YZ, He P, Hu BY, et al. Genome-wide transcriptional analysis of temperature shift in *L. interrogans* serovar lai strain 56601. BMC Microbiol. 2006;6:51.
70. Lo M, Cordwell SJ, Bulach DM, Adler B. Comparative transcriptional and translational analysis of leptospiral outer membrane protein expression in response to temperature. PLoS Negl Trop Dis. 2009;3(12):e560.
71. Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW, Aebersold R. Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. Nature. 2009;460(7256):762–5.
72. Nally JE, Timoney JF, Stevenson B. Temperature-regulated protein synthesis by *Leptospira interrogans*. Infect Immun. 2001;69(1):400–4.
73. Eshghi A, Cullen PA, Cowen L, Zuerner RL, Cameron CE. Global proteome analysis of *Leptospira interrogans*. J Proteome Res. 2009;8(10):4564–78.
74. Matsunaga J, Sanchez Y, Xu X, Haake DA. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect Immun. 2005;73(1):70–8.
75. Matsunaga J, Schlax PJ, Haake DA. Role for cis-acting RNA sequences in the temperature-dependent expression of the multiadhesive lig proteins in *Leptospira interrogans*. J Bacteriol. 2013;195(22):5092–101.
76. Eshghi A, Pinne M, Haake DA, Zuerner RL, Frank A, Cameron CE. Methylation and in vivo expression of the surface-exposed *Leptospira interrogans* outer-membrane protein OmpL32. Microbiology. 2012;158(Pt 3):622–35.
77. Cao XJ, Dai J, Xu H, Nie S, Chang X, Hu BY, et al. High-coverage proteome analysis reveals the first insight of protein modification systems in the pathogenic spirochete *Leptospira interrogans*. Cell Res. 2010;20(2):197–210.
78. Ricaldi JN, Matthias MA, Vinetz JM, Lewis AL. Expression of sialic acids and other nonulosonic acids in Leptospira. BMC Microbiol. 2012;12:161.
79. Trueba G, Zapata S, Madrid K, Cullen P, Haake D. Cell aggregation: a mechanism of pathogenic Leptospira to survive in fresh water. Int Microbiol. 2004;7(1):35–40.
80. Andre-Fontaine G, Aviat F, Thorin C. Waterborne Leptospirosis: survival and preservation of the virulence of pathogenic *Leptospira* spp. in fresh water. Curr Microbiol. 2015;71 (1):136–42.
81. Mwachui MA, Crump L, Hartskeerl R, Zinsstag J, Hattendorf J. Environmental and behavioural determinants of Leptospirosis transmission: a systematic review. PLoS Negl Trop Dis. 2015;9(9):e0003843.
82. Haake DA, Levett PN. Leptospirosis in humans. Curr Top Microbiol Immunol. 2015;387:65–97.
83. Segura ER, Ganoza CA, Campos K, Ricaldi JN, Torres S, Silva H, et al. Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden. Clin Infect Dis. 2005;40(3):343–51.
84. Truccolo J, Serais O, Merien F, Perolat P. Following the course of human leptospirosis: evidence of a critical threshold for the vital prognosis using a quantitative PCR assay. FEMS Microbiol Lett. 2001;204(2):317–21.
85. Fernandes LG, Siqueira GH, Teixeira AR, Silva LP, Figueredo JM, Cosate MR, et al. *Leptospira* spp.: novel insights into host-pathogen interactions. Vet Immunol Immunopathol. 2016;176:50–7.
86. Bourhy P, Louvel H, Saint Girons I, Picardeau M. Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. J Bacteriol. 2005;187(9):3255–8.
87. Murray GL, Srikram A, Hoke DE, Wunder EA Jr, Henry R, Lo M, et al. Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*. Infect Immun. 2009;77(3):952–958.

88. Croda J, Figueira CP, Wunder EA, Jr., Santos CS, Reis MG, Ko AI, et al. Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the LigB gene does not affect virulence in animal models of leptospirosis. Infect Immun. 2008;76(12):5826–5833.
89. Zhang L, Zhang C, Ojcius DM, Sun D, Zhao J, Lin X, et al. The mammalian cell entry (Mce) protein of pathogenic *Leptospira* species is responsible for RGD motif-dependent infection of cells and animals. Mol Microbiol. 2012;83(5):1006–23.
90. Kassegne K, Hu W, Ojcius DM, Sun D, Ge Y, Zhao J, et al. Identification of collagenase as a critical virulence factor for invasiveness and transmission of pathogenic *Leptospira* species. J Infect Dis. 2014;209(7):1105–15.
91. Liao S, Sun A, Ojcius DM, Wu S, Zhao J, Yan J. Inactivation of the fliY gene encoding a flagellar motor switch protein attenuates mobility and virulence of *Leptospira interrogans* strain Lai. BMC Microbiol. 2009;9:253.
92. Ristow P, Bourhy P, da Cruz McBride FW, Figueira CP, Huerre M, Ave P, et al. The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog. 2007;3(7):e97.
93. Murray GL, Srikram A, Henry R, Hartskeerl RA, Sermswan RW, Adler B. Mutations affecting *Leptospira interrogans* lipopolysaccharide attenuate virulence. Mol Microbiol. 2010;78(3):701–9.
94. Lambert A, Picardeau M, Haake DA, Sermswan RW, Srikram A, Adler B, et al. FlaA proteins in *Leptospira interrogans* are essential for motility and virulence but are not required for formation of the flagellum sheath. Infect Immun. 2012;80(6):2019–25.
95. Fontana C, Lambert A, Benaroudj N, Gasparini D, Gorgette O, Cachet N, et al. Analysis of a spontaneous non-motile and avirulent mutant shows that FliM is required for full endoflagella assembly in *Leptospira interrogans*. PLoS One. 2016;11(4):e0152916.
96. Wunder EA, Jr., Figueira CP, Benaroudj N, Hu B, Tong BA, Trajtenberg F, et al. A novel flagellar sheath protein, FcpA, determines filament coiling, translational motility and virulence for the Leptospira spirochete. Mol Microbiol. 2016;101(3):457-470.
97. Murray GL, Srikram A, Henry R, Puapairoj A, Sermswan RW, Adler B. *Leptospira interrogans* requires heme oxygenase for disease pathogenesis. Microbes Infect. 2009;11 (2):311–4.
98. Lourdault K, Cerqueira GM, Wunder EA, Jr., Picardeau M. Inactivation of clpB in the pathogen *Leptospira interrogans* reduces virulence and resistance to stress conditions. Infect Immun. 2011;79(9):3711-3717.
99. King AM, Pretre G, Bartpho T, Sermswan RW, Toma C, Suzuki T, et al. High-temperature protein G is an essential virulence factor of *Leptospira interrogans*. Infect Immun. 2014;82 (3):1123–31.
100. Zhang K, Murray GL, Seemann T, Srikram A, Bartpho T, Sermswan RW, et al. Leptospiral LruA is required for virulence and modulates an interaction with mammalian apolipoprotein AI. Infect Immun. 2013;81(10):3872–9.
101. Eshghi A, Lourdault K, Murray GL, Bartpho T, Sermswan RW, Picardeau M, et al. Leptospira interrogans catalase is required for resistance to H_2O_2 and for virulence. Infect Immun. 2012;80(11):3892–9.
102. King AM, Bartpho T, Sermswan RW, Bulach DM, Eshghi A, Picardeau M, et al. Leptospiral outer membrane protein LipL41 is not essential for acute leptospirosis but requires a small chaperone protein, lep, for stable expression. Infect Immun. 2013;81(8):2768–76.
103. Murray GL. The molecular basis of leptospiral pathogenesis. Curr Top Microbiol Immunol. 2015;387:139–85.
104. Xue F, Zhao X, Yang Y, Zhao J, Yang Y, Cao Y, et al. Responses of murine and human macrophages to leptospiral infection: a study using comparative array analysis. PLoS Negl Trop Dis. 2013;7(10):e2477.
105. Toma C, Murray GL, Nohara T, Mizuyama M, Koizumi N, Adler B, et al. Leptospiral outer membrane protein LMB216 is involved in enhancement of phagocytic uptake by macrophages. Cell Microbiol. 2014;16(9):1366–77.

106. Scharrig E, Carestia A, Ferrer MF, Cedola M, Pretre G, Drut R, et al. Neutrophil extracellular traps are involved in the innate immune response to infection with Leptospira. PLoS Negl Trop Dis. 2015;9(7):e0003927.
107. Chassin C, Picardeau M, Goujon JM, Bourhy P, Quellard N, Darche S, et al. TLR4- and TLR2-mediated B cell responses control the clearance of the bacterial pathogen, *Leptospira interrogans*. J Immunol. 2009;
108. Viriyakosol S, Matthias MA, Swancutt MA, Kirkland TN, Vinetz JM. Toll-like receptor 4 protects against lethal *Leptospira interrogans* serovar icterohaemorrhagiae infection and contributes to in vivo control of leptospiral burden. Infect Immun. 2006;74(2):887–95.
109. Castiblanco-Valencia MM, Fraga TR, Breda LC, Vasconcellos SA, Figueira CP, Picardeau M, et al. Acquisition of negative complement regulators by the saprophyte Leptospira biflexa expressing LigA or LigB confers enhanced survival in human serum. Immunol Lett. 2016;173:61–8.
110. Fraga TR, Courrol Ddos S, Castiblanco-Valencia MM, Hirata IY, Vasconcellos SA, Juliano L, et al. Immune evasion by pathogenic Leptospira strains: the secretion of proteases that directly cleave complement proteins. J Infect Dis. 2014;209(6):876–86.
111. Kyriakidis I, Samara P, Papa A. Serum TNF-alpha, sTNFR1, IL-6, IL-8 and IL-10 levels in Weil's syndrome. Cytokine. 2011;
112. Reis EA, Hagan JE, Ribeiro GS, Teixeira-Carvalho A, Martins-Filho OA, Montgomery RR, et al. Cytokine response signatures in disease progression and development of severe clinical outcomes for leptospirosis. PLoS Negl Trop Dis. 2013;7(9):e2457.
113. Mikulski M, Boisier P, Lacassin F, Soupe-Gilbert ME, Mauron C, Bruyere-Ostells L, et al. Severity markers in severe leptospirosis: a cohort study. Eur J Clin Microbiol Infect Dis. 2015;34(4):687–95.
114. Adler B, Faine S. Susceptibility of mice treated with cyclophosphamide to lethal infection with *Leptospira interrogans* Serovar pomona. Infect Immun. 1976;14(3):703–8.
115. Lessa-Aquino C, Lindow JC, Randall A, Wunder E, Pablo J, Nakajima R, et al. Distinct antibody responses of patients with mild and severe leptospirosis determined by whole proteome microarray analysis. PLoS Negl Trop Dis. 2017;11(1):e0005349.
116. Adler B, Faine S. Host immunological mechanisms in the resistance of mice to leptospiral infections. Infect Immun. 1977;17(1):67–72.
117. Jost BH, Adler B, Vinh T, Faine S. A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. J Med Microbiol. 1986;22(3):269–75.
118. Jost BH, Adler B, Faine S. Experimental immunisation of hamsters with lipopolysaccharide antigens of *Leptospira interrogans*. J Med Microbiol. 1989;29(2):115–20.
119. Sonrier C, Branger C, Michel V, Ruvoen-Clouet N, Ganiere JP, Andre-Fontaine G. Evidence of cross-protection within *Leptospira interrogans* in an experimental model. Vaccine. 2000;19(1):86–94.
120. Srikram A, Zhang K, Bartpho T, Lo M, Hoke DE, Sermswan RW, et al. Cross-protective immunity against leptospirosis elicited by a live, attenuated lipopolysaccharide mutant. J Infect Dis. 2011;203(6):870–9.
121. WHO. Leptospirosis: an emerging public health problem. Wkly Epidemiol Rec. 2011;86 (6):45–50.
122. Yang CW, Hung CC, Wu MS, Tian YC, Chang CT, Pan MJ, et al. Toll-like receptor 2 mediates early inflammation by leptospiral outer membrane proteins in proximal tubule cells. Kidney Int. 2006;69(5):815–22.
123. Visith S, Kearkiat P. Nephropathy in leptospirosis. J Postgrad Med. 2005;51(3):184–8.
124. Seguro AC, Andrade L. Pathophysiology of leptospirosis. Shock. 2013;39(Suppl 1):17–23.
125. Daher Ede F, Zanetta DM, Abdulkader RC. Pattern of renal function recovery after leptospirosis acute renal failure. Nephron Clin Pract. 2004;98(1):c8–14.
126. Dolhnikoff M, Mauad T, Bethlem EP, Carvalho CR. Pathology and pathophysiology of pulmonary manifestations in leptospirosis. Braz J Infect Dis. 2007;11(1):142–8.

127. Gulati S, Gulati A. Pulmonary manifestations of leptospirosis. Lung India. 2012;29 (4):347–53.
128. Gouveia EL, Metcalfe J, de Carvalho AL, Aires TS, Villasboas-Bisneto JC, Queirroz A, et al. Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. Emerg Infect Dis. 2008;14(3):505–8.
129. De Brito T, Aiello VD, da Silva LF, Goncalves da Silva AM, Ferreira da Silva WL, Castelli JB, et al. Human hemorrhagic pulmonary leptospirosis: pathological findings and pathophysiological correlations. PLoS One. 2013;8(8):e71743.
130. Panicker JN, Mammachan R, Jayakumar RV. Primary neuroleptospirosis. Postgrad Med J. 2001;77(911):589–90.
131. Puca E, Majko J, Puca E, Qyra E, Gega A, Pipero P. Acute encephalitis as initial presentation of leptospirosis. J Infect Dev Ctries. 2017;11(4):361–3.
132. Shah K, Amonkar GP, Kamat RN, Deshpande JR. Cardiac findings in leptospirosis. J Clin Pathol. 2010;63(2):119–23.
133. Shukla D, Rathinam SR, Cunningham ET Jr. Leptospiral uveitis in the developing world. Int Ophthalmol Clin. 2010;50(2):113–24.
134. De Silva NL, Niloofa M, Fernando N, Karunanayake L, Rodrigo C, De Silva HJ, et al. Changes in full blood count parameters in leptospirosis: a prospective study. Int Arch Med. 2014;7:31.
135. Miyahara S, Saito M, Kanemaru T, Villanueva SY, Gloriani NG, Yoshida SI. Destruction of the hepatocyte junction by intercellular invasion of Leptospira causes jaundice in a hamster model of Weil's disease. Int J Exp Pathol. 2014;95(4):271–81.
136. Andrade L, Rodrigues AC Jr, Sanches TR, Souza RB, Seguro AC. Leptospirosis leads to dysregulation of sodium transporters in the kidney and lung. Am J Physiol Renal Physiol. 2007;292(2):F586–92.
137. Goris M, Leeflang M, Boer K, Goeijenbier M, van Gorp E, Wagenaar J, et al. Establishment of valid laboratory case definition for human leptospirosis. J Bacteriol Parasitol. 2011;3(2).
138. Ahmed A, Engelberts MF, Boer KR, Ahmed N, Hartskeerl RA. Development and validation of a real-time PCR for detection of pathogenic leptospira species in clinical materials. PLoS One. 2009;4(9):e7093.
139. Desvars A, Gigan J, Hoarau G, Gerardin P, Favier F, Michault A. Short report: Seroprevalence of human leptospirosis in Reunion Island (Indian Ocean) assessed by microscopic agglutination test on paper disc-absorbed whole blood. Am J Trop Med Hyg. 2011;85 (6):1097–9.
140. Bourhy P, Bremont S, Zinini F, Giry C, Picardeau M. Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. J Clin Microbiol. 2011;49(6):2154–60.
141. Denipitiya DT, Chandrasekharan NV, Abeyewickreme W, Hartskeerl CM, Hartskeerl RA, Jiffrey AM, et al. Application of a real time polymerase chain reaction (PCR) assay for the early diagnosis of human leptospirosis in Sri Lanka. Biologicals. 2016;
142. Stoddard RA. Detection of pathogenic *Leptospira* spp. through real-time PCR (qPCR) targeting the LipL32 gene. Methods Mol Biol. 2013;943:257–66.
143. Agampodi SB, Moreno AC, Vinetz JM, Matthias MA. Utility and limitations of direct multilocus sequence typing on qPCR-positive blood to determine infecting Leptospira strain. Am J Trop Med Hyg. 2013;88(1):184–5.
144. Ahmed SA, Sandai DA, Musa S, Hoe CH, Riadzi M, Lau KL, et al. Rapid diagnosis of leptospirosis by multiplex PCR. Malay J Med Sci. 2012;19(3):9–16.
145. Pelaez Sanchez RG, Quintero JAL, Pereira MM, Agudelo-Florez P. High-resolution melting curve analysis of the 16S ribosomal gene to detect and identify pathogenic and saprophytic Leptospira species in Colombian isolates. Am J Trop Med Hyg. 2017;96(5):1031–8.
146. Naze F, Desvars A, Picardeau M, Bourhy P, Michault A. Use of a new high resolution melting method for genotyping pathogenic *Leptospira* spp. PLoS One. 2015;10(7):e0127430.

147. Tulsiani SM, Craig SB, Graham GC, Cobbold RC, Dohnt MF, Burns MA, et al. High-resolution melt-curve analysis of random amplified polymorphic DNA (RAPD-HRM) for the characterisation of pathogenic leptospires: intra-serovar divergence, inter-serovar convergence, and evidence of attenuation in Leptospira reference collections. Ann Trop Med Parasitol. 2010;104(5):427–37.
148. Sengupta M, Prabhakar AK, Satyendra S, Thambu D, Abraham OC, Balaji V, et al. Utility of Loop-mediated isothermal amplification assay, polymerase chain reaction, and ELISA for diagnosis of Leptospirosis in South Indian patients. J Glob Infect Dis. 2017;9(1):3–7.
149. Lin X, Chen Y, Lu Y, Yan J. Application of a loop-mediated isothermal amplification method for the detection of pathogenic Leptospira. Diagn Microbiol Infect Dis. 2009;63(3):237–42.
150. Sonthayanon P, Chierakul W, Wuthiekanun V, Thaipadungpanit J, Kalambaheti T, Boonsilp S, et al. Accuracy of loop-mediated isothermal amplification for diagnosis of human leptospirosis in Thailand. Am J Trop Med Hyg. 2011;84(4):614–20.
151. Chandy S, Kirubanandhan L, Hemavathy P, Khadeeja AM, Kurian SJ, Venkataraman K, et al. Serovar prevalence of Leptospira in semirural India and the development of an IgM-based indirect ELISA. J Infect Dev Ctries. 2017;11(3):234–41.
152. Niloofa R, Fernando N, de Silva NL, Karunanayake L, Wickramasinghe H, Dikmadugoda N, et al. Diagnosis of Leptospirosis: comparison between microscopic agglutination test, IgM-ELISA and IgM rapid immunochromatography test. PLoS One. 2015;10(6):e0129236.
153. Vedhagiri K, Velineni S, Timoney JF, Shanmughapriya S, Vijayachari P, Narayanan R, et al. Detection of LipL32-specific IgM by ELISA in sera of patients with a clinical diagnosis of leptospirosis. Pathog Glob Health. 2013;107(3):130–5.
154. Natarajaseenivasan K, Vijayachari P, Sharma S, Sugunan AP, Selvin J, Sehgal SC. Serodiagnosis of severe leptospirosis: evaluation of ELISA based on the recombinant OmpL1 or LipL41 antigens of *Leptospira interrogans* serovar autumnalis. Ann Trop Med Parasitol. 2008;102(8):699–708.
155. Suputtamongkol Y, Pongtavornpinyo W, Lubell Y, Suttinont C, Hoontrakul S, Phimda K, et al. Strategies for diagnosis and treatment of suspected leptospirosis: a cost-benefit analysis. PLoS Negl Trop Dis. 2010;4(2):e610.
156. Goarant C, Bourhy P, D'Ortenzio E, Dartevelle S, Mauron C, Soupe-Gilbert ME, et al. Sensitivity and specificity of a new vertical flow rapid diagnostic test for the serodiagnosis of human leptospirosis. PLoS Negl Trop Dis. 2013;7(6):e2289.
157. Bryceson AD. Clinical pathology of the Jarisch-Herxheimer reaction. J Infect Dis. 1976;133 (6):696–704.
158. Guerrier G, D'Ortenzio E. The Jarisch-Herxheimer reaction in leptospirosis: a systematic review. PLoS One. 2013;8(3):e59266.
159. Vaughan C, Cronin CC, Walsh EK, Whelton M. The Jarisch-Herxheimer reaction in leptospirosis. Postgrad Med J. 1994;70(820):118–21.
160. Fekade D, Knox K, Hussein K, Melka A, Lalloo DG, Coxon RE, et al. Prevention of Jarisch-Herxheimer reactions by treatment with antibodies against tumor necrosis factor alpha. N Engl J Med. 1996;335(5):311–5.
161. Schulze MH, Raschel H, Langen HJ, Stich A, Tappe D. Severe *Leptospira interrogans* serovar Icterohaemorrhagiae infection with hepato-renal-pulmonary involvement treated with corticosteroids. Clin Case Rep. 2014;2(5):191–6.
162. Abdulkader RC, Seguro AC, Malheiro PS, Burdmann EA, Marcondes M. Peculiar electrolytic and hormonal abnormalities in acute renal failure due to leptospirosis. Am J Trop Med Hyg. 1996;54(1):1–6.
163. Seguro AC, Lomar AV, Rocha AS. Acute renal failure of leptospirosis: nonoliguric and hypokalemic forms. Nephron. 1990;55(2):146–51.
164. Andrade L, de Francesco DE, Seguro AC. Leptospiral nephropathy. Semin Nephrol. 2008;28 (4):383–94.

165. Amato MB, Barbas CS, Medeiros DM, Magaldi RB, Schettino GP, Lorenzi-Filho G, et al. Effect of a protective-ventilation strategy on mortality in the acute respiratory distress syndrome. N Engl J Med. 1998;338(6):347–54.
166. Costa F, Wunder EA Jr, De Oliveira D, Bisht V, Rodrigues G, Reis MG, et al. Patterns in Leptospira shedding in Norway rats (*Rattus norvegicus*) from Brazilian slum communities at high risk of disease transmission. PLoS Negl Trop Dis. 2015;9(6):e0003819.
167. Martins G, Lilenbaum W. Control of bovine leptospirosis: aspects for consideration in a tropical environment. Res Vet Sci. 2017;112:156–60.
168. Costa F, Ribeiro GS, Felzemburgh RD, Santos N, Reis RB, Santos AC, et al. Influence of household rat infestation on Leptospira transmission in the urban slum environment. PLoS Negl Trop Dis. 2014;8(12):e3338.
169. Reis RB, Ribeiro GS, Felzemburgh RD, Santana FS, Mohr S, Melendez AX, et al. Impact of environment and social gradient on Leptospira infection in urban slums. PLoS Negl Trop Dis. 2008;2(4):e228.
170. Felzemburgh RD, Ribeiro GS, Costa F, Reis RB, Hagan JE, Melendez AX, et al. Prospective study of leptospirosis transmission in an urban slum community: role of poor environment in repeated exposures to the Leptospira agent. PLoS Negl Trop Dis. 2014;8(5):e2927.
171. Hartskeerl RA, Collares-Pereira M, Ellis WA. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. Clin Microbiol Infect. 2011;17 (4):494–501.
172. Faine SB, Adler B, Bolin C, Perolat P. Leptospira and leptospirosis. 2nd ed. MediSci: Melbourne; 1999.
173. Sehgal SC, Sugunan AP, Murhekar MV, Sharma S, Vijayachari P. Randomized controlled trial of doxycycline prophylaxis against leptospirosis in an endemic area. Int J Antimicrob Agents. 2000;13(4):249–55.
174. Takafuji ET, Kirkpatrick JW, Miller RN, Karwacki JJ, Kelley PW, Gray MR, et al. An efficacy trial of doxycycline chemoprophylaxis against leptospirosis. N Engl J Med. 1984;310(8):497–500.
175. Schneider MC, Velasco-Hernandez J, Min KD, Leonel DG, Baca-Carrasco D, Gompper ME, et al. The use of chemoprophylaxis after floods to reduce the occurrence and impact of leptospirosis outbreaks. Int J Environ Res Public Health. 2017;14(6).
176. Adler B. Vaccines against leptospirosis. Curr Top Microbiol Immunol. 2015;387:251–72.
177. Ido Y, Hoki R, Ito H, Wani H. The prophylaxis of Weil's disease (Spirochaetosis Icterohaemorrhagica). J Exp Med. 1916;24(5):471–83.
178. Chen T. Development and present status of a leptospiral vaccine and the technology of vaccine production in China. Nihon Saikingaku Zasshi. 1985;40(4):755–62.
179. Martinez Sanchez R, Perez Sierra A, Baro Suarez M, Alvarez AM, Menendez Hernandez J, Diaz Gonzalez M, et al. Evaluation of the effectiveness of a new vaccine against human leptospirosis in groups at risk. Rev Panam Salud Publica. 2000;8(6):385–92.
180. Yanagihara Y, Villanueva SY, Yoshida S, Okamoto Y, Masuzawa T. Current status of leptospirosis in Japan and Philippines. Comp Immunol Microbiol Infect Dis. 2007;30 (5-6):399–413.
181. Rodriguez-Gonzalez I, Fillonneau C, Blanchet B, Suard I, Catilina P, Andre-Fontaine G. Efficacy of Spirolept vaccine against human leptospirosis as estimated by passive protection of laboratory rodents. Med Mal Infect. 2004;34(5):196–200.
182. Laurichesse H, Gourdon F, Smits HL, Abdoe TH, Estavoyer JM, Rebika H, et al. Safety and immunogenicity of subcutaneous or intramuscular administration of a monovalent inactivated vaccine against *Leptospira interrogans* serogroup Icterohaemorrhagiae in healthy volunteers. Clin Microbiol Infect. 2007;13(4):395–403.
183. Ikoev VN, Gorbunov MA, Vachaev BF, Yagovkin EA, Kondratenko VF, Ananyina YV, et al. Evaluation of the reactogenicity and immunogenic activity of a new concentrated inactivated leptospirosis vaccine. Zhurnal Mikrobiologii Epidemiologii i Immunobiologii. 1999;4:39–43.

184. Yan Y, Chen Y, Liou W, Ding J, Chen J, Zhang J, et al. An evaluation of the serological and epidemiological effects of the outer envelope vaccine to Leptospira. J Chin Med Assoc. 2003;66(4):224–30.
185. Mailloux M, Lambert R, Chenu M. Human vaccination against leptospirosis icterohaemorrhagiae. Med Hyg (Geneve). 1983;41:1025–30.
186. Wani H. The prophylaxis of Spirochaetosis icterohaemorrhagica Inada (Weil's disease) by vaccination. Z Immunitatsforsch. 1933;79:1–26.
187. da Cunha CE, Felix SR, Neto AC, Campello-Felix A, Kremer FS, Monte LG, et al. Infection with Leptospira kirschneri Serovar Mozdok: first report from the Southern Hemisphere. Am J Trop Med Hyg. 2016;94(3):519–21.
188. Shenberg E, Torten M. A new leptospiral vaccine for use in man. I. Development of a vaccine from Leptospira grown on a chemically defined medium. J Infect Dis. 1973;128(5):642–6.
189. Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, Matsunaga J, et al. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun. 1999;67(12):6572–82.
190. Dellagostin OA, Grassmann AA, Hartwig DD, Felix SR, da Silva EF, McBride AJ. Recombinant vaccines against leptospirosis. Hum Vaccin. 2011;7(11):1215–24.
191. Grassmann AA, Souza JD, McBride AJ. A universal vaccine against leptospirosis: are we going in the right direction? Front Immunol. 2017;8:256.
192. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, et al. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun. 2000;68(4):2276–85.
193. Haake DA, Suchard MA, Kelley MM, Dundoo M, Alt DP, Zuerner RL. Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. J Bacteriol. 2004;186(9):2818–28.
194. Branger C, Chatrenet B, Gauvrit A, Aviat F, Aubert A, Bach JM, et al. Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. Infect Immun. 2005;73(7):4062–9.
195. Lucas DS, Cullen PA, Lo M, Srikram A, Sermswan RW, Adler B. Recombinant LipL32 and LigA from Leptospira are unable to stimulate protective immunity against leptospirosis in the hamster model. Vaccine. 2011;29(18):3413–8.
196. Grassmann AA, Felix SR, dos Santos CX, Amaral MG, Seixas Neto AC, Fagundes MQ, et al. Protection against lethal leptospirosis after vaccination with LipL32 coupled or coadministered with the B subunit of *Escherichia coli* heat-labile enterotoxin. Clin Vaccine Immunol. 2012;19(5):740–5.
197. Habarta A, Abreu PA, Olivera N, Hauk P, Cedola MT, Ferrer MF, et al. Increased immunogenicity to LipL32 of *Leptospira interrogans* when expressed as a fusion protein with the cholera toxin B subunit. Curr Microbiol. 2011;62(2):526–31.
198. Seixas FK, da Silva EF, Hartwig DD, Cerqueira GM, Amaral M, Fagundes MQ, et al. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. Vaccine. 2007;26(1):88–95.
199. Branger C, Sonrier C, Chatrenet B, Klonjkowski B, Ruvoen-Clouet N, Aubert A, et al. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infect Immun. 2001;69 (11):6831–8.
200. Pinne M, Haake DA. LipL32 is a subsurface lipoprotein of *Leptospira interrogans*: presentation of new data and reevaluation of previous studies. PLoS One. 2013;8(1):e51025.
201. Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvao RM, et al. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun. 2001;69(8):4958–68.
202. Adler B, Lo M, Seemann T, Murray GL. Pathogenesis of leptospirosis: the influence of genomics. Vet Microbiol. 2011;153(1–2):73–81.
203. Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, Siqueira I, et al. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol. 2003;49(4):929–45.

204. Cerqueira GM, McBride AJ, Picardeau M, Ribeiro SG, Moreira AN, Morel V, et al. Distribution of the leptospiral immunoglobulin-like (lig) genes in pathogenic *Leptospira* species and application of ligB to typing leptospiral isolates. J Med Microbiol. 2009;58 (Pt 9):1173–81.
205. McBride AJ, Cerqueira GM, Suchard MA, Moreira AN, Zuerner RL, Reis MG, et al. Genetic diversity of the Leptospiral immunoglobulin-like (Lig) genes in pathogenic *Leptospira* spp. Infect Genet Evol. 2009;9(2):196–205.
206. Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, Haake DA. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. Infect Immun. 2007;75(5):2441–50.
207. Choy HA, Kelley MM, Croda J, Matsunaga J, Babbitt JT, Ko AI, et al. The multifunctional LigB adhesin binds homeostatic proteins with potential roles in cutaneous infection by pathogenic *Leptospira interrogans*. PLoS One. 2011;6(2):e16879.
208. Hsieh CL, Chang E, Tseng A, Ptak C, Wu LC, Su CL, et al. Leptospira immunoglobulin-like protein B (LigB) binds to both the C-terminal 23 amino acids of fibrinogen alphaC domain and Factor XIII: insight into the mechanism of LigB-mediated blockage of fibrinogen alpha chain cross-linking. PLoS Negl Trop Dis. 2016;10(9):e0004974.
209. Choy HA. Multiple activities of LigB potentiate virulence of *Leptospira interrogans*: inhibition of alternative and classical pathways of complement. PLoS One. 2012;7(7):e41566.
210. Hartwig DD, Bacelo KL, Oliveira PD, Oliveira TL, Seixas FK, Amaral MG, et al. Mannosylated LigANI produced in *Pichia pastoris* protects hamsters against leptospirosis. Curr Microbiol. 2014;68(4):524–30.
211. Coutinho ML, Choy HA, Kelley MM, Matsunaga J, Babbitt JT, Lewis MS, et al. A LigA three-domain region protects hamsters from lethal infection by *Leptospira interrogans*. PLoS Negl Trop Dis. 2011;5(12):e1422.
212. Silva EF, Medeiros MA, McBride AJ, Matsunaga J, Esteves GS, Ramos JG, et al. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine. 2007;25 (33):6277–86.
213. Conrad NL, Cruz McBride FW, Souza JD, Silveira MM, Felix S, Mendonca KS, et al. LigB subunit vaccine confers sterile immunity against challenge in the hamster model of leptospirosis. PLoS Negl Trop Dis. 2017;11(3):e0005441.
214. Cao Y, Faisal SM, Yan W, Chang YC, McDonough SP, Zhang N, et al. Evaluation of novel fusion proteins derived from extracellular matrix binding domains of LigB as vaccine candidates against leptospirosis in a hamster model. Vaccine. 2011;29(43):7379–86.
215. Yan W, Faisal SM, McDonough SP, Divers TJ, Barr SC, Chang CF, et al. Immunogenicity and protective efficacy of recombinant Leptospira immunoglobulin-like protein B (rLigB) in a hamster challenge model. Microbes Infect. 2009;11(2):230–7.
216. Yan W, Faisal SM, McDonough SP, Chang CF, Pan MJ, Akey B, et al. Identification and characterization of OmpA-like proteins as novel vaccine candidates for leptospirosis. Vaccine. 2010;28(11):2277–83.
217. Umthong S, Buaklin A, Jacquet A, Sangjun N, Kerdkaew R, Patarakul K, et al. Immunogenicity of a DNA and recombinant protein vaccine combining LipL32 and Loa22 for leptospirosis using chitosan as a delivery system. J Microbiol Biotechnol. 2015;25(4):526–36.
218. Garcon N, Chomez P, Van Mechelen M. GlaxoSmithKline adjuvant systems in vaccines: concepts, achievements and perspectives. Expert Rev Vaccines. 2007;6(5):723–39.
219. Faisal SM, Yan W, McDonough SP, Mohammed HO, Divers TJ, Chang YF. Immune response and prophylactic efficacy of smegmosomes in a hamster model of leptospirosis. Vaccine. 2009;27(44):6129–36.
220. Faisal SM, Yan W, McDonough SP, Chang YF. Leptospira immunoglobulin-like protein A variable region (LigAvar) incorporated in liposomes and PLGA microspheres produces a robust immune response correlating to protective immunity. Vaccine. 2009;27(3):378–87.

221. Faisal SM, Yan W, McDonough SP, Chang CF, Pan MJ, Chang YF. Leptosome-entrapped leptospiral antigens conferred significant higher levels of protection than those entrapped with PC-liposomes in a hamster model. Vaccine. 2009;27(47):6537–45.
222. Oliveira TL, Bacelo KL, Schuch RA, Seixas FK, Collares T, Rodrigues OE, et al. Immune response in hamsters immunised with a recombinant fragment of LigA from *Leptospira interrogans*, associated with carrier molecules. Mem Inst Oswaldo Cruz. 2016:0.
223. Bacelo KL, Hartwig DD, Seixas FK, Schuch R, Moreira Ada S, Amaral M, et al. Xanthan gum as an adjuvant in a subunit vaccine preparation against leptospirosis. BioMed Res Int. 2014;2014:636491.
224. Monaris D, Sbrogio-Almeida ME, Dib CC, Canhamero TA, Souza GO, Vasconcellos SA, et al. Protective immunity and reduced renal colonization induced by vaccines containing recombinant *Leptospira interrogans* outer membrane proteins and flagellin adjuvant. Clin Vaccine Immunol. 2015;22(8):965–73.
225. Rappuoli R. Reverse vaccinology, a genome-based approach to vaccine development. Vaccine. 2001;19(17-19):2688–91.
226. Kremer FS, Eslabao MR, Jorge S, Oliveira NR, Labonde J, Santos MN, et al. Draft genome of the *Leptospira interrogans* strains, Acegua, RCA, Prea, and Capivara, obtained from wildlife maintenance hosts and infected domestic animals. Mem Inst Oswaldo Cruz. 2016;111 (4):280–3.
227. Dormitzer PR, Grandi G, Rappuoli R. Structural vaccinology starts to deliver. Nat Rev Microbiol. 2012;10(12):807–13.
228. Umamaheswari A, Pradhan D, Hemanthkumar M. Computer aided subunit vaccine design against pathogenic Leptospira serovars. Interdiscip Sci. 2012;4(1):38–45.
229. Zeng L, Wang D, Hu N, Zhu Q, Chen K, Dong K, et al. A novel pan-genome reverse vaccinology approach employing a negative-selection strategy for screening surface-exposed antigens against leptospirosis. Front Microbiol. 2017;8:396.
230. Blumerman SL, Herzig CT, Wang F, Coussens PM, Baldwin CL. Comparison of gene expression by co-cultured WC1+ gammadelta and CD4+ alphabeta T cells exhibiting a recall response to bacterial antigen. Mol Immunol. 2007;44(8):2023–35.
231. Hsu H, Chen C, Nenninger A, Holz L, Baldwin CL, Telfer JC. WC1 is a hybrid gammadelta TCR coreceptor and pattern recognition receptor for pathogenic bacteria. J Immunol. 2015;194(5):2280–8.
232. Baldwin CL, Telfer JC. The bovine model for elucidating the role of gammadelta T cells in controlling infectious diseases of importance to cattle and humans. Mol Immunol. 2015;66 (1):35–47.

Overview on Japanese Encephalitis in South and Southeast Asia

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Contents

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1 Japanese Encephalitis: An Introduction

Arboviruses (arthropod-borne viruses) are common causes of debilitating fever syndromes in susceptible human population. Out of more than 500 known arboviruses, about 100 are relevant to human infections and mostly belong to the *Flaviviridae*, *Bunyaviridae*, and *Togaviridae* families [1]. For more than a century, countries in South and Southeast Asia have been under the shadow of one such disease—Japanese encephalitis. As the name suggests, this disease was first reported from the islands of Japan in the year 1871 [2]. Following large-scale epidemic in 1924, the causative agent was isolated in 1933 [3]. The virus, initially named as Japanese encephalitis B virus, was later on characterized to belong to the family *Flaviviridae* (Latin *flavus*—yellow) named after the yellow fever virus which was the first member of this family. Apart from yellow fever virus and Japanese encephalitis virus (JEV), this family currently includes over 85 species of viruses including dengue virus, West Nile virus, and the Zika virus [4].

The JEV is a single-stranded positive-sense RNA virus with 10,976 nucleotides, inside a nucleocapsid and is surrounded by a glycoprotein-containing envelope. The RNA comprises a short 5′ untranslated region (UTR), a longer 3′ UTR lacking a polyadenylation sequence, and a single open reading frame of 10,296 nucleotides between them. It codes for a single polyprotein, of 3432 amino acid residues which is translationally and posttranslationally cleaved by viral and host proteases into 3 structural proteins (core (C), pre-membrane (PrM) or membrane (M), and envelope (E)) and 7 nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [5]. The C protein (~120 amino acids; ~14 kDa) is hydrophobic and has abundances of basic amino acid residues and very little amino acid homology to other flaviviruses. The C protein also contains a nuclear localization signal which helps viral transmission into nucleus of infected cells [6]. The C protein is also reported to inhibit stress granule formation in infected cells so as to aid viral replication [7]. The prM (~165 amino acids; ~26 kDa) is a precursor of the membrane-anchored M protein [8]. IN immature virions, prM serves as a chaperon for folding and assembly of the E protein. It is cleaved to form a soluble Pr peptide and virion-associated M protein (~8 kDa) by trans-Golgi resident furin or related enzyme [9]. The viral E protein (~500 amino acids; ~54 kDa) consists of two helical transmembrane domains. It is responsible for cellular attachment and possesses a hydrophobic loop that mediates fusion of viral and host membranes [10]. Among the nonstructural proteins, NS1 is a glycosylated protein; NS3 and NS5 are hydrophilic, whereas, NS2A, NS2B, NS4A, and NS4B are hydrophobic.

NS1 (~350 amino acids; ~40 kDa) is a multifunctional protein that is conserved across many other flaviviruses. They are involved in viral replication and assembly as well as modulation of host's innate immune response. However, in contrast with other flavivirus NS1 proteins that show a single hexameric presentation, JEV NS1 exists in two oligomeric forms in roughly equal proportion. A ribosomal frameshift event leads to the production of another larger NS1-related protein denoted as NS1′ (~53 kDa) which may play a role in viral neuroinvasiveness [11]. NS2A (~230 amino acids; ~24 kDa) is a hydrophobic, multifunctional, membrane-associated protein that binds with the 3′ UTR of viral RNA and to other components of the replication complex to regulate viral replication. Additionally, it is also involved in the regulation of antiviral interferon response of the host cells [12]. NS2B (~131 amino acids; ~14 kDa) on the other hand serves as a cofactor to promote or modulate NS3 (serine protease) activity [13]. NS3 (~610 amino acids; ~46 kDa) and NS5 (~905 amino acids; ~48 kDa) are believed to be enzymatic components of the viral RNA replicase. The NS3 and NS5 are reported to interact with heat shock protein 70, eukaryotic elongation factor 1-alpha, and ras-related nuclear protein to form the replicase complex [14]. NS5 also interacts with mitochondrial hydroxyacyl-CoA dehydrogenase α and β subunits thereby impairing β-oxidation and promoting pro-inflammatory cytokine production in host cells [15]. NS4A (~125 amino acids; ~14 kDa) and NS4B (~255 amino acids; ~27 kDa) are membrane proteins that supposedly serve as the scaffold for the formation of replication complexes. These two proteins interact with each other at the genetic level to facilitate viral replication [16]. NS4A is also reported to inhibit interferon-mediated host antiviral defense [17].

The clinical features of this disease are similar to many other encephalitides and can be conveniently divided into three stages [18]:

(a) A prodromal stage characterized by general malaise, headache, fever, and other symptoms which are common to various other diseases that may be unrelated to flaviviral infections
(b) An acute encephalitic stage marked by continuing fever, convulsions, nuchal rigidity, and altered sensorium, progressing in many cases to coma
(c) A late stage marked by recovery or the persistence of cognitive dysfunction as a result of irreversible CNS damage

There are several other viruses, bacteria, fungus, parasites, spirochetes, chemicals, and toxins which may also result in similar clinical features in affected individuals. In 2006, the World Health Organization (WHO) coined the term "acute encephalitis syndrome" (AES) for surveillance purposes of all such cases which manifests the abovementioned clinical features. Apart from JEV, other viruses which may result in AES are West Nile virus, Eastern equine encephalitis virus, Venezuelan equine encephalitis virus, Hendra virus, enteroviruses, Chandipura virus, Nipah virus, Kyasanur forest disease virus, St. Louis encephalitis virus, herpes simplex virus, poliovirus, and measles virus [19]. To identify JE cases among AES cases, laboratory diagnosis is necessary which is accomplished by testing of serum or cerebrospinal fluid (CSF) to detect virus-specific IgM

antibodies. These antibodies are usually detectable 3–8 days after onset of illness and may persist for 30–90 days, but longer persistence has been documented [20]. If serum collected within 10 days of onset does not have IgM at detectable levels, the test should be repeated on a convalescent sample [21]. JEV IgM-positive cases could be further confirmed by neutralizing antibody testing.

2 The Life Cycle of Japanese Encephalitis Virus

By 1938, it was clear that mosquitoes are involved in the spread of the disease; however it was not until the 1950s when the life cycle of the virus was described in detail [22–28]. The virus has been known to replicate within the salivary glands of mosquitoes belonging to the *Culicine* species such as *Culex tritaeniorhynchus*, *Culex fuscocephala*, *Culex vishnui*, *Culex sitiens*, *Culex annulirostris*, *Culex gelidus*, *Culex bitaeniorhynchus*, *Culex epidesmus*, *Culex pseudovishnui*, and *Culex whitmorei*. Apart from these, JEV has also been isolated from four species of anophelines, *Anopheles annularis*, *Anopheles barbirostris*, *Anopheles hyrcanus*, and *Anopheles subpictus*, and five species of other mosquito genera *Armigeres subalbatus*, *Mansonia annulifera*, *Mansonia bonneael dives*, *Mansonia uniformis*, and *Aedes vigilax* [29–33]. *Cx. tritaeniorhynchus* is considered as the major vector owing to its vast distribution over the JEV-endemic regions. Apart from mosquitoes JEV has only been successfully isolated from two other arthropods—*Lasiohelea taiwana* (bloodsucking midge) [34] and *Haemaphysalis japonica* (ixodid tick) [35]. However, these two reports are now considered to be exceptions as there have been no other reports of isolation from non-mosquito arthropods since then.

The virus is transmitted by the mosquito to vertebrate hosts through its saliva while engaged in blood-feeding activity. Within the mosquito salivary gland, JEV remains entrapped in intracellular vacuoles and is later released into the apical cavity through the fusion of these vacuoles with the apical plasma membrane. Post blood-feeding, this helps in the resynthesis of saliva in the mosquito. Another type of shedding involves virus particles, either singly or in mass, being released directly through the apical plasma membrane [36]. Sialokinin I and II, which are vasodilatory tachykinins present in mosquito saliva, have also been reported to be involved in modulating cytokine milieu at the site of feeding [37].

Stagnant pools of water are necessary for increase in the population of vector and in South or Southeast Asian countries; rice paddy fields provide the perfect environment [38–40]. As rice cultivation is a critical component in the agro-economic setup of most of the countries in this region, vector and disease control efforts have been difficult to achieve. These paddy fields as well as other water bodies such as perennial lakes and swamps also make for ideal wintering and staging ground for several migratory waterfowl and wading ardeid water birds, particularly the black-crowned night heron (*Nycticorax nycticorax*) and the Asiatic cattle egret (*Bubulcus ibis coromandus*). Owing to this close proximity with the disease vector, it is not surprising that these birds play a critical role in the viral life cycle. These birds have

been reported to serve as reservoirs or maintenance hosts of the virus but are never symptomatic for the disease. Interestingly, the evolution and spread of the more recent JEV genotypes can be correlated with the expansion of the Asiatic cattle egret's range across Asia in the nineteenth century following changes in agricultural practices [41]. Apart from these, other birds can also be infected with the virus, though it is unclear whether the virus persists in them long enough to be clinically relevant in human epidemics [42]. Other than birds, bats have frequently been reported to also serve as maintenance host of JEV [43–46]. Like birds, bats are asymptomatic for the disease but do manifest viremia. Till date, five species of bats/flying foxes, viz., *Rousettus leschenaultii*, *Murina aurata*, *Myotis ricketti*, *Miniopterus schreibersii*, and *Rhinolophus affinis*, have been reported to carry JEV, whereas *Pteropus alecto* has been reported to be able to transmit the virus to mosquitoes in experimental infection models [47].

Another critical link in the life cycle of JEV involves pigs. Like rice cultivation, pig farming is also an important source of income in many South or Southeast Asian countries. Pig has got highest feed conversion efficiency, i.e., they produce more live weight gain from a given weight of feed than any other class of meat-producing animals except broilers. Owing to the growing domestic and export demands, there has been substantial growth of the swine industry in many JE-endemic Asian countries (Table 1) which unfortunately has also led to the increase in human health risks [48–52]. Unlike the maintenance hosts, JEV amplifies rapidly in pigs

Table 1 Change in rice cultivation and pork production in JE-endemic South-Southeast Asian countries over a 15-year period (adapted from Tobias et al. [56])

Endemic countries	% change in paddy area (1990–2005)	% change in pork production (1990–2005)
Bangladesh	1	NA
Cambodia	30	46
China	−13	87
India	−2	−8
Indonesia	12	−13
Japan	−18	−23
North Korea	−2	−35
South Korea	−21	69
Laos	NA	21
Malaysia	−1	−47
Myanmar	47	381
Nepal	6	12
Pakistan	24	NA
Philippines	27	18
Singapore	0	NA
Sri Lanka	10	21
Thailand	14	80
Vietnam	21	147

NA not available

and results in high viremia lasting for up to 4 days with peak at day 1 postinfection [53]. In pigs, JEV can be found in the bone marrow, thymus, kidney, liver, and skeletal muscles for the duration of viremia but disappear soon afterward. It however persists in the brain as well as in secondary lymphoid tissue including the spleen and lymph nodes long after the viremia phase [54]. Infected- pig-to-non-infected-pig transmission is usually carried out by the mosquitoes. However, a recent investigation reports that viral transfer between pigs could be independent of the vector. Oronasal secretions of infected pigs contain JEV at sufficiently high titer, so as to infect another naive animal staying in close proximity [55]. This could explain the viral persistence in pigs over winter when there is a decrease in vector population.

Due to the close proximity of pigs with human dwellings, these animals are considered main component in the transmission cycle with respect to human infection [57]. JEV is spread to humans by infected mosquitoes as a result of their blood-feeding activity. However, as humans have a transient viremia, they are actually dead-end hosts which means further propagation from them is not possible. Other domestic animals such as horses, cows, dogs, and goats [58–62] and wild animals such as raccoons (*Procyon lotor*), wild boars (*Sus scrofa*), and raccoon dogs (*Nyctereutes procyonoides*) [63] have been reported to be infected with the virus and are dead-end hosts too but could serve as sentinels to assesses for risk of human infections. This enzootic life cycle of the virus is summarized in Fig. 1.

3 The Realm of Japanese Encephalitis

According to the WHO, there are approximately 67,900 estimated global cases of JE annually, of which 20–30% are fatal and 30–50% of survivors have significant neurologic sequelae [64]. There are two distinct patterns of transmission of JE—epidemic and endemic. The epidemic pattern typically corresponds with seasonal outbreaks linked to increased rainfall, whereas the endemic pattern results in sporadic cases occurring throughout the year [65]. Currently, almost all the countries in South and Southeast Asia fall under the JE-endemic zone, and it is believed that population in rural Asia has been infected with the virus by early adulthood [66]. This region is also the most densely populated region in the world with estimated 57% of total human population leave here [67]. As stated earlier, JE was first reported from the islands of Japan. However, within a century, it has spread its realm over the current endemic zone. JEV can be introduced to virgin territories either by transferring vectors or other vertebrate reservoirs. During the mid-1990s, JE cases were reported from Torres Strait islands in Australia. This was believed to have been imported by migratory birds and/or windblown mosquitoes from a place with well-documented viral activity such as Papua New Guinea [68]. Transport of carrier mosquitoes that gets trapped inside airplanes is also a method to virus dispersal such as in Nepal [69]. Amplifying hosts, i.e., pigs carrying the virus if introduced to newer territories, may also be a cause of initiation of the

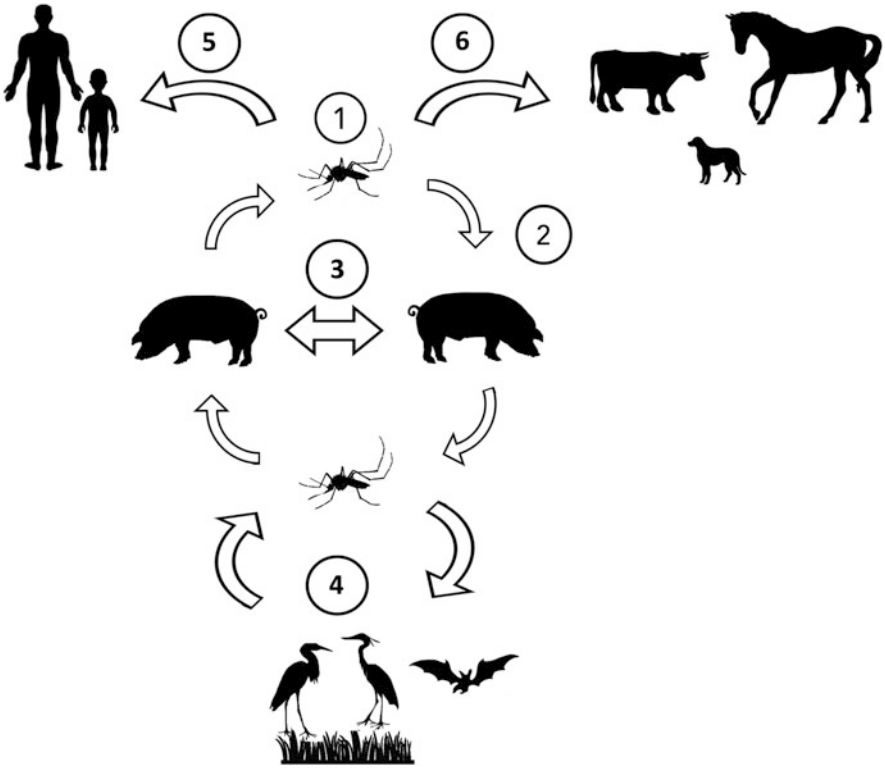

Fig. 1 The life cycle of JEV. JEV is carried by mosquito vectors (1) can persist in pigs which serve as amplification hosts (2). Infection in pigs results in viremia with high titre. The virus can be transmitted from an infected pig to another naive pig via oronasal secretions (3), which explains the viral persistence in pigs even when there is a lack of mosquitoes. Blood feeding activity from the amplification hosts by mosquitoes (previously infected or un-infected) results is reloading the virus into vectors from which it can be transmitted to reservoir birds or bats (4) or dead-end hosts like human (5) and other domestic mammals (6)

disease. An example of this is the sudden spate of JE in children in the National Capital Region in India where infected pigs were brought from the city of Indore [70].

In the following sections, we provide brief descriptions of status of JE in the South or Southeast Asian countries that falls under the endemic zone. The countries are arranged in alphabetical order of their official names. We begin with Table 2, showing number of reported cases to the WHO from respective countries in this region.

Table 2 List of recorded cases of JE in South or Southeast Asian countries for a 10-year period

Country name	2015	2014	2013	2012	2011	2010	2009	2008	2007	2006
Afghanistan	0	–	0	–	–	0	–	–	–	–
Bangladesh	76	183	23	52	103	15	15	702	204	–
Bhutan	5	2	0	27	3	0	0	–	–	–
Brunei Darussalam	1	0	–	0	0	–	0	0	–	–
Cambodia	48	60	41	55	45	41	193	372	295	–
Democratic People's Republic of Korea (North Korea)	0	0	0	0	0	–	10	124	0	0
India	1620	1657	1078	–	1214	555	653	427	4017	–
Indonesia	39	72	–	–	–	–	–	–	–	–
Lao People's Democratic Republic	13	4	9	23	24	82	26	–	44	12
Malaysia	36	47	12	22	12	0	9	17	–	–
Maldives	0	0	0	0	0	0	0	–	–	–
Myanmar	113	50	3	14	20	18	8	5	28	0
Nepal	937	1304	118	75	126	183	146	329	435	290
Pakistan	–	–	–	–	–	–	–	–	–	–
Philippines	115	69	24	–	–	181	5	34	–	–
People's Republic of China[a]	624	858	2178	1763	1625	2541	3913	2975	4330	7647
Republic of Korea (South)	40	26	14	20	3	26	3	6	7	0
Singapore	–	–	–	0	0	1	0	1	1	1
Sri Lanka	17	21	70	60	30	27	72	118	45	29
Thailand	23	31	59	54	52	40	36	64	43	49
Timor-Leste	0	0	5	0	0	0	3	0	0	–
Vietnam	368	421	224	183	126	140	68	17	38	–

Table shows recorded data as per the WHO for a period between 2006 and 2015. No reported cases are denoted by "0." No available data is represented as "–." The data is updated as of 1 December 2016 (data received as of 18 November 2016). Accessed from http://apps.who.int/immunization_monitoring/globalsummary/timeseries/tsincidencejapenc.html

[a]Includes data from Republic of China (Taiwan) also

3.1 Democratic Socialist Republic of Sri Lanka

Sri Lanka is an island country situated in the Indian Ocean at the South of India. There was an outbreak of encephalitis in 1948 which was suspected to be JE, but in 1968 the first confirmed case of JE was reported and the outbreak was reported in 1971 [71]. This was followed by major outbreaks between 1984 (November) and 1985 (February) in the North Central Province with 385 individuals affected out of which 64 succumbed to the disease (case fatality rate of 17%). In the following

year, outbreaks were reported from other places in the North Central Province which also spread to the North Western Province. Incidentally, this was the largest outbreak reported so far with 812 cases, 192 deaths with a CFR of 24% [72]. Since then, JE cases have been identified from various parts of the country, and the virus is routinely found in vectors as well as vertebrate hosts [73–75]. There are over 11 species of *Culex* mosquitoes found in Sri Lanka [76] out of which *Cx. tritaeniorhynchus*, *Cx. gelidus*, *Cx. fuscocephala*, and *Cx. Whitmorei* have been shown to serve as vectors of JEV [75]. A cause of concern regarding vector control efforts was raised when it was reported that Sri Lankan *Cx. tritaeniorhynchus* mosquitoes were resistant to organophosphorus insecticides due to a gene amplification resulting in increased esterase activity [77].

An annual immunization campaign launched in 1988 with the mouse brain-derived inactivated vaccine (raised against the Beijing-1 strain) resulted in decline of JE cases from 812 (incidence rate of 4.7/100,000) in 1987 to 26 sporadic cases (incidence rate of 0.1/100,000) in 2006. However, along with the increased coverage of the immunization program each year, the number of cases reporting adverse events following immunization also increased. This prompted a decline in the number of children receiving either the first dose of the vaccine or subsequent booster doses [71]. Finally the government was forced to replace island-wide, routine immunization with the live attenuated JE vaccine (raised against the SA 14-14-2 strain) in 2009. The live attenuated vaccine has proven to be a safe alternative even when administered as a booster to those who had been previously immunized with the mouse brain-derived inactivated vaccine [78–80].

3.2 Federal Democratic Republic of Nepal

The first cases of JE in the landlocked Himalayan country of Nepal were believed to have migrated from neighboring India [81]. The first clinical diagnosis was done in 1978 from the Terai district of Rupandehi. Since then the entire Terai region has been reported to be endemic for JE [82]. The Terai region experiences tropical climate with high temperature, high humidity, vast rice fields, and flourishing pig rearing—all of which increases the risk of JE in that particular region. Surveillance studies have shown that 4 western Terai districts are more affected than 20 - non-western districts [83]. Outside the Terai, an outbreak of JE in Kathmandu valley in the hill region was confirmed in 1997 [84, 85], and JE endemicity was also reported in 2006 [69]. Since then there has been reports of JE from many other districts, and currently JE has been confirmed in 54 of the 75 districts in Nepal [86].

Nepal has the second highest prevalence of JE in Southeast Asia. Between 1978 and 2003, 26,667 cases and 5381 deaths have been reported with case fatality rates ranging from 9.8 to 46.3% during that time [82], and between 2005 and 2010, 2040 cases and 205 deaths have been reported [87]. Since the introduction of immunization with the live attenuated vaccine in 2006, mass campaigns had been implemented in 23 districts within next 3 years. As a result, the observed incidence

rate of JE in those districts fell by 72–84% than the expected incidence rates thereby marking the success of the program [88].

Apart from mass immunization, various steps have been proposed to control the threat of JE in Nepal. Other than vector control, an important step would be to educate pig farmers regarding the risks of JE. Pig rearing is encouraged by the government as a mean of poverty alleviation and as a source of income which requires little investment. However, surveys have showed that majority of the pig farmers are either unaware of this disease or nonchalant about its seriousness [89]. The economic burden of JE could also be significant. In Nepal, the associated costs to families, including medical bills, medication, and lost earnings, have been calculated to be US$1151 (10 times median monthly income) for children with severe/moderate sequelae and $524 (4.6 times median monthly income) for those with mild/no impairment [90].

3.3 *Islamic Republic of Pakistan*

Pakistan is another South Asian country that falls under the endemic zone. The earliest report of JE comes from 114 analyzed cases of encephalitis in Karachi city between 1971 and 1983. Out of 114, only 2 cases could be confirmed as JE by serological investigation [91]. In the early 1980s, it was also reported that antibodies against JEV could be detected in serum samples from humans, domesticated animals, as well as rodents [92]. In 1992 following an outbreak of encephalitis in Karachi, the JEV genome was detected from the cerebrospinal fluid of one patient where most of the other cases were attributed to West Nile virus, JEV's closest genetic cousin [93]. Recently, serologic investigation performed on 467 patients suffering from undifferentiated fever revealed that JEV IgM antibodies were present in 32 patients [94]. Studies to identify vector species for diseases have reveled presence of the major vector of JEV, *Culex tritaeniorhynchus* among other *Culicine* species in Pakistan [95, 96]. Recent geopolitical and socioeconomic changes have led to migration of people and livestock in large numbers across the Afghanistan-Pakistan border regions such as the Khyber Pakhtunkhwa Province and the Federally Administered Tribal Areas where vector-borne diseases in people and livestock are common [97]. Though there are yet no reports of JE in such places, other flaviviruses are common. A combination of increasing population, poverty, and poor healthcare facilities with close proximity to vectors has thus increased the risk an outbreak in the near future.

3.4 *Japan*

A brief history of the advent of JE in Japan has already been mentioned in the introduction section of this chapter. JE used to be a major public health concern in

Japan with recurrent epidemics of summer encephalitis recorded from 1871 with major epidemics in 1924 (6000 cases, with a 60% case fatality rate), 1929, 1935, 1937, and 1948 [98, 99]. Following the isolation of the Nakayama strain of JEV, an inactivated mouse brain-derived vaccine was developed in 1954 which was used for mass immunization purposes. However, since May 2005, the inactivated mouse brain-derived vaccine has been replaced by inactivated Vero cell-derived vaccine [100]. As a result of immunization efforts, there was a gradual decrease in the number of JE cases from the islands of Japan. From more than 100 reported cases annually in the 1960s, less than 100 cases were reported from 1982 to 1991, and fewer than 10 cases have been reported annually since the 1990s. This is apparently also due to changes in agricultural and animal husbandry practices, apart from the successful program of JEV vaccination [63]. Among the reported cases since the 1990s, some cases were from nonimmunized US servicemen stationed in Japan [101].

A detailed analysis of all recorded cases (324) between 1982 and 1996 in Japan showed that 13% of the cases were reported from children below the age of 10 years, 9% in the age group of 10–39 years, 50% in the 40–69 years age group, and 29% in the age group of over 70 years. Most of the patients (95%) were living in the western parts of Japan with no reports from the southernmost or northern parts of Japan. JE was prevalent from the end of July to the end of October, with the highest incidence observed in the end of August. Of the 324 cases, complete recovery was reported from 31% patients, while 48% displayed moderate and 17% high degree of neurological sequelae. However, it is to be noted that among these cases, nearly 56% were non-vaccinated. The study thus points that non-vaccinated adults are at highest risk of the disease and underscores the necessity to increasing the vaccine coverage [102].

Even though annual number of reported human cases further decreased in the recent years (two in 2012, nine in 2013; www.mhlw.go.jp/english/database/db-hh/xls/2-12.xls), JEV remains in circulation in reservoirs and vectors all across Japan [103]. The virus has been routinely isolated from swine [104], and a high percentage of all tested animals remain seropositive for JE [105]. Apart from pigs, anti-JEV antibodies have also been detected from wild boars [106], dogs [61], horses [107], raccoons, and raccoon dogs [63] in Japan. Among vectors *Cx. tritaeniorhynchus* remains widely distributed throughout Japan [108].

3.5 Kingdom of Cambodia

The earliest report of isolation of JEV from mosquitoes in Cambodia is from 1965 [65]. Existing JE data from Cambodia are based on reports from several sentinel hospitals. Between 1996 and 1998, among 50 pediatric encephalitis cases diagnosed in one hospital, 18% patients were found to be JE positive [109]. In another study conducted in a different hospital between 1999 and 2000 out of 52 pediatric cases, 31% were JEV positive [110]. A third study conducted on patients aged

15 years or less suffering from encephalitis, at six different hospitals over a 2-year period, concluded that on an average, 19% of all the cases was JE (range of 13–35% across the six hospitals). The estimated incidence of JE was 11.1 per 100,000 in children under 15 years of age, and the mean age of JE-affected children was 6.2 years, with 95% of cases in children aged 12 years or less, and there was no particular seasonal variation for contracting the disease [111]. Another study conducted to estimate the disability from JEV infection in Cambodia showed that 11% of survivors had severe, 39% had moderate, and 45% had mild sequelae [112]. However, hospital-based sentinel surveillance studies may not be true representation of country-wide disease incidences. Thus a later study, employing the latest modeling technique, has estimated that Cambodia has 563 cases of JE every year with an estimated 1 in 250–500 infections resulting in clinical disease [113]. To assess the role of pigs in the JEV infection cycle in Cambodia, serum samples were collected from eight provinces. Tests revealed that more than 95% of the pigs sampled were positive for anti-JEV antibodies suggesting that human JE disease also had the potential for high prevalence [114, 115]. Following the success of a cost-effectiveness analysis of immunization program with the SA 14-14-2 vaccine [116], the Cambodian government has initiated routine immunization against JE from October 2009 onward covering children aged 10–25 months, and within a year, almost 50,000 children had been covered under this program.

3.6 Kingdom of Thailand

Thailand is a country in Southeast Asia bordering Myanmar, Laos, Cambodia, and Malaysia—all of which are endemic for JE. Like many other Asian countries, agriculture, especially rice cultivation, forms a major economic pillar for Thailand. As per data available from the Asian Development Bank, Thailand was the largest exporter of rice in the world till 2012 (https://www.adb.org/sites/default/files/project-document/73082/43430-012-reg-tacr-03.pdf) and the sixth largest producer. The large swaths of rice cultivation fields correlate with the high concentration of various mosquito species, including several known vectors of JEV [117]. Indeed, among all other species of mosquitoes identified in Thailand, *Culex* sp. is the most common taxon [118].

JE was first reported from Thailand in the early 1960s. The virus was isolated from mosquitoes [119], and epidemics were reported from 1969 to 1970 [120]. Since then there has been sporadic reports of JE every year with 1500–2500 cases reported annually throughout the 1970s and 1980s [121, 122]. JEV transmission cycle is believed to last year-round in Thailand with the peak between the months of May and October. Many cases of JE have been reported from the Chiang Mai valley in the northern part of the country with a high incidence rate (14.7/100,000); however a more recent study predicted cases to be concentrated in central or southern part of the country [123–127].

Immunization against JE was first introduced in the early 1970s [128], and in 1990s onward locally produced inactivated mouse brain-derived vaccine was introduced into the routine immunization schedule [129]. A vaccination coverage survey conducted in 2003 concluded that more than 80% of children between the age group 1 and 3 were vaccinated against the disease. As a result of this program along with vector control efforts, data available from the number of encephalitis cases reported nationally between 2002 and 2008 was significantly reduced and ranged from 297 to 418 per year [130]. However, despite all efforts, JE remains an important cause of encephalitis in Thailand, responsible for an estimated 15%–39% of hospitalized encephalitis cases [130, 131].

3.7 Lao People's Democratic Republic (Laos)

Laos is a landlocked country bordered by Myanmar, China, Vietnam, and Thailand. Earliest records of JE from this country are available from the World Health Statistics in 1974 that reports about 34 cases [125]. In a study conducted in 1994, an endemic pattern of JE was proposed in Laos in which prevalence rates of JE antibodies increased gradually with age in residents and changed seasonally in slaughtered swine [132]. However, till then JEV activity was considered to be low as many of the human patients screened was negative for anti-JEV antibody. Later studies on 729 swine blood/serum samples collected between 2008 and 2009 revealed anti-JEV antibodies in 74.7% cases [133]. Other studies performed later on have indicated higher prevalence of the disease in urbanized regions of Laos [134]. Following the construction of the Nam Theun 2 Hydroelectric Project, located in the south-central part of Laos on the Nakai Plateau, a serosurveillance study conducted on the resettlement zones near the dam/reservoir area showed that about 10% of the sample population were positive for anti-JEV antibodies. This also correlated with the increased numbers of mosquitoes in that areas belonging to the *Culex* species, specifically those that are known vectors for the disease [135]. Another recent seroprevalence study from human population in northern Laos reported that of 1136 samples analyzed, antibodies to JEV were detected in 39.4% of the population [136]. In 2015, Laos became the first country to start mass immunization against JE with the cooperation of the Global Alliance for Vaccines and Immunization. An estimated 1.5 million children under the age of 14 were proposed to be covered under the program which was to be incorporated into the national immunization schedule by 2016 (http://www.who.int/immunization/newsroom/press/lao_japanese_encephalitis_apr2015/en/).

3.8 Malaysia

Malaysia is a Southeast Asian country with year-round transmission peaking between October and December. The earliest JE cases were reported in the early 1950s. Sporadic cases of JE are reported now and then from the country with the Ministry of Health surmising that between 9 and 82 cases have been reported each year since 1985, mainly in the states of Perak, Penang, and Sarawak. Between 1989 and 1998, another 531 cases were reported with 33 deaths [137]. There was an epidemic between October 1998 and March 1999 with 157 cases (42 confirmed and 115 suspected) in Peninsular Malaysia, out of which there were 58 fatalities. As pig farming is a thriving industry in the country (more than 544 pig farms exist in Malaysia (http://www.agnet.org/library.php?func=view&style=type&id=20160224103337)), most of JE-affected persons have been adults living in the vicinity of pig farms in the states of Perak (Kinta District) and Negeri Sembilan in the west of Peninsular Malaysia [138]. Mass vaccination against JEV is conducted in the country and a survey of JE cases between 1997 and 2006 showed a 45% drop in JE risk post vaccination [139]. However, few cases are still reported every year. A long-term follow-up study involving 118 serologically confirmed cases of JE conducted over a period of more than 8 years based in the state of Sarawak reported that 44% patients made a full recovery, and 3% had mild, 26% had moderate, and 31% had severe neurological sequelae. About 86 patients were monitored for a median time duration of approximately 53 months (range, 0.9–114.9 months) which showed varying degrees of permanent neuropsychological sequelae and behavioral disorders [140].

3.9 Myanmar (Formerly Burma)

The earliest records of presence of JE in Myanmar are from two serological investigations carried out in 1968 and 1970. Results from both investigations indicated the presence of JEV-neutralizing antibodies in some cases (accurate figures unavailable). In contrast, these antibodies were present in 91% of sera collected from pigs across the country [141]. A JE outbreak was reported in 1974 in Shan State in which 5 cases (4 deaths) were reported followed by 42 cases (32 deaths) in the following year. The disease was at a peak between July and October, and 83.3% of the patients were under 20 years old. Subsequent investigation showed that *Cx. tritaeniorhynchus* was likely to have been the main vector, and the JE antibody positive rates were 81.5% and 42% in domestic animal and human populations, respectively [142]. However, a later serological survey conducted in the Rangoon region from children aged 8 years or less and piglets aged 8 months failed to detect significant number of JEV-positive human samples even though more than 52% of swine samples tested positive. This study also went on to report the presence of important JEV vectors such as *Culex tritaeniorhynchus*, *Culex*

gelidus, *Culex vishnui*, and *Culex fuscocephalus* in Myanmar [143]. More recent studies have reported on the continued presence of JEV-carrying vectors at places bordering China and Laos [144, 145]. Immunization against JEV is not part of the regular mandatory vaccination schedule in Myanmar (as per the Demographic and Health Survey 2015–2016, Key Indicators Report, Government of Myanmar accessed from: http://www.moh.gov.mm/file/MDHS%202015-16%20KIR.pdf). However, as per reports available in news media, 40,000 children are supposed to be vaccinated in 2016 as a response to 19 JE-related deaths in Myanmar (accessed from http://outbreaknewstoday.com/myanmar-japanese-encephalitis-kills-19-this-year-40000-to-be-vaccinated-41275/).

3.10 People's Republic of Bangladesh

In spite of bordering India and Myanmar, two countries with very high prevalence of JE, Bangladesh till date has had very few reported cases of JE. The earliest report of JE was from an outbreak in 1977 near Mymensingh in central Bangladesh. A surveillance program based on serological investigations showed that 1.9% of 1046 healthy persons tested were positive for antibody against the virus [146]. Mosquitoes collected from different pools also tested negative for JEV. Since then there were no other reported cases of the disease from anywhere in the country. However, a prospective hospital-based surveillance study carried out by the Centre for Health and Population Research and the Centers for Disease Control and Prevention (CDC), Atlanta and Ft. Collins, USA, between June 2003 and 2005 at Dhaka, Mymensingh, and Rajshahi Medical College Hospitals, evaluated 2609 patients with 492 meeting criteria for screening for JEV. The study results showed that approximately 4% of the encephalitis cases could be attributed specifically to JEV [147]. The estimated incidence of JE found rates as high as 2.7 per 100,000 populations in the division of Rajshahi, 1.4 in Khulna, and 0.6 in Chittagong. However, a drawback to the study was their non-inclusion of negative data from early symptomatic patients. Recommended diagnosis based on IgM responses to JEV is best done after 10 days of appearance of symptoms. Any negative result obtained from samples collected prior to that period should ideally be classified as JE unknown. Thus the data presented by Hossain et al. could underestimate the actual incidence rate of the disease [148]. Currently, immunization against JE is not part of the regular vaccination program in Bangladesh, but in 2014 a clinical trial was conducted to evaluate the efficacy of SA 14-14-2 JE vaccine and their lot-to-lot variability so that it could be recommended to be routine component of immunization programs [149].

3.11 People's Republic of China

With a population of over 1.381 billion, China is the world's most populous country. JE cases have been reported from all over the country except the three neighboring provinces—Qinghai, Xinjiang Uyghur Autonomous Region, and Tibet. However, seropositivity for JEV antibodies has now been reported from human and swine samples in Tibet, along with an increase in pig farming, and presence of primary vector, *Cx. tritaeniorhynchus*, from which genotype V of JEV was isolated [150, 151]. The provinces of Shaanxi, Sichuan, Guizhou, and Yunnan are reported to be highly endemic regions. The first cases of JE from this country occurred in the 1940s [152] though their reports are sketchy. Since mandatory JE case reporting system was initiated in 1951, there have been two major JE epidemics in China. The first one was in 1966 that had an annual incidence of >15/100,000 nationwide, whereas the second, in 1971, was associated with 174,932 cases of morbidity and an incidence of 20.92/100,000 [153]. The inactivated mouse brain-derived vaccine raised against the P-3 strain was introduced for mass immunization purposes in the 1970s that was replaced by the live attenuated vaccine based on the SA 14-14-2 strain 1989. By 2006 an estimated 300,000,000 people living in 16 provinces were supposed to have been covered by the anti-JE immunization program, and since then the scope of vaccination has been further extended to cover various rural regions [154]. Due to these rigorous vaccination efforts, there has been a gradual decrease in JE incidence across China. Prior to the 1990s, the annual morbidities numbered between 20,000 and 40,000 and between 1996 and 1999 varied from 8556 to 12,490. However, between 2000 and 2005, JE morbidities decreased from 11,779 to 5097. The annual incidence rate declined from 0.9489/100,000 to 0.3898/100,000, whereas the mortality rate also decreased from 0.0302/100,000 to 0.0164/100,000. Over this 6-year period, the annual mortality rate ranged from 2.51 to 4.66% [155]. However, small-scale epidemics are still reported from some regions in the country sporadically. A study conducted in the Dehong Prefecture in western Yunnan Province in 2010 found the incidence rate to be 10.4/100,000 children <15 years of age which was manyfold higher than the national average. This high incidence of JE was attributed to irregular immunization programs conducted in that region [156]. Interestingly, the Wuhan province remained JE-free for three consecutive years between 2005 and 2008; however, the disease reemerged in 2009 and 2010 [157].

As the largest developing country, China has experienced considerable changes in climate over during the last decade with more rapid changes in the past 50 years [158] that are hypothesized to be detrimental on frequency and distribution of mosquito-borne diseases which are a major cause of hazard to public health in China [159]. A positive relationship between climatic variables (monthly maximum temperature, minimum temperature, and total rainfall) and JE transmission in both rural and urban regions in China has been reported [160, 161]. As mosquito vectors for JE are known to be night feeders, a correlation between duration of sunshine and incidences of JE has also been reported from multiple studies in China [162–

164]. As in many other highly endemic countries, JE in China is a seasonal epidemic disease with approximately 90% of cases recorded in July, August, and September each year. The peak of JE onset is 1 month earlier in South China than in the north of the country [152]. This corresponds with the rainy season whence mosquito population thrive. *Cx. tritaeniorhynchus* is the major JE vector circulating in China which is predominantly rice field breeder. As China is currently the largest producer of rice in the world, large swaths of paddy areas are available for breeding of the mosquito. Surveys conducted over years have indicated that over 40 different species of mosquitoes are found in Chinese rice fields and life cycle of *Cx. tritaeniorhynchus* is reported to be intricately associated with rice cultivation as adult mosquito population peaks with every number of crops per year [165]. A recent study reported mutational changes in *Cx. tritaeniorhynchus* rendered it immune to pesticides like deltamethrin, beta-cypermethrin, and permethrin [166].

As is wont with endemic countries, swine population across China are affected with JE, and seropositivity for JEV antibodies has been reported from almost all provinces. An investigation into a JE epidemic at a pig farm in the Sichuan province in 2012 resulted in the isolation of a new strain named as SCYA201201 that was classified as belonging to genotype I of JEV [167]. Another study conducted in the Henan province around the same time also yielded the same strain along with other older genotype III strains. Further analysis of data from other sites has led to the belief that swine JEV genotype III is predominant in swine population in China but the genotype I is emerging and spreading rapidly in recent years [168].

3.12 Republic of China (Taiwan)

The Republic of China is mainly composed of the island of Taiwan and some smaller islands off the coast of People's Republic of China. Encephalitis during the summer months had been a common feature in Taiwan since the early 1930s which were suspected to be JE based on clinical features of the disease. However, definite proof of the involvement of JEV was not obtained until 1958 when researchers from the US Naval Medical Research Unit Number 2 based in Taiwan succeeded in isolating the virus [169]. Since then JE is considered to be endemic in Taiwan and has been designated as a notifiable infectious disease since 1955. The highest incidence rate of confirmed JE cases (2.05 per 100,000) was recorded in 1967 [170]. After mass vaccination program was implemented in 1968, the incidence rate of confirmed JE cases declined significantly to 0.052 (in age group <30 years) to 0.167 (in age group >30 years) per 100,000 population between 2001 and 2012 [171]. From 1998 to 2012, the annual number of confirmed JE cases ranged from 13 to 37 [172], and in 2016, till November, there has been 23 recorded cases of JE in Taiwan where, with the exception of 1, all other cases are from persons above the age of 35 years (http://www.cdc.gov.tw/english/surveillancesmore.aspx?treeid=00ed75d6c887bb27&nowtreeid=f0131176aa46d5db&tid=7B98079D47D2133B: 3rd category report of every month).

Although the incidence of JE in children less than 10 years of age had decreased significantly over the years, a disturbing trend currently observed is the sporadic cases of JE in adults from all over the island. In 2004 a seroepidemiological study of JE in Taiwan was commissioned by the CDC Taiwan whose aim was to evaluate the vaccination program in Taiwan by assessing the neutralizing antibody among people of various age groups from different living environments. A total of 2800 residual serum samples, collected from hospitals around the island, were stratified by 7 age groups and 4 living areas. Each age group included 400 samples equally distributed among areas. The seroprevalence of anti-JEV antibody was compared among cohorts as well as among areas. In northern Taiwan, the cohorts born during 1953–1962 and 1963–1972 were found to have the lowest seroprevalence of 44%. In central Taiwan, the cohort born during 1953–1962 had the lowest seroprevalence of 35%, followed by the 1973–1982 cohort (40%). In southern Taiwan, the population with the lowest seroprevalence (33%) was surprisingly found in the 1983–1992 cohort, followed by the 1963–1972 cohort (39%). In eastern Taiwan, the population with the lowest seroprevalence was also the 1963–1972 cohort (32%), followed by the 1973–1982 cohort (42%). Overall, the 1963–1972 cohort had the lowest seroprevalence of 40.8%, followed by the 1953–1962 cohort of 44.5%. The 1983–1992 and 1973–1982 cohorts also had relatively low seroprevalence of 45%. The study inferred that anti-JEV antibody seroprevalence was lower among the 10–50-year-old age groups, suggesting the opportunity of natural infection has largely decreased, the persistence of vaccine has reduced, and the size of susceptible population might increase as well (http://www.cdc.gov.tw/english/programresultinfo.aspx?treeid=9d909a43ebf2819d&nowtreeid=7b5b3c66a1ff6e25&tid=7F829A8E0F776FA1).

In Taiwan, a total of 11 species of mosquitoes are reported to carry JEV including *Cx. tritaeniorhynchus*, *Cx. annulus*, *Anopheles sinensis*, *Armigeres subalbatus*, and *Cx. fuscocephala*. Among them, *Cx. tritaeniorhynchus* is the most frequently identified JEV-positive species followed by *Cx. annulus* and *An. Sinensis*. *Cx. tritaeniorhynchus* and *An. sinensis* are the most frequently identified mosquito species on the pig farms, whereas *Cx. tritaeniorhynchus* and *Cx. sitiens* are most frequent in the wetlands [172].

3.13 Republic of India

India is the second most populous country in the world with over 1.2 billion people and is the world's seventh largest economy. India's recent growth and development has resulted in improved health conditions but still faces considerable challenges in the form of "triple burden of disease"—unfinished agenda of communicable diseases, emerging noncommunicable diseases related to lifestyle, and emerging infectious diseases. India is endemic for JE, and frequent epidemics have been reported from parts of the country. Approximately 597,542,000 people in India have been estimated live in JE-endemic regions, and 500 to 4000 cases are reported

Table 3 Number of recorded JE cases and deaths in the last 7 years in India (data as per [184] and Directorate of National Vector Borne Disease Control Program of India)

	Recorded JE cases	Deaths
2004	1714	367
2005	6727	1682
2006	2842	658
2007	4024	963
2008	3839	684
2009	4521[a] (653 JE)	774[a]
2010	555	112
2011	1214	181
2012	745	140
2013	1086[b]	202
2014	1661[b]	293
2015	1730[b]	291
2016 (provisional)	1627	278

[a]AES
[b]Data similar to that reported by the World Health Organization but not exactly the same (see Table 2)

every year [173] (see Table 3). Though JE is categorized as an emerging infectious disease, in India the presence of the virus was first indicated in a serological investigation that took place in 1952. Serum samples collected from different places in Southern, Western, and Central India showed positive results for the presence of anti-JEV antibodies (19 out of 588 samples) [174]. The first human case was reported from Vellore district of Tamil Nadu in 1955 [175], and in between 1955 and 1966, a total of approximately 65 sporadic cases were reported [176]. The incidence rate was later estimated to be 15/100,000 population of children belonging to the age group of 5–9 years in Tamil Nadu [177]. The virus was first isolated in 1958 from a JE case [178]. In 1973, a major outbreak resulting in a 42.6% fatality rate was reported in the Bankura and Burdwan districts of West Bengal [179, 180] followed by another in 1976 in Burdwan only [181]. Since then, JE cases have been reported from many other states and union territories (21 till date) and are currently reported from the states of Andhra Pradesh, Assam, Bihar, Goa, Haryana, Karnataka, Kerala, Maharashtra, Manipur, Odisha, Tamil Nadu, Uttar Pradesh, West Bengal, and Nagaland and the National Capital Region [70, 182]. In 1978, a major JE epidemic occurred in Gorakhpur, Uttar Pradesh, with 1002 cases and 297 deaths reported [183]. Multiple subsequent outbreaks reported from this region since then with varying intensity and magnitude thereby led this region to be designated as the "JE epicenter" of India. Data of the recorded JE cases over three decades show that between 1978–1987 and 1988–1997, 20–25% of total cases in India originated from this region; between 1998 and 2007, this was dramatically increased when more than 50% of total Indian cases were from Uttar Pradesh.

The overall incidence rates varied from 0.01 to 9.87/100,000 between 1978 and 1987 but were increased in several districts in the next decade. The Kushinagar district reported the highest incidence rate of 29.90/100,000 population, followed

by Maharajganj (21.80/100,000) and Gorakhpur (10.01/100,000) [185]. As per recorded case history between 1978 and 2004, more than 10,000 lives have been lost to JE in Uttar Pradesh state of India. In 2005 there was another severe JE epidemic in the same region with 6061 cases and 1500 deaths, followed by another in 2006, with 2320 cases and 528 deaths, and in 2007, with 3024 cases and 645 deaths [184, 186]. However, the case fatality ratio has been decreasing in Uttar Pradesh with every epidemic. In the 2005 JE outbreak, the overall CFR was 24.9% as compared with earlier outbreaks in 1978 (31.5%), 1985 (34.5%), and 1988 (31.5%) [185].

Assam is another state with high number of JE cases. Initial cases of JE were reported in the early 1980s [187] followed by a major outbreak in 1989 with a recorded case fatality ratio of 50% [188]. Since then, cases of JE have been reported regularly from Assam which were mostly in the age group of <12 years [189]. Since the mid-2000s, a shift in the primary age group of JE-affected cases has been observed. A cross-sectional study on patients with AES in Assam between 2007 and 2009 showed majority of the confirmed cases of JE were being reported from persons above the age group of 15 years [190, 191]. Though adult cases of JE are not unheard of in India, the situation in Assam is unique where adult cases outnumber pediatric cases.

Apart from the states that frequently report JE cases, the state of Odisha (formerly Orissa) has seen a spike in JE cases in 2016. The only previously known outbreak in Odisha was in 1989 when 41 cases with a case fatality rate of 36.6% were reported from the city of Rourkela [192]. No significant reports of JE outbreaks were available till 2016 when tentatively 242 cases of JE were reported along with 42 deaths in the Malkangiri district (http://www.nvbdcp.gov.in/Doc/je-aes-cd-31Dec16.pdf).

There is an abundance of vectors as well as amplification host and reservoirs of JEV available in India. The most common mosquito species responsible for the spread of JE in India is *Cx. tritaeniorhynchus* though the virus has been isolated from other species of mosquito also. Table 4 lists all such species and the regions of the country from where they were isolated. India also has a very high population of pigs which, as per the 19th livestock census conducted by the Department of Animal Husbandry, Dairy & Fisheries of the Ministry of Agriculture & Farmers Welfare, Government of India, constitutes around 1.30% of the total world's population (http://dahd.nic.in/sites/default/files/Livestock%20%205.pdf). The close proximity of mosquito vectors, pigs, and ardeid birds does contribute to the epidemiology of JE in India [193].

An indigenous version of the inactivated mouse brain-derived vaccine was produced by the Central Research Institute, Kasauli. Prior to 2006, JE vaccines were available at a subnational level in places which reported disease outbreaks. The anti-JE vaccination campaign was launched during 2006 wherein 11 most sensitive districts in Assam, Karnataka, and Uttar Pradesh were covered. In 2007 and 2008, the program was expanded to include Andhra Pradesh, Bihar, Haryana, Maharashtra, and Tamil Nadu that has resulted in reduced incidence of JE in these states [182]. Till date altogether 86 JE-endemic districts in the states of Assam,

Table 4 Japanese encephalitis vectors in India (data as per National Vector Borne Disease Control Programme of India)

Species	No. of isolations	State
Cx. tritaeniorhynchus	79	Tamil Nadu, Karnataka, Kerala
Cx. vishnui	30	Tamil Nadu, Karnataka, West Bengal
Cx. pseudovishnui	8	Karnataka, Goa
Cx. bitaeniorhynchus	3	Karnataka, West Bengal
Cx. epidesmus	1	West Bengal
Cx. fuscocephala	7	Tamil Nadu, Karnataka
Cx. gelidus	8	Tamil Nadu, Karnataka
Cx. quinquefasciatus	1	Karnataka
Cx. whitmorei	4	Tamil Nadu, Karnataka, Andhra Pradesh, West Bengal
An. barbirostris	1	West Bengal
An. paeditaeniatus	1	Karnataka
An. subpictus	9	Tamil Nadu, Karnataka, Kerala
Ma. annulifera	2	Kerala, Assam
Ma. indiana	3	Kerala
Ma. uniformis	4	Karnataka, Kerala

Cx. Culex; *An.* Anopheles, *Ma.* Mansonia

Andhra Pradesh, Bihar, Haryana, Goa, Karnataka, Kerala, Maharashtra, Tamil Nadu, Uttar Pradesh, and West Bengal have been covered.

3.14 Republic of Indonesia

Indonesia is the world's largest archipelagic state consisting of more than 1000 islands lying between the Indian and the Pacific oceans. Currently Indonesia is the world's third most populous democracy and the largest economy in Southeast Asia (http://www.worldbank.org/en/country/indonesia/overview). The first data suggesting JE virus transmission in Indonesia were from animal and human serosurveys conducted in the mid- to late 1960s. However, almost all the investigations utilized hemagglutination inhibition (HI) assays which show significant cross-reactivity, and thus the results are considered inconclusive [194–196]. The presence of JE virus was confirmed when it was isolated in 1972 from *Cx. tritaeniorhynchus* mosquitoes near Jakarta [197] and later from *Anopheles annularis* and *Anopheles vagus* from Lombok island [198]. Subsequently JEV was also isolated from vectors obtained from West and Central Java [199] and from pigs and mosquitoes near Jakarta and Bali [200–202]. Since the early 1970s, there have been reports of intermittent serologic surveys that have suggested JEV

transmission in several areas of Indonesia [203–206]. However, reports of confirmed human cases were infrequent and from limited areas. In 1981–1982 in Jakarta, about one in four children with clinically suspected viral encephalitis in two hospitals showed a fourfold rise in JEV titer with HI and immune adherence hemagglutination test. In 1985, seven JE cases with IgM in CSF were identified among hospitalized patients with encephalitis in Yogyakarta, Central Java. Other sporadic reports have come from Timika, Irian Jaya, and Sumatera which could not be serologically confirmed to be JE [207]. The first laboratory-confirmed cases of JE were reported in 1996 from patients suffering from viral encephalitis using IgM capture ELISA both on serum and CSF samples in Bali [208]. Subsequently, hospital-based surveillance in Bali from 2001 to 2003 showed a total of 86 JE cases among 239 pediatric patients with 10% mortality rate, and 37% of survivors suffered from severe sequelae. The estimated incidence rate was calculated to be 8.2 per 100,000 in children under 10 years of age [209]. Another sentinel surveillance involving 15 hospitals in 6 provinces from 2005 to 2006 further confirmed the presence of JE cases in all provinces throughout the year, with 95% of cases occurring in children under 10 years of age, and thus confirmed that JE was endemic in Indonesia [207]. However, currently there are no active anti-JE immunization programs in Indonesia even though modeling studies have predicted them to be cost-effective [210].

The importance of Indonesia with respect to JE could also be appreciated from the fact that according to current understanding, this is the place of origin of the virus. The tropical climate of the Indonesia/Malaysia region, together with its plethora of distinct fauna and flora, may have driven the emergence and evolution of JEV as evidenced by the genetic diversity of JEV found here. This is also the only region where all five genotypes of JEV can be found [211].

3.15 Republic of Korea (South Korea)

Though recorded evidences are lacking, a long association with Japan—politically, culturally, as well as socioeconomically—is possibly the reason for the introduction and rapid propagation of JE into the Korean Peninsula. Amidst the geopolitical upheavals of the 1940s and 1950s, a large number of foreign military personnel, most belonging to the United States, were stationed in the Republic of Korea. It is from these nonimmunized personnel that the first recorded cases of JE are reported from this region [212, 213]. However, the population of the country as a whole also did not escape the ravages of this disease. The epidemic of 1949 reported 5616 cases and 2729 deaths [214]; the epidemic of 1958 recorded 2177 mortalities among 6897 incidences. Between 1960 and 1968, the annual incidence of JE was 1000–3000 cases per year, with an annual mortality of 300–900 [215].

Measures to control the menace of JE were adopted from the early 1970s. Apart from vector control efforts [216], vaccination against JEV was first introduced in 1971. Even though it was not widely available across the country, the number of JE

cases steadily declined throughout the 1970s and 1980s with the only aberration of 1983 when 1197 cases and 40 consequential deaths were reported [217]. Since then immunization against JE was incorporated into the national vaccination program which includes annual booster vaccine for all children less than or equal to 15 years of age [218]. The success of the vaccination program can be ascertained from the fact that from the mid-1980s till 2009, there has been very limited reports of JE from the country with incidence rates as low as <0.02 cases per 100,000 population [218]. However, since 2010 JE has reemerged in South Korea, and available records show the reported number of cases has been steadily increasing till 2015 (except for 2011, when only 3 cases were reported) when incidence was highest (40 cases). A total of 19 patients died during 2010–2014 (overall case fatality rate 21.3%), whereas during the previous 25 years (1985–2009), only five deaths were attributable to JE [219]. Interestingly, the median age of the persons affected from JE during this reemergence was found to be 53 years. Though the possibility of non-vaccinated adults getting affected by JEV is not remote, in case of South Korea, 98.1% of adults at risk had neutralizing antibodies against the virus, probably resulting from low-grade natural infections at younger age [220]. This raises questions about the lifelong efficacy of childhood immunization, and further booster doses may be required to effectively curb the menace of JE.

3.16 Republic of the Philippines

The Philippines is a western Pacific archipelago (approximately 7107 islands) situated in Southeast Asia. A serosurveillance study conducted in the early 1940s to detect arbovirus infections first reported the presence of JEV-neutralizing antibodies in Philippine horses [221], and the first isolation of the virus from *Culicine* vectors was made in the late 1970s [32, 222]. Recent studies also document the abundant presence of JEV vectors in the Philippines [223]. This in part is supported by the fact that as typical in Asia, rice cultivation is a major contributor to the local economy. Although not sufficient to sustain the domestic need, the Philippines is still the 8th largest producer of rice in the world, and interestingly pork also forms a major constituent of local diet. In fact according to recent data available from the Philippine Statistics Authority, there has been a constant increase in pork production in the country over the last decade (~4% increase in 2016 from 2015) (https://psa.gov.ph/content/swine-industry-performance-report-2). It is likely that a combination of these two factors (rice cultivation and swine rearing) contributes to the persistence of JEV in the Philippines.

There have been several reports of JE from different regions/islands of the archipelago which have been reported in various scientific communications with the first clinically recorded cases in 1972. A thorough compilation of all such reports can be found in a recently published review article [224]. From 29 published reports and presentations on JE in the Philippines, Lopez et al. concluded that between 1972 and 2013 there were 257 laboratory-confirmed cases of JE. The JEV

was found to be the causative agent in 16–40% of clinical encephalitis cases and also in 7–18% of cases representing a combination of meningitis and encephalitis. Like many other places, children in the Philippines less than 15 years of age were predominantly affected by JE, and 6–7% of cases resulted in death. Recent surveillance data between January 2011 and March 2014 identified 73 (15%) laboratory-confirmed JE cases out of 497 cases tested. The peak season for disease transmission was found to cover the months of June–July. Currently a proposal to include vaccination against JE in the Expanded Program on Immunization by the Department of Health of the Philippine government is under consideration (http://www.pchrd.dost.gov.ph/index.php/news/library-health-news/4878-doh-to-include-je-vaccine-in-immunization-program).

3.17 Republic of Singapore

Singapore is a small island city-state in Southeast Asia with Malaysia and Indonesia as its neighbors. Singapore is used to be under the endemic zone of JE with multiple reported cases [225–229]. Since phasing out of pig farming between the early 1980s and 1992, incidence of reported cases became very low. Between 1991 and 2005, there are reports of only six cases, out of which three were imported but in the case of three other patients, the disease seemed to be contracted within the country [230]. Seroepidemiological studies on the prevalence of neutralizing antibodies to JEV conducted on a variety of domestic or peridomestic animals showed a prevalence of 46.5% in dogs and 60% in chickens [231, 232]. Thus, even after abolition of pig farming, the virus seems to persist in other vertebrate hosts. However, as the reported number of cases is low and infrequent, currently vaccination against JEV is not part of the immunization schedule in the country.

3.18 Socialist Republic of Vietnam

Vietnam is a Southeast Asian country bordering China, Laos, and Cambodia. Postcolonial rule and Civil War era Vietnam was marked with economic backwardness and political isolation that took a toll on its healthcare sector. The virus was first isolated from vectors in 1951 [233], and epidemics of JE have been frequently reported since the 1960s, with reported incidence rates as high as 22 per 100,000 population [125]. In Vietnam acute encephalitis syndrome (AES) cases are counted and reported as a surrogate for JE surveillance. From 1985 through 1993, annual AES incidence rates in Vietnam, reported as JE incidence, ranged from 1 to 8 cases per 100,000 population [234, 235]. The highest incidence rates were reported from provinces in the Red River Delta region in the northern part of the country. In South Vietnam, the highest morbidity of 936 patients was recorded in 1980, while the highest mortality of 339 deaths was recorded in 1977.

The lowest figures of morbidity were 197 cases in 1990 and lowest mortality was 34 deaths in 1985. Sporadic cases were reported throughout the year, but small annual outbreaks with low peaks were usually reported in February and July [236]. Between 1998 and 2007, the incidence rates in this region have been reported to be 1.9 cases annually per 100,000 inhabitants, with a mean case fatality of 6.4% [235]. Rates were also high in some provinces in the Mekong River Delta region in the southern part of the country. Both Red River and Mekong Delta regions are major rice-producing areas which make Vietnam the fifth largest rice producer in the world. Among mosquito species, *Cx. tritaeniorhynchus*, *Cx. vishnui complex*, *Cx. fuscocephala*, and *Cx. gelidus* are abundantly found in rice agroecosystems (rice fields, ditches, ponds, and wetlands) in Northern Vietnam, and their numbers increased during the rainy season [237]. Also these regions have flourishing pig industries, which make them ideal enclave of vectors and vertebrate hosts of JEV [238]. Apart from rural areas, JEV has been reported to circulate in urban areas of Southern Vietnam. A study in 2013 detected both genotypes I and III of JEV from mosquitoes caught from Ninh Kieu District of Can Tho City. Serological analyses from pigs also revealed that 100% of the samples were positive for anti-JEV antibodies [239].

Since 1997, a locally produced mouse brain-derived inactivated vaccine has been administered to children exclusively under the age of 5 years and was also included in routine childhood immunization program in selected high-risk districts. As a result of vaccination, a decrease in incidence rates was observed in the high-risk regions. Data available from the Hatay Province (a high-risk region) showed that the JE incidence rate dropped from >20 per 100,000 in 1996 to around 5 per 100,000 in 1999, with the majority of cases still occurring among children aged <15 years. However, no further decline was noted during 1999–2004 [240].

3.19 Timor-Leste

Timor-Leste is the first new sovereign state of the twenty-first century after it gained its independence from Indonesia in 2002. As part of the island chains that form the Indonesian archipelago, this country is also considered to be under the JE-endemic zone. An Australian Quarantine and Inspection Service (AQIS) and Australian Defense Force (ADF) collaboration study in 2000 in conjunction with the WHO reported the presence of anti-JEV antibodies in serum samples from pigs in Timor-Leste. Later on human patients manifesting encephalitis-like symptoms were reported to the WHO by doctors associated with Médecins Sans Frontières which were later confirmed to be positive for JEV by laboratory tests (www.who.int/disasters/repo/5362.doc). Since then as per WHO records, there have been few identified cases of JE over the years in Timor-Leste (Table 2).

3.20 Other Countries

There are some other South or Southeast Asian countries from where there is a lack of reliable data regarding confirmed cases of JE or seroprevalence of anti-JEV antibodies in human or other vertebrate hosts or in the vectors. Hence, brief mentions of such countries are given below:

(a) **Afghanistan** falls under the endemic zone of JE [241], even though there are no reported cases available. Serological studies from patients suffering from acute febrile illness have reported the presence of other vector-borne flaviviruses such as West Nile virus, dengue virus, and tick-borne encephalitis virus [242]. The presence of JEV-specific vectors, i.e., mosquitoes belonging to *Culex* sp. including *Culex tritaeniorhynchus*, has been reported from Afghanistan [243, 244], and thus the risk of future emergence of the disease remains.

(b) The **Democratic People's Republic of Korea** (DPRK, North Korea) is isolated from almost the rest of the world, and thus practically no information regarding status of JE are available in the public domains. Data available from the WHO shows that there were 134 reported cases between 2006 and 2015 (see Table 2). A joint effort by the WHO, the United Nations Children's Fund, the Global Alliance for Vaccines and Immunization, and the International Vaccine Institute with the Government of DPRK has resulted in the supply of vaccines for multiple diseases including one for JE to this reclusive country [245].

(c) Owing to the proximity with India, specifically with the states of West Bengal and Assam (two states with frequently reported cases of JE), the risk of spillover to the **Kingdom of Bhutan** is always present. There is only one report of a 20-year-old man succumbing to JE in the year 2014 available (https://flutrackers.com/forum/forum/emerging-diseases-other-health-threats-alphabetical-a-thru-h/encephalitis/168569-bhutan-japanese-encephalitis-claims-a-life-in-punakha) which could not be verified from any other source.

(d) The **Negara Brunei Darussalam** is a nation on the island of Borneo, surrounded by Malaysia and the South China Sea. The first report of three laboratory-confirmed cases of JE from this country was available in the year 2013 (http://www.promedmail.org/post/2026099). Interestingly, this does not make it to the list of the WHO (*Table 2)* where one confirmed case is reported in 2015. However, owing to its close proximity with the neighboring Sarawak district of Malaysia from where multiple cases of JE have been reported, future occurrences are possible.

(e) The **Republic of Maldives** is comprised of a group of islands in the Indian Ocean with frequent reports of arboviral diseases (Chikungunya and Dengue) [246]. Even though it also falls under the endemic zone of JE, there has been no report from this island nation till date though it should not be a cause for complacency as tropical islands could emerge as hubs for arboviruses [247].

Beyond the realm of endemic/epidemic zone, JE is scarcely reported. From 1973 through 2013, only 68 cases of JE cases were reported to the US Centers for Disease

Control and Prevention. These cases of JE from North American or European countries are from travelers or expatriates who visit endemic zone long enough to be exposed to the virus [248–254]. Among US military personnel who were serving in South Korea during the 1950s, there were reports of many people suffering from JE [212, 255, 256]. Currently the numbers of reported cases have reduced dramatically but are yet to be non-zero [257]. As humans are dead-end hosts of the virus, the possibility of its spread in Europe or North America by native mosquito species is remote. However, studies have reported the detection of JEV RNA from mosquitoes in Italy [258] and also from birds in Italy and Spain [259, 260]. Hypothetically speaking, there is a potential risk of JEV introduction to the West Coast of the United States where competent vectors and amplifying vertebrate hosts are available [261–263].

4 Shift in Japanese Encephalitis Virus Genotypes

The genotypes of JEV are determined by the nucleotide sequences of the structural core (C)/pre-membrane (prM) and envelope (E) protein genes. Based on variations in the *prM* gene, JEV is classified into four genotypes (I, II, III, IV); when variations in the *E* gene and full-length genome were phylogenetically analyzed, the occurrence of genotype V was established [264]. Genotypes IV and V are believed to be the oldest, whereas I, II, and III are newer [265]. All five genotypes have been detected in vectors and/or reservoirs, while the number of genotypes detected in humans is limited. While multiple strains of JEV GI and III have been reported from humans, only two of JEV GII strains—one in Australia [266] and the other in Korea [267]—and one of JEV GV strain from Malaysia [268] have been detected so far. JEV GIII was once dominant across the entire South and Southeast Asia and was most frequently isolated in JE-endemic areas until the 1990s. However, a gradual shift from GIII to GI occurred in many such regions over the last three or four decades. Phylogenetic analysis of JEV isolated from vectors and pigs in Thailand indicates that the GIII-GI shift could have begun by the 1980s [269] followed by Japan [270, 271] and South Korea [272] in the early 1990s. Since then GIII-GI shift has been reported from Northern Vietnam [273, 274], India [275], China [276], and Taiwan [172]. The migration pattern of JEV GI can be visualized in Fig. 2. JEV GI co-circulated with JEV GIII as a minor strain in some of the countries even before the GIII-GI shift. Comprehensive phylogenetic analysis of the JEV GI strains has demonstrated that the old and the new GI strains form distinct lineages [277, 278]. This suggests that genetic differences in the new GI genome might be responsible for the spread of GI viruses. Recent findings indicate that JEV GI exhibits a higher replicative ability in mosquitoes than JEV GIII [278] and also differ in antigenicity [279].

The factors influencing emergence and geographical spread of JEV genotypes are not clearly understood. Some emerge in areas where they were absent before (e.g., genotype V in Tibet, a cold and high-altitude region that was considered free

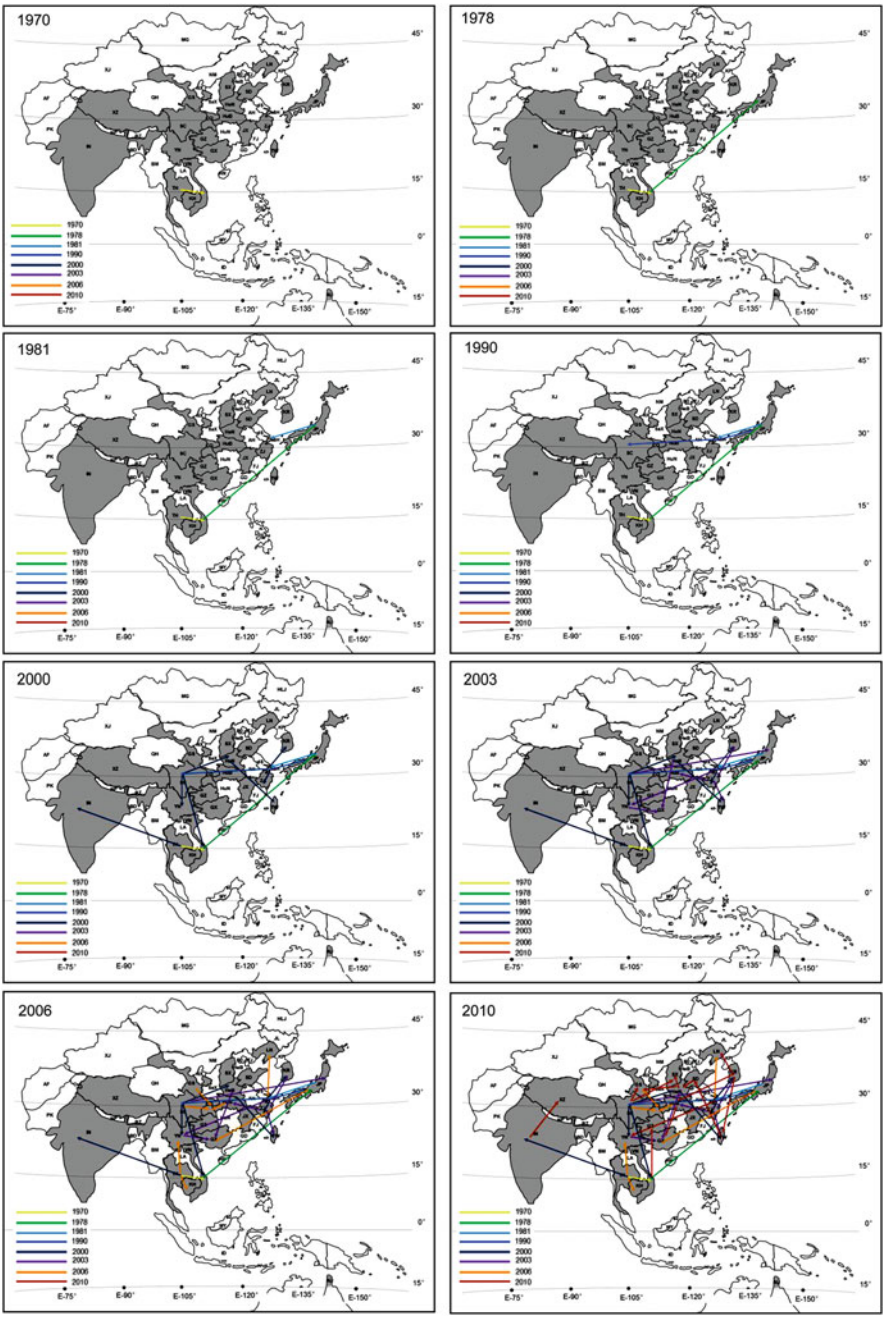

Fig. 2 The spatiotemporal migration of Genotype 1 JEV since the 1970s. The different panels represent temporal projections of reconstructed migration events in last 40 years (1970, 1978, 1981, 1990, 2000, 2003, 2006 and 2010, respectively). The different colors, *yellow*, *green*, *light blue*, *blue*, *dark blue*, *purple*, *orange*, *red*, *dark red* demonstrates for 1970, 1978, 1981, 1990, 2000, 2003, 2006 and 2010, respectively. The *panels* only show the tendency of migration events or partial migration events that have occurred up to a particular date, assuming that the virus

of JEV [150, 151], and genotype I in the Australasian region [280]), whereas some replace the dominant genotype (e.g., GIII-GI shift). The role of environmental factors has been suggested to be associated with the complex pattern of emergence and spread of the virus. Some reports indicated that GI and GIII viruses were collected mostly in temperate zones, while GII and GIV isolates were collected mostly in tropical zones [281, 282]. Paucity of comprehensive dataset of isolates from these regions prevented rigorous analysis to confirm this hypothesis until recently when a large-scale study reported that GIII and the recently emerged GI are temperate genotypes likely maintained year-round in northern latitudes, while older GI and GII are tropical genotypes likely maintained primarily through mosquito-avian and mosquito-swine transmission cycles [283]. Wind dispersal of mosquitoes across the Torres Strait into Australia is another example of involvement of environmental factors. Other factors such as animal ecology (e.g., migration of animals), human activity (e.g., pig trade introducing genotype I in Australia or increased pig farming in Tibet), and climatic changes are variables independent to JEV genetics that may modify the distribution area of JEV and its genotypes [284]. Another factor could be the fitness of the virus to a new competent vector; JEV GV, besides being isolated from *Cx. tritaeniorhynchus*, seems to be associated with a new vector in the Republic of Korea, *Cx. bitaeniorhynchus*.

An important cause of concern regarding the shift in genotype of JEV involves immunization efforts to control or prevent the spread of the disease. Since the isolation of the Nakayama strain of JEV in Japan, all vaccines are based on JEV GIII strain (*see the following section*). Though till date there have been no reports of vaccine ineffectiveness due to the GIII-GI shift, this paradigm could be altered in case of future epidemics involving JEV GV. A recent study has reported that current GIII-based JE vaccine derived does not provide adequate levels of protection against the emerging JEV GV infections [285]. This is a cause of concern as although GV is estimated to be the most ancestral JEV lineage, it showed a highly active dispersal capacity (Tibet and Malaysia, *Cx. tritaeniorhynchus* and *Cx. bitaeniorhynchus*) following its reemergence almost after 60 years since its first isolation. Also the GV Maur strain has been reported to be more neurovirulent than JEV GI strains [286]. If JEV GV follows a dispersal pattern similar to that of GI, it

Fig. 2 (continued) migrates at a constant rate over the inferred time span of the branch. *Blue circles* mark the hot point of migration events. *AF* Afghanistan, *PK* Pakistan, *IN* India, *NP* Nepal, *BT* Bhutan, *BG* Bangladesh, *BM* Burma, *TH* Thailand, *LA* Laos, *VN* Vietnam, *KH* Cambodia, *MY* Malaysia, *ID* Indonesia, *PP* Papua New Guinea, *AU* Australia, *KP* North Korea, *KR* South Korea, *JP* Japan. Chinese provinces: *HLJ* Heilongjiang Province, *JL* Jilin Province, *LN* Liaoning Province, *NM* Neimenggu, *XJ* Xinjiang, *BJ* Beijing, *TJ* Tianjin, *HeB* Hebei Province, *SX* Shanxi Province, *SaX* Shaanxi Province, *GS* Gansu Province, *QH* Qinghai Province, *NX* Ningxia, *SD* Shandong Province, *SH* Shanghai, *JS* Jiangsu Province, *AH* Anhui Province, *HeN* Henan Province, *XZ* Xizang, *ZJ* Zhejiang Province, *JX* Jiangxi Province, *HuB* Hubei Province, *CQ* Chongqing, *SC* Sichuan Province, *HuN* Hunan Province, *GZ* Guizhou Province, *YN* Yunnan Province, *FJ* Fujian Province, *GD* Guangdong Province, *GX* Guangxi, *HN* Hainan, *TW* Taiwan, *MG* Mongolia (figure is from [277])

could have the potential to spread all over the endemic regions rapidly. Thus it is clear that GV needs to be monitored closely throughout JEV-endemic regions.

5 Prophylaxis and Therapeutics

5.1 Vaccination Against JE

JE is a vaccine-preventable disease. Human immunization programs conducted in various countries such as Japan and South Korea have successfully controlled the spread of the disease, and many countries in the endemic zone report decreasing number of cases every year. Currently available JE vaccines fall into four classes:

(a) *Inactivated mouse brain-derived vaccines:* The Nakayama strain of JEV, isolated from the CSF of a patient in 1935 and maintained by continuous mouse brain passage, has been the principal strain used in mouse brain-derived vaccines produced throughout Asia. This class of vaccine was initially prepared in Japan and licensed for human treatment in 1954. A later purified version was reintroduced by the Biken foundation in 1965 which was later also produced in Taiwan and South Korea since 1968 [287]. Later on, the Beijing-1 strain of virus has also been used for this type of vaccine production. However, uncertainty over duration of protection, requirement of multiple booster doses and rare reports of acute disseminated encephalomyelitis temporally associated with this type of vaccine resulted in the search for newer generation of vaccines with greater safety profile.

(b) *Inactivated Vero cell-derived vaccines:* Inactivated, Vero cell-derived, alum-adjuvanted vaccine (SA 14-14-2 strain, attenuated, IXIARO and JESPECT) was licensed in 2009 based on non-inferior immunogenicity to a field effective, mouse brain-derived JE vaccine [288] and is licensed in several countries. Production of this vaccine was transferred by technology agreement to another manufacturer and was licensed in 2012 in India (JEEV) and since then in other countries in Asia (http://www.who.int/immunization_standards/vaccine_quality/pq266_je_1dose_biologicale/en/). Other inactivated Vero cell-derived vaccines are produced in China, India, and Japan using different viral strains; these have limited or no international distribution.

(c) *Live attenuated vaccines:* A primary hamster kidney (PHK) cell-derived, live attenuated vaccine based on the SA 14-14-2 strain of the JEV is licensed and has been used widely in China since 1988 (CD.JEVAX) [289]. This vaccine has similar immunogenicity and efficacy as compared to the inactivated mouse brain-derived vaccine but has no or minimal side effects in comparison [290]. The vaccine is now licensed and used in an increasing number of countries in Asia. Two other live attenuated vaccines based on the same attenuated strain are manufactured in China but not exported.

(d) *Live recombinant vaccine:* A live attenuated, recombinant (chimeric) JE vaccine was developed by Sanofi Pasteur by replacing the prM and E coding sequences of the yellow fever live attenuated 17D vaccine virus with the analogous sequences coding for the antigenic determinants from the SA 14-14-2 live attenuated JEV strain [291, 292]. The vaccine virus is produced in Vero cells. This vaccine was first licensed in Australia and Thailand in 2010 and since then licensed and used in a growing number of Asian countries under the brand names IMOJEV, JE-CV, and ChimeriVax-JE. Multiple phase II and phase III studies conducted in adults and children have demonstrated the safety and immunogenicity of this type of vaccine, and a recent phase IV trial failed to identify any new safety concerns and confirmed its good safety profile [293].

The mouse brain-derived inactivated vaccine has been slowly phased out from most countries in Asia and now replaced with live attenuated, inactivated, or the recombinant chimeric vaccine. The vaccine availability in various countries that are under the purview of discussion of this chapter is depicted in Table 5. As countries transition from the use of one product to another or use multiple products requiring more than one dose, the potential exists for vaccines to receive more than one product to finish out a series or for the purposes of a booster. Limited data exist on vaccine interchangeability, and the few studies that have been done do not report any adverse effects [294–297]. Interestingly, approximately all of the licensed JE vaccines currently in use are based on genotype III virus strains. While these vaccines reportedly retain the protective efficacy against genotypes I, II, and IV [298], a recent report suggests that they may not do so for genotype V [285]. As discussed earlier in this chapter, JEV genotype V could be an important pathogen in the recent future as it is considered as an emerging genotype. Thus without an effective vaccine, it could lead to a major problem in South-Southeast Asia.

5.2 Therapy Options

At present, there are no specific therapies targeted against the virus available. Therapeutic options have been and even currently are being explored in many places; however, till date there are limited options available. Most of the drugs/compounds reported in academic researches either work in vitro or to a limited extent in animal models. A list of all such drugs/compounds and their mechanisms of action can be found in a recent review article by us [300]. However, only a few of them has been deemed suitable enough to take for human clinical trials. Even then most of them have failed to elicit sufficient curative efficacy. The anti-inflammatory steroid dexamethasone was one such compound that was used for a clinical trial in Thailand. A relatively high dose of dexamethasone (600 mg/kg body weight) failed to elicit any beneficial response in the treated group as compared to placebo-treated group. There were no differences in mortality rate as well as neurologic symptoms, 3 months post discharge among survivors of either group [301]. Interferon alpha is a

Table 5 Current JE vaccine used in South and Southeast Asian countries (adapted from [299])

Country	Vaccine (public market)	National/ subnational	Vaccine (private market)
Bangladesh	None	NA	None
Bhutan	None	NA	None
Brunei Darussalam	Chimeric	Subnational	Chimeric
Cambodia	Live attenuated	Subnational	Mouse brain (inactivated)
China	Live attenuated	National[a]	Vero cell (inactivated); live attenuated
Democratic People's Republic of Korea	None	NA	Unknown
India	Live attenuated; inactivated Vero cell (adults)	Subnational	Mouse brain (inactivated); Vero cell (inactivated)
Indonesia	None	NA	None
Japan	Vero cell (inactivated)	National	Vero cell (inactivated)
Lao People's Democratic Republic	Live attenuated	Subnational	None
Malaysia	Chimeric	Subnational	None
Myanmar	None	NA	Chimeric (expected 2015)
Nepal	Live attenuated	Subnational	Live attenuated
Pakistan	None	NA	Unknown
Philippines	None	NA	Chimeric
Republic of Korea	Mouse brain (inactivated); live attenuated	National	Vero cell (inactivated); live attenuated
Singapore	None	NA	Vero cell (inactivated)
Sri Lanka	Live attenuated	National	Mouse brain (inactivated)
Thailand	Mouse brain (inactivated); live attenuated[b]	National	Live attenuated; chimeric
Timor-Leste	None	NA	None
Vietnam	Mouse brain (inactivated)	Subnational	None

[a]Excluding non-endemic provinces
[b]Distribution limited geographically

member of the cytokine family that many reports have shown to the protective in case of viral infections. In the mid-1980s, there was a report from Thailand about the positive effect of human recombinant interferon alpha administration on two patients suffering from JE [302]. The study reported that one patient recovered from comatose state posttreatment with interferon alpha. This was thus touted to be the effective cure for JE. However, a much larger (112 patients), controlled trial conducted many years later in Vietnam failed to see any significant alteration either in survival rates or severe sequelae post discharge from hospital [303]. The broad-spectrum antiviral drug ribavirin (1-b-d-ribofuranosyl-1,2,4-triazole-3-carboxamide) was also used in human patients in a clinical trial conducted in

India. Ribavirin was administered at a dosage of 10 mg/kg body weight to serologically confirmed cases of JE in patients ranging from 6 months to 15 years of age. However, a 7-day treatment with the drug failed to show significant difference in early mortality rate or secondary outcome measures [304].

Despite the abovementioned failures, there have been some new developments in the field of JE therapy that are quite promising. Our group in the National Brain Research Centre, India, has been investigating the role of minocycline, a second-generation tetracycline antibiotic, in various models of JE, for nearly a decade. A concise description of the probable anti-JE mechanisms of action of minocycline can be found in a review article authored by us a few years back [305]. Based on the compelling laboratory-based evidence, two randomized, placebo-controlled clinical trials of minocycline administration to patients suffering from encephalitis have been conducted recently. The first of these was conducted in the pediatrics department of King George's Medical University, in the Indian state of Uttar Pradesh with support from the National Brain Research Centre, India. Owing to logistic constraints, the subject recruitment criteria encompassed all cases falling under the acute encephalitis syndrome. Minocycline (or placebo) was administered as a suspension through nasogastric tube for 7 days at a dosage of 5 mg/kg/day followed by 2.5 mg/kg every 12 hours in children (<12 years of age). In adults the starting dosage was 200 mg followed by 100 mg every 12 hours. A total of 140 patients received minocycline and 141 received placebo. Even though there was not a statistically significant difference in survival between the drug and placebo groups, when the Glasgow Outcome Score at 3 months post discharge was compared between survivors of the two groups, there was a clear significant improvement with minocycline. Moreover, if the data from patients who succumbed within 24 h of hospitalization/enrollment were excluded, then significantly better overall outcome was observed at 3 months in those receiving minocycline along with a trend toward lower cumulative mortality [306]. The second trial was conducted in the Baba Raghav Das Medical College, also in the Indian state of Uttar Pradesh, and on a smaller population with 44 patients enrolled. However, this study specifically included serologically confirmed cases of JE. The dosage of minocycline used for this study was 5–6 mg/kg in two divided doses administered for 10 days through feeding tube, which was started from the day of hospitalization/enrollment. Minocycline was found to be effective in reducing duration of symptoms like fever and unconsciousness and mean duration of hospitalization. However, owing to the small sample size and availability of advanced life support and early referral facility of patients from remote areas, decreased mortality and increased full clinical recovery observed in drug-treated group could not be statistically correlated with treatment alone [307]. When data from the trials are taken together, minocycline does seem to impart significant beneficial effect in clinical cases of JE. At this point, studies with larger population and probably with other dosages are needed to optimize the application of this drug as a standard anti-JE therapeutic option.

Another promising therapeutic approach involves the administration of intravenous immunoglobulin (IVIg) to patients suffering from JE [308, 309]. It is believed

that a large part of the population living in the endemic zone is exposed to the virus at some point in life and has suffered mild subclinical-grade infection. Thus IVIg purified from pooled plasma of healthy donors from JE-endemic zones would theoretically have high titers of specific JEV-neutralizing antibodies [177, 310]. In fact, IVIg, which is not hyperimmune to JEV, has already been reported to impart therapeutic benefits in the recovery from JE [311]. Conceptually, this practice of IVIg administration is not novel as this has already been adopted for West Nile virus infection with some success [312, 313]. A small group of children in Nepal (11 per group, aged between 1 and14 years) manifesting symptoms of AES was recruited for the study and randomly divided into IVIg and placebo groups. The AES-affected patients received either IVIg, intravenously, at a dose of 400 mg/kg/day for 5 days or an equivalent volume of 0.9% normal saline. Initial infusion rate was kept at 0.01–0.02 ml/kg body weight/minute and if well tolerated was gradually increased over 30–60 min to a maximum rate of 0.08 ml/kg body weight/minute. At hospital discharge, most of the patients belonging to either group demonstrated major sequelae; at 3–6 months follow-up, 45% in IVIg group and 18% in placebo group exhibited complete recovery (no neurological sequelae). However, no significant difference was observed between the two groups when analyzed by intention-to-treat to determine the proportion of patients exhibiting complete recovery either at hospital discharge or at follow-up. JEV-neutralizing antibody titers were expectedly higher in patients who received IVIg compared to placebo. The other interesting aspect with IGIV is their ability to induce anti-inflammatory responses in the subjects non-specifically, usually by suppression of various pro-inflammatory mediators, including cytochemokines/chemokines, and metalloproteinases [314]. In this trial, the investigators report that the level of IL-4 was found to be significantly elevated in IVIg-treated patients. IL-4 is a complex cytokine that affects various regulatory pathways [315], and its higher levels have been detected in survivors of JE as compared to non-survivors [316].

6 Conclusion

Despite more than a centuries' effort in immunization of populace and vector control, JE still manages to rear its ugly head in the endemic zones that sometimes leads to epidemics. One explanation for this persistence of the disease despite the wide use of vaccines against JE is that childhood vaccination confers no herd immunity to adults or other susceptible populations because humans are not the primary hosts. This calls for further investigation into the lifelong or long-term efficacy of the current vaccines available for immunization against JEV. The role of mass immunization can be directly correlated with the significant decrease in the number of reported cases of JE in every country over the last century. However, there are many countries where vaccination against JE is not part of the national mandatory immunization regime. This probably benefits the virus by aiding it to remain in circulation. Compounding the problem is the lack of viable therapeutic

options to treat JE. The current supportive therapeutic regime is targeted to stabilize the patient rather to counter the virus itself which in many cases is devastating for the survivors. Long-lasting or permanent neurological deficits add to the human cost of the disease. As JE belongs to the group of diseases that are commonly clubbed as neglected tropical diseases, encouraging pharmaceutical and biotechnology companies to invest in developing much-needed treatments remains a challenge due to a lack of commercial incentive [317]. Thus to fill the void academic, researches need to step up that in turn needs to be encouraged and supported by both public and private sectors.

Moreover, being a zoonotic virus, JEV can persist in nonhuman vertebrate hosts, and thus a surveillance mechanism of such animals needs to be instituted in every country under the endemic zone. This also calls for better cooperation between medical and health facilities in every affected country and international health organization to combat this disease. Even in countries where JE does not pose a significant health risk yet or from where there are no new reports available, the threat of emergence or reemergence remains. The recent reemergence and spread of Zika virus [4] and the associated human costs should serve as a reminder regarding other neglected tropical diseases such as JE.

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References

1. Gan VC, Leo YS. Current epidemiology and clinical practice in arboviral infections—implications on blood supply in South-East Asia. ISBT Sci Ser. 2014;9:262–7.
2. Miyake M. The pathology of Japanese encephalitis. A review. Bull World Health Organ. 1964;30:153–60.
3. Hayashi M. Ubertragung des virus von encephalitis epidemica auf Affen. Proc Imp Acad Tokyo. 1934;10:41–4.
4. Vasilakis N, Weaver SC. Flavivirus transmission focusing on Zika. Curr Opin Virol. 2016;22:30–5.
5. Sumiyoshi H, Mori C, Fuke I, Morita K, Kuhara S, Kondou J, Kikuchi Y, Nagamatu H, Igarashi A. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. Virology. 1987;161:497–510.
6. Mori Y, Okabayashi T, Yamashita T, Zhao Z, Wakita T, Yasui K, Hasebe F, Tadano M, Konishi E, Moriishi K, et al. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. J Virol. 2005;79:3448–58.
7. Katoh H, Okamoto T, Fukuhara T, Kambara H, Morita E, Mori Y, Kamitani W, Matsuura Y. Japanese encephalitis virus core protein inhibits stress granule formation through an interaction with Caprin-1 and facilitates viral propagation. J Virol. 2013;87:489–502.
8. Lee HJ, Min KI, Lee J, Kang SH, Jeon W, Nam JH, YR J, Kim YB. The prM-independent packaging of pseudotyped Japanese encephalitis virus. Virol J. 2009;6:115.
9. Unni SK, Růžek D, Chhatbar C, Mishra R, Johri MK, Singh SK. Japanese encephalitis virus: from genome to infectome. Microbes Infect. 2011;13:312–21.

10. Luca VC, AbiMansour J, Nelson CA, Fremont DH. Crystal structure of the Japanese encephalitis virus envelope protein. J Virol. 2012;86:2337–46.
11. Melian EB, Hinzman E, Nagasaki T, Firth AE, Wills NM, Nouwens AS, Blitvich BJ, Leung J, Funk A, Atkins JF, et al. NS1' of flaviviruses in the Japanese encephalitis virus serogroup is a product of ribosomal frameshifting and plays a role in viral neuroinvasiveness. J Virol. 2010;84:1641–7.
12. Leung JY, Pijlman GP, Kondratieva N, Hyde J, Mackenzie JM, Khromykh AA. Role of nonstructural protein NS2A in flavivirus assembly. J Virol. 2008;82:4731–41.
13. Jan LR, Yang CS, Trent DW, Falgout B, Lai CJ. Processing of Japanese encephalitis virus non-structural proteins: NS2B-NS3 complex and heterologous proteases. J Gen Virol. 1995;76(Pt 3):573–80.
14. Ye J, Chen Z, Zhang B, Miao H, Zohaib A, Xu Q, Chen H, Cao S. Heat shock protein 70 is associated with replicase complex of Japanese encephalitis virus and positively regulates viral genome replication. PLoS One. 2013;8:e75188.
15. Kao YT, Chang BL, Liang JJ, Tsai HJ, Lee YL, Lin RJ, Lin YL. Japanese encephalitis virus nonstructural protein NS5 interacts with mitochondrial trifunctional protein and impairs fatty acid beta-oxidation. PLoS Pathog. 2015;11:e1004750.
16. Li XD, Ye HQ, Deng CL, Liu SQ, Zhang HL, Shang BD, Shi PY, Yuan ZM, Zhang B. Genetic interaction between NS4A and NS4B for replication of Japanese encephalitis virus. J Gen Virol. 2015;96:1264–75.
17. Lin CW, Cheng CW, Yang TC, Li SW, Cheng MH, Wan L, Lin YJ, Lai CH, Lin WY, Kao MC. Interferon antagonist function of Japanese encephalitis virus NS4A and its interaction with DEAD-box RNA helicase DDX42. Virus Res. 2008;137:49–55.
18. Dutta K, Nazmi A, Basu A. Japanese encephalitis virus and human CNS infection. In: Singh SK, Ruzek D, editors. Neuroviral infections: RNA viruses and retroviruses. London: CRC Press; 2013. p. 193–210.
19. Tyler KL. Emerging viral infections of the central nervous system: part 1. Arch Neurol. 2009;66:939–48.
20. Burke DS, Nisalak A, Ussery MA, Laorakpongse T, Chantavibul S. Kinetics of IgM and IgG responses to Japanese encephalitis virus in human serum and cerebrospinal fluid. J Infect Dis. 1985;151:1093–9.
21. Solomon T, Thao LT, Dung NM, Kneen R, Hung NT, Nisalak A, Vaughn DW, Farrar J, Hien TT, White NJ, et al. Rapid diagnosis of Japanese encephalitis by using an immunoglobulin M dot enzyme immunoassay. J Clin Microbiol. 1998;36:2030–4.
22. Buescher EL, Scherer WF. Ecologic studies of Japanese encephalitis virus in Japan. IX. Epidemiologic correlations and conclusions. Am J Trop Med Hyg. 1959;8:719–22.
23. Buescher EL, Scherer WF, Mc CH, Moyer JT, Rosenberg MZ, Yoshii M, Okada Y. Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. Am J Trop Med Hyg. 1959;8:678–88.
24. Buescher EL, Scherer WF, Rosenberg MZ, Gresser I, Hardy JL, Bullock HR. Ecologic studies of Japanese encephalitis virus in Japan. II. Mosquito infection. Am J Trop Med Hyg. 1959;8:651–64.
25. Scherer WF, Buescher EL, Mc CH. Ecologic studies of Japanese encephalitis virus in Japan. V. Avian factors. Am J Trop Med Hyg. 1959;8:689–97.
26. Scherer WF, Buescher EL, Southam CM, Flemings MB, Noguchi A. Ecologic studies of Japanese encephalitis virus in Japan. VIII. Survey for infection of wild rodents. Am J Trop Med Hyg. 1959;8:716–8.
27. Scherer WF, Kitaoka M, Okuno T, Ogata T. Ecologic studies of Japanese encephalitis virus in Japan. VII. Human infection. Am J Trop Med Hyg. 1959;8:707–15.
28. Scherer WF, Moyer JT, Izumi T, Gresser I, Mc CJ. Ecologic studies of Japanese encephalitis virus in JapanVI. Swine infection. Am J Trop Med Hyg. 1959;8:698–706.
29. Muangman D, Edelman R, Sullivan MJ, Gould DJ. Experimental transmission of Japanese encephalitis virus by Culex fuscocephala. Am J Trop Med Hyg. 1972;21:482–6.

30. Reid M, Mackenzie D, Baron A, Lehmann N, Lowry K, Aaskov J, Guirakhoo F, Monath TP. Experimental infection of Culex annulirostris, Culex gelidus, and Aedes vigilax with a yellow fever/Japanese encephalitis virus vaccine chimera (ChimeriVax-JE). Am J Trop Med Hyg. 2006;75:659–63.
31. Rosen L. The natural history of Japanese encephalitis virus. Annu Rev Microbiol. 1986;40:395–414.
32. Trosper JH, Ksiazek TG, Cross JH. Isolation of Japanese encephalitis virus from the Republic of the Philippines. Trans R Soc Trop Med Hyg. 1980;74:292–5.
33. Vythilingam I, Oda K, Tsuchie H, Mahadevan S, Vijayamalar B. Isolation of Japanese encephalitis virus from Culex sitiens mosquitoes in Selangor, Malaysia. J Am Mosq Control Assoc. 1994;10:228–9.
34. Wu CJ, Wu SY. Isolation of virus of B type encephalitis from Lasiohelea taiwana Shiraki-a blood sucking midge. Acta Microbial Sin. 1957;5:22–6.
35. Lvov DK. The role of ixodid ticks in the reservation and transmission of arboviruses in the USSR. In: Wilde TKH, editor. Tick-borne diseases and their vectors. Tonbridge, UK: Lewis Reprints; 1978. p. 482–6.
36. Takahashi M, Suzuki K. Japanese encephalitis virus in mosquito salivary glands. Am J Trop Med Hyg. 1979;28:122–35.
37. Zeidner NS, Higgs S, Happ CM, Beaty BJ, Miller BR. Mosquito feeding modulates Th1 and Th2 cytokines in flavivirus susceptible mice: an effect mimicked by injection of sialokinins, but not demonstrated in flavivirus resistant mice. Parasite Immunol. 1999;21:35–44.
38. Innis BL. Japanese encephalitis. In: Porterfield JS, editor. Kass handbook of infectious diseases: exotic viral infections. London: Chapman and Hall Medical; 1995. p. 147–74.
39. Lacey LA, Lacey CM. The medical importance of riceland mosquitoes and their control using alternatives to chemical insecticides. J Am Mosq Control Assoc Suppl. 1990;2:1–93.
40. Smith CE. Studies on arbovirus epidemiology associated with established and developing rice culture. Introduction. Trans R Soc Trop Med Hyg. 1970;64:481–2.
41. Hancock J, Kushlan J. The herons handbook. New York, NY: Harper and Row; 1984.
42. Hasegawa T, Takehara Y, Takahashi K. Natural and experimental infections of Japanese tree sparrows with Japanese encephalitis virus. Arch Virol. 1975;49:373–6.
43. Cui J, Counor D, Shen D, Sun G, He H, Deubel V, Zhang S. Detection of Japanese encephalitis virus antibodies in bats in Southern China. Am J Trop Med Hyg. 2008;78:1007–11.
44. Jiang L, Chen S, Zheng X, Ma S, Zhou J, Zhang Q, Li X, Xiong Y, Zhong X, Wang Z, et al. Detection of serum antibodies against Japanese encephalitis virus in bats in Hainan and Guangdong Provinces of China. Nan Fang Yi Ke Da Xue Xue Bao. 2015;35:720–3.
45. Liu S, Li X, Chen Z, Chen Y, Zhang Q, Liao Y, Zhou J, Ke X, Ma L, Xiao J, et al. Comparison of genomic and amino acid sequences of eight Japanese encephalitis virus isolates from bats. Arch Virol. 2013;158:2543–52.
46. Miura T, Kitaoka M. Viruses isolated from bats in Japan. Arch Virol. 1977;53:281–6.
47. van den Hurk AF, Smith CS, Field HE, Smith IL, Northill JA, Taylor CT, Jansen CC, Smith GA, Mackenzie JS. Transmission of Japanese Encephalitis virus from the black flying fox, Pteropus alecto, to Culex annulirostris mosquitoes, despite the absence of detectable viremia. Am J Trop Med Hyg. 2009;81:457–62.
48. Chew-Lim M, Ng CY. Recurrent viruses in a Singapore intensive pig farming estate. Ann Acad Med Singapore. 1987;16:651–4.
49. Dhakal S, Joshi DD, Ale A, Sharma M, Dahal M, Shah Y, Pant DK, Stephen C. Regional variation in pig farmer awareness and actions regarding Japanese encephalitis in Nepal: implications for public health education. PLoS One. 2014;9:e85399.
50. Holt HR, Inthavong P, Khamlome B, Blaszak K, Keokamphe C, Somoulay V, Phongmany A, Durr PA, Graham K, Allen J, et al. Endemicity of zoonotic diseases in pigs and humans in lowland and upland Lao PDR: identification of socio-cultural risk factors. PLoS Negl Trop Dis. 2016;e0003913:10.

51. Okello AL, Burniston S, Conlan JV, Inthavong P, Khamlome B, Welburn SC, Gilbert J, Allen J, Blacksell SD. Prevalence of endemic pig-associated zoonoses in Southeast Asia: a review of findings from the Lao people's democratic republic. Am J Trop Med Hyg. 2015;92:1059–66.
52. Riedel S, Schiborra A, Huelsebusch C, Huanming M, Schlecht E. Opportunities and challenges for smallholder pig production systems in a mountainous region of Xishuangbanna, Yunnan Province, China. Trop Anim Health Prod. 2012;44:1971–80.
53. Sasaki O, Karoji Y, Kuroda A, Karaki T, Takenokuma K, Maeda O. Protection of pigs against mosquito-borne Japanese encephalitis virus by immunization with a live attenuated vaccine. Antivir Res. 1982;2:355–60.
54. Ricklin ME, Garcìa-Nicolàs O, Brechbühl D, Python S, Zumkehr B, Posthaus H, Oevermann A, Summerfield A. Japanese encephalitis virus tropism in experimentally infected pigs. Vet Res. 2016;47:34.
55. Ricklin ME, Garcia-Nicolas O, Brechbuhl D, Python S, Zumkehr B, Nougairede A, Charrel RN, Posthaus H, Oevermann A, Summerfield A. Vector-free transmission and persistence of Japanese encephalitis virus in pigs. Nat Commun. 2016;7:10832.
56. Tobias EE, Svenja W, Jennifer K, Jürg U, Karin W. Past, present, and future of Japanese encephalitis. Emerg Infect Dis J. 2009;15(1):1333.
57. Ghosh D, Basu A. Japanese encephalitis-a pathological and clinical perspective. PLoS Negl Trop Dis. 2009;3:e437.
58. Gulati BR, Singha H, Singh BK, Virmani N, Kumar S, Singh RK. Isolation and genetic characterization of Japanese encephalitis virus from equines in India. J Vet Sci. 2012;13:111–8.
59. Kako N, Suzuki S, Sugie N, Kato T, Yanase T, Yamakawa M, Shirafuji H. Japanese encephalitis in a 114-month-old cow: pathological investigation of the affected cow and genetic characterization of Japanese encephalitis virus isolate. BMC Vet Res. 2014;10:63.
60. Rajendran R, Thenmozhi V, Tewari SC, Balasubramanian A, Ayanar K, Manavalan R, Gajanana A, Kabilan L, Thakare JP, Satyanarayana K. Longitudinal studies in South Indian villages on Japanese encephalitis virus infection in mosquitoes and seroconversion in goats. Trop Med Int Health. 2003;8:174–81.
61. Shimoda H, Ohno Y, Mochizuki M, Iwata H, Okuda M, Maeda K. Dogs as sentinels for human infection with Japanese encephalitis virus. Emerg Infect Dis. 2010;16:1137–9.
62. Yang DK, Kweon CH, Kim BH, Hwang IJ, Kang MI, So BJ, Cho KO. The seroprevalence of Japanese encephalitis virus in goats raised in Korea. J Vet Sci. 2007;8:197–9.
63. Ohno Y, Sato H, Suzuki K, Yokoyama M, Uni S, Shibasaki T, Sashika M, Inokuma H, Kai K, Maeda K. Detection of antibodies against Japanese encephalitis virus in raccoons, raccoon dogs and wild boars in Japan. J Vet Med Sci. 2009;71:1035–9.
64. Campbell GL, Hills SL, Fischer M, Jacobson JA, Hoke CH, Hombach JM, Marfin AA, Solomon T, Tsai TF, Tsu VD, et al. Estimated global incidence of Japanese encephalitis: a systematic review. Bull World Health Org. 2011;89:766–74. 774A–774E
65. Wang H, Liang G. Epidemiology of Japanese encephalitis: past, present, and future prospects. Ther Clin Risk Manag. 2015;11:435–48.
66. Solomon T. Recent advances in Japanese encephalitis. J Neurovirol. 2003;9:274–83.
67. LaBeaud A, Bashir F, King CH. Measuring the burden of arboviral diseases: the spectrum of morbidity and mortality from four prevalent infections. Popul Health Metrics. 2011;9(1):1.
68. Hanna JN, Ritchie SA, Phillips DA, Shield J, Bailey MC, Mackenzie JS, Poidinger M, McCall BJ, Mills PJ. An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. Med J Aust. 1996;165:256–60.
69. Partridge J, Ghimire P, Sedai T, Bista MB, Banerjee M. Endemic Japanese encephalitis in the Kathmandu valley, Nepal. Am J Trop Med Hyg. 2007;77:1146–9.
70. Adhya D, Dutta K, Basu A. Japanese encephalitis in India: risk of an epidemic in the National Capital region. Int Health. 2013;5:166–8.

71. De Alwis KN, Abeysinghe MR, Wickramesinghe AR, Wijesinghe PR. A cohort event monitoring to determine the adverse events following administration of mouse brain derived, inactivated Japanese Encephalitis vaccine in an endemic district in Sri Lanka. Vaccine. 2014;32:924–30.
72. Wijesinghe PR. 2012. Japanese encephalitis: a manual for medical officers of Health. Epidemiology unit, Ministry of Health care and Nutrition, Government of Sri Lanka. 1:15.
73. Peiris JS, Amerasinghe FP, Amerasinghe PH, Ratnayake CB, Karunaratne SH, Tsai TF. Japanese encephalitis in Sri Lanka—the study of an epidemic: vector incrimination, porcine infection and human disease. Trans R Soc Trop Med Hyg. 1992;86:307–13.
74. Peiris JS, Amerasinghe FP, Arunagiri CK, Perera LP, Karunaratne SH, Ratnayake CB, Kulatilaka TA, Abeysinghe MR. Japanese encephalitis in Sri Lanka: comparison of vector and virus ecology in different agro-climatic areas. Trans R Soc Trop Med Hyg. 1993;87:541–8.
75. Peiris JS, Amerasinghe PH, Amerasinghe FP, Calisher CH, Perera LP, Arunagiri CK, Munasingha NB, Karunaratne SH. Viruses isolated from mosquitoes collected in Sri Lanka. Am J Trop Med Hyg. 1994;51:154–61.
76. Yasuoka J, Levins R. Ecology of vector mosquitoes in Sri Lanka—suggestions for future mosquito control in rice ecosystems. Southeast Asian J Trop Med Public Health. 2007;38:646–57.
77. Karunaratne SH, Vaughan A, Paton MG, Hemingway J. Amplification of a serine esterase gene is involved in insecticide resistance in Sri Lankan Culex tritaeniorhynchus. Insect Mol Biol. 1998;7:307–15.
78. Sanchayan K, Fernandopulle R, Amarasinghe A, Thiyahiny SN, Sri Ranganathan S. Safety of live attenuated Japanese encephalitis vaccine given at the age of 9 months in National Immunisation Programme of Sri Lanka. Ceylon Med J. 2016;61:99–105.
79. Wijesinghe PR, Abeysinghe MR, Yoksan S, Yao Y, Zhou B, Zhang L, Fleming JA, Marfin AA, Victor JC. Immunogenicity of live attenuated Japanese encephalitis SA 14-14-2 vaccine among Sri Lankan children with previous receipt of inactivated JE vaccine. Vaccine. 2016;34:5923–8.
80. Wijesinghe PR, Abeysinghe MR, Yoksan S, Yao Y, Zhou B, Zhang L, Yaich M, Neuzil KM, Victor JC. Safety and immunogenicity of live-attenuated Japanese encephalitis SA 14-14-2 vaccine co-administered with measles vaccine in 9-month-old infants in Sri Lanka. Vaccine. 2014;32:4751–7.
81. Joshi D. Incidence of Japanese encephalitis in children 1978, 1979, and 1980 outbreaks. NEPAS J. 1983;2:18–25.
82. Bista MB, Shrestha JM. Epidemiological situation of Japanese encephalitis in Nepal. JNMA J Nepal Med Assoc. 2005;44:51–6.
83. Wierzba TF, Ghimire P, Malla S, Banerjee MK, Shrestha S, Khanal B, Sedai TR, Gibbons RV. Laboratory-based Japanese encephalitis surveillance in Nepal and the implications for a national immunization strategy. Am J Trop Med Hyg. 2008;78:1002–6.
84. Anuj B, Sumi A, Tika Ram S, Shyam Raj U, Jeffrey P. Japanese encephalitis in hill and mountain districts, Nepal. Emerg Infect Dis J. 2009;15:1691.
85. Zimmerman MD, Scott RM, Vaughn DW, Rajbhandari S, Nisalak A, Shrestha MP. Short report: an outbreak of Japanese encephalitis in Kathmandu, Nepal. Am J Trop Med Hyg. 1997;57:283–4.
86. Ghimire S, Dhakal S. Japanese encephalitis: challenges and intervention opportunities in Nepal. Vet World. 2015;8:61–5.
87. Dumre SP, Shakya G, Na-Bangchang K, Eursitthichai V, Rudi Grams H, Upreti SR, Ghimire P, Kc K, Nisalak A, Gibbons RV, et al. Dengue virus and Japanese encephalitis virus epidemiological shifts in Nepal: a case of opposing trends. Am J Trop Med Hyg. 2013;88:677–80.
88. Upreti SR, Janusz KB, Schluter WW, Bichha RP, Shakya G, Biggerstaff BJ, Shrestha MM, Sedai TR, Fischer M, Gibbons RV, et al. Estimation of the impact of a Japanese encephalitis

immunization program with live, attenuated SA 14-14-2 vaccine in Nepal. Am J Trop Med Hyg. 2013;88:464–8.
89. Dhakal S, Stephen C, Ale A, Joshi DD. Knowledge and practices of pig farmers regarding Japanese encephalitis in Kathmandu, Nepal. Zoonoses Public Health. 2012;59:568–74.
90. Griffiths MJ, Lemon JV, Rayamajhi A, Poudel P, Shrestha P, Srivastav V, Kneen R, Medina-Lara A, Singh RR, Solomon T. The functional, social and economic impact of acute encephalitis syndrome in Nepal—a longitudinal follow-up study. PLoS Negl Trop Dis. 2013;7:e2383.
91. Takasu T, Ahmed A, Kono R, Kondo K, Aoyama Y, Sugamata M, Chida K, Kame S. Neurovirological research of encephalitides in and around Karachi. The clinical aspects (interim report). J Pak Med Assoc. 1986;36:159–69.
92. Darwish MA, Hoogstraal H, Roberts TJ, Ahmed IP, Omar F. A sero-epidemiological survey for certain arboviruses (Togaviridae) in Pakistan. Trans R Soc Trop Med Hyg. 1983;77:442–5.
93. Igarashi A, Tanaka M, Morita K, Takasu T, Ahmed A, Akram DS, Waqar MA. Detection of West Nile and Japanese encephalitis viral genome sequences in cerebrospinal fluid from acute encephalitis cases in Karachi, Pakistan. Microbiol Immunol. 1994;38:827–30.
94. Khan E, Farooqi JQ, Barr KL, Prakoso D, Nasir A, Kanji A, Shakoor S, Malik FR, Hasan R, Lednicky JA, et al. Flaviviruses as a cause of undifferentiated fever in Sindh Province, Pakistan: a preliminary report. Front Public Health. 2016;4:8.
95. Ashfaq M, Hebert PD, Mirza JH, Khan AM, Zafar Y, Mirza MS. Analyzing mosquito (Diptera: culicidae) diversity in Pakistan by DNA barcoding. PLoS One. 2014;9:e97268.
96. Kamimura K, Takasu T, Ahmed A. A survey of mosquitoes in Karachi area, Pakistan. J Pak Med Assoc. 1986;36:182–8.
97. Nieto NC, Khan K, Uhllah G, Teglas MB. The emergence and maintenance of vector-borne diseases in the khyber pakhtunkhwa province, and the federally administered tribal areas of Pakistan. Front Physiol. 2012;3:250.
98. Lewis L, Taylor HG, et al. Japanese B encephalitis clinical observations in an outbreak on Okinawa Shima. Arch Neurol Psychiatr. 1947;57:430–63.
99. Manitoff A. Japanese B encephalitis; current epidemic in Tokyo. Surg Circ Lett US Army Far East Command Med Sect. 1948;3:7–9.
100. Takeshita N, Lim CK, Mizuno Y, Shimbo T, Kotaki A, Ujiie M, Hayakawa K, Kato Y, Kanagawa S, Kaku M, et al. Immunogenicity of single-dose Vero cell-derived Japanese encephalitis vaccine in Japanese adults. J Infect Chemother. 2014;20:238–42.
101. Saito M, Sunagawa T, Makino Y, Tadano M, Hasegawa H, Kanemura K, Zamami Y, Killenbeck BJ, Fukunaga T. Three Japanese encephalitis cases in Okinawa, Japan, 1991. Southeast Asian J Trop Med Public Health. 1999;30:277–9.
102. Matsunaga Y, Yabe S, Taniguchi K, Nakayama M, Kurane I. Current status of Japanese encephalitis in Japan. Kansenshogaku Zasshi. 1999;73:97–103.
103. Matsunaga T, Shoda M, Konishi E. Japanese encephalitis viral infection remains common in Japan. Pediatr Infect Dis J. 2008;27:769–70.
104. Tang WF, Ogawa M, Eshita Y, Aono H, Makino Y. Molecular evolution of Japanese encephalitis virus isolates from swine in Oita, Japan during 1980-2009. Infect Genet Evol. 2010;10:329–36.
105. Arai S, Matsunaga Y, Takasaki T, Tanaka-Taya K, Taniguchi K, Okabe N, Kurane I. Japanese encephalitis: surveillance and elimination effort in Japan from 1982 to 2004. Jpn J Infect Dis. 2008;61:333–8.
106. Nidaira M, Kyan H, Taira K, Okano S, Oshiro T, Kato T, Kudo N, Azama Y, Mahoe Y, Kudaka J, et al. Survey of Japanese encephalitis virus in pigs and wild boars on Ishigaki and Iriomote Islands in Okinawa, Japan. Epidemiol Infect. 2014;142:856–60.
107. Konishi E, Shoda M, Kondo T. Analysis of yearly changes in levels of antibodies to Japanese encephalitis virus nonstructural 1 protein in racehorses in central Japan shows high levels of natural virus activity still exist. Vaccine. 2006;24:516–24.

108. Murakami M, Hori K, Kitagawa Y, Oikawa Y, Kamimura K, Takagemi T. An ecological survey of mosquitoes and the distribution of Japanese encephalitis virus in Ishikawa Prefecture, Japan between 2010 and 2014. Jpn J Infect Dis. 2017;70(4):362–7.
109. Chhour YM, Ruble G, Hong R, Minn K, Kdan Y, Sok T, Nisalak A, Myint KS, Vaughn DW, Endy TP. Hospital-based diagnosis of hemorrhagic fever, encephalitis, and hepatitis in Cambodian children. Emerg Infect Dis. 2002;8:485–9.
110. Srey VH, Sadones H, Ong S, Mam M, Yim C, Sor S, Grosjean P, Reynes JM. Etiology of encephalitis syndrome among hospitalized children and adults in Takeo, Cambodia, 1999-2000. Am J Trop Med Hyg. 2002;66:200–7.
111. Touch S, Hills S, Sokhal B, Samnang C, Sovann L, Khieu V, Soeung SC, Toda K, Robinson J, Grundy J. Epidemiology and burden of disease from Japanese encephalitis in Cambodia: results from two years of sentinel surveillance. Trop Med Int Health. 2009;14:1365–73.
112. Hills SL, Van Cuong N, Touch S, Mai HH, Soeung SC, Lien TT, Samnang C, Sovann L, Van Diu P, Lac LD, et al. Disability from Japanese encephalitis in Cambodia and Viet Nam. J Trop Pediatr. 2011;57:241–4.
113. Tarantola A, Goutard F, Newton P, de Lamballerie X, Lortholary O, Cappelle J, Buchy P. Estimating the burden of Japanese encephalitis virus and other encephalitides in countries of the Mekong region. PLoS Negl Trop Dis. 2014;8:e2533.
114. Cappelle J, Duong V, Pring L, Kong L, Yakovleff M, Prasetyo DB, Peng B, Choeung R, Duboz R, Ong S, et al. Intensive circulation of Japanese Encephalitis virus in peri-urban Sentinel Pigs near Phnom Penh, Cambodia. PLoS Negl Trop Dis. 2016;10:e0005149.
115. Duong V, Sorn S, Holl D, Rani M, Deubel V, Buchy P. Evidence of Japanese encephalitis virus infections in swine populations in 8 provinces of Cambodia: implications for national Japanese encephalitis vaccination policy. Acta Trop. 2011;120:146–50.
116. Touch S, Suraratdecha C, Samnang C, Heng S, Gazley L, Huch C, Sovann L, Chhay CS, Soeung SC. A cost-effectiveness analysis of Japanese encephalitis vaccine in Cambodia. Vaccine. 2010;28:4593–9.
117. Sucharit S, Surathin K, Shrestha SR. Vectors of Japanese encephalitis virus (JEV): species complexes of the vectors. Southeast Asian J Trop Med Public Health. 1989;20:611–21.
118. Thongsripong P, Green A, Kittayapong P, Kapan D, Wilcox B, Bennett S. Mosquito vector diversity across habitats in central Thailand endemic for dengue and other arthropod-borne diseases. PLoS Negl Trop Dis. 2013;7:e2507.
119. Robin Y, Yenbutra D, Dasaneyavaja A. A method of isolating viruses from vector mosquitoes. Isolation of the 1st strain of Japanese B encephalitis virus in Thailand. Med Trop (Mars). 1963;23:781–7.
120. Yamada T, Rojanasuphot S, Takagi M, Wungkobkiat S, Hirota T. Studies on an epidemic of Japanese encephalitis in the northern region of Thailand in 1969 and 1970. Biken J. 1971;14:267–96.
121. Chunsuttiwat S. Japanese encephalitis in Thailand. Southeast Asian J Trop Med Public Health. 1989;20:593–7.
122. Thongcharoen P. Japanese encephalitis in Thailand. J Med Assoc Thailand. 1985;68:534–45.
123. Fukunaga T, Igarashi A, Okuno Y, Ishimine T, Tadano M, Okamoto Y, Fukai K. A seroepidemiological study of Japanese encephalitis and dengue virus infections in the Chiang Mai area, Thailand. Biken J. 1984;27:9–17.
124. Mwandawiro C, Boots M, Tuno N, Suwonkerd W, Tsuda Y, Takagi M. Heterogeneity in the host preference of Japanese encephalitis vectors in Chiang Mai, northern Thailand. Trans R Soc Trop Med Hyg. 2000;94:238–42.
125. Okuno T. An epidemiological review of Japanese encephalitis. World Health Stat Q. 1978;31:120–33.
126. Poneprasert B. Japanese encephalitis in children in northern Thailand. Southeast Asian J Trop Med Public Health. 1989;20:599–603.
127. Wiwanitkit V. Japanese encephalitis and rainfall in Thailand: a study on clinic-geographical correlation. Ann Trop Med Public Health. 2012;5:45–6.

128. Fukunaga T, Rojanasuphot S, Wungkorbkiat S, Thammanichanon A, Kan T. Japanese encephalitis vaccination in Thailand. Biken J. 1974;17:21–31.
129. Pongpairoj S, Choakouychai B, Boonrueng C, Kutirakan P, Ahandrik S, Leelasiri K, Phanthumachinda B. A test production of inactivated mouse brain JE vaccine in Thailand. Southeast Asian J Trop Med Public Health. 1989;20:647–52.
130. Olsen SJ, Supawat K, Campbell AP, Anantapreecha S, Liamsuwan S, Tunlayadechanont S, Visudtibhan A, Lupthikulthum S, Dhiravibulya K, Viriyavejakul A, et al. Japanese encephalitis virus remains an important cause of encephalitis in Thailand. Int J Infect Dis. 2010;14: e888–92.
131. Olsen SJ, Campbell AP, Supawat K, Liamsuwan S, Chotpitayasunondh T, Laptikulthum S, Viriyavejakul A, Tantirittisak T, Tunlayadechanont S, Visudtibhan A, et al. Infectious causes of encephalitis and meningoencephalitis in Thailand, 2003-2005. Emerg Infect Dis. 2015;21:280–9.
132. Makino Y, Saito M, Phommasack B, Vongxay P, Kanemura K, Pothawan T, Bounsou, Insisiengmay S, Sompaw, Fukunaga T. Arbovirus infections in pilot areas in Laos. Trop Med. 1995;36:131–9.
133. Conlan JV, Vongxay K, Jarman RG, Gibbons RV, Lunt RA, Fenwick S, Thompson RC, Blacksell SD. Serologic study of pig-associated viral zoonoses in Laos. Am J Trop Med Hyg. 2012;86:1077–84.
134. Vallee J, Dubot-Peres A, Ounaphom P, Sayavong C, Bryant JE, Gonzalez JP. Spatial distribution and risk factors of dengue and Japanese encephalitis virus infection in urban settings: the case of Vientiane, Lao PDR. Trop Med Int Health. 2009;14:1134–42.
135. Hiscox A, Winter CH, Vongphrachanh P, Sisouk T, Somoulay V, Phompida S, Kaul S, Sananikhom P, Nguyen TY, Paul RE, et al. Serological investigations of flavivirus prevalence in Khammouane Province, Lao People's Democratic Republic, 2007-2008. Am J Trop Med Hyg. 2010;83:1166–9.
136. Conlan JV, Vongxay K, Khamlome B, Jarman RG, Gibbons RV, Fenwick SG, Thompson RC, Blacksell SD. Patterns of Flavivirus seroprevalence in the human population of Northern Laos. Am J Trop Med Hyg. 2015;93:1010–3.
137. Kreidl P, De Mateo S, Rasch G, Desenclos JC, Hemmer R, Thornton L, Tielinen M, Tucker R. Japanese encephalitis in Malaysia and vaccines. Euro Surveill. 1999;3:1.
138. Easton A. Outbreak of Japanese encephalitis hits Malaysia. BMJ. 1999;318:893.
139. Impoinvil DE, Ooi MH, Diggle PJ, Caminade C, Cardosa MJ, Morse AP, Baylis M, Solomon T. The effect of vaccination coverage and climate on Japanese encephalitis in Sarawak, Malaysia. PLoS Negl Trop Dis. 2013;7:e2334.
140. Ooi MH, Lewthwaite P, Lai BF, Mohan A, Clear D, Lim L, Krishnan S, Preston T, Chieng CH, Tio PH, et al. The epidemiology, clinical features, and long-term prognosis of Japanese encephalitis in Central Sarawak, Malaysia, 1997-2005. Clin Infect Dis. 2008;47:458–68.
141. Swe T, Thein S, Myint MS. Pilot sero-epidemiological survey on Japanese encephalitis in North-Western Burma. Biken J. 1979;22:125–9.
142. Ming CK, Swe T, Thaung U, Lwin TT. Recent outbreaks of Japanese encephalitis in Burma. Southeast Asian J Trop Med Public Health. 1977;8:113–20.
143. Thein S, Aung H, Sebastian AA. Study of vector, amplifier, and human infection with Japanese encephalitis virus in a Rangoon community. Am J Epidemiol. 1988;128:1376–82.
144. Feng Y, Fu S, Zhang H, Li M, Zhou T, Wang J, Zhang Y, Wang H, Tang Q, Liang G. Distribution of mosquitoes and mosquito-borne viruses along the China-Myanmar border in Yunnan Province. Jpn J Infect Dis. 2012;65:215–21.
145. Wang J, Zhang H, Sun X, Fu S, Wang H, Feng Y, Tang Q, Liang GD. Distribution of mosquitoes and mosquito-borne arboviruses in Yunnan Province near the China-Myanmar-Laos border. Am J Trop Med Hyg. 2011;84:738–46.
146. Khan AM, Khan AQ, Dobrzynski L, Joshi GP, Myat A. A Japanese encephalitis focus in Bangladesh. J Trop Med Hyg. 1981;84:41–4.

147. Hossain MJ, Gurley ES, Montgomery S, Petersen L, Sejvar J, Fischer M, Panella A, Powers AM, Nahar N, Uddin AK, et al. Hospital-based surveillance for Japanese encephalitis at four sites in Bangladesh, 2003-2005. Am J Trop Med Hyg. 2010;82:344–9.
148. Lewthwaite P, Solomon T. Response to Hossain and others: hospital-based surveillance for Japanese encephalitis at four sites in Bangladesh, 2003-2005. Am J Trop Med Hyg. 2010;83:445. author reply 446
149. Zaman K, Naser AM, Power M, Yaich M, Zhang L, Ginsburg AS, Luby SP, Rahman M, Hills S, Bhardwaj M, et al. Lot-to-lot consistency of live attenuated SA 14-14-2 Japanese encephalitis vaccine manufactured in a good manufacturing practice facility and non-inferiority with respect to an earlier product. Vaccine. 2014;32:6061–6.
150. Li MH, Fu SH, Chen WX, Wang HY, Guo YH, Liu QY, Li YX, Luo HM, Da W, Duo Ji DZ, et al. Genotype v Japanese encephalitis virus is emerging. PLoS Negl Trop Dis. 2011; e1231:5.
151. Li YX, Li MH, Fu SH, Chen WX, Liu QY, Zhang HL, Da W, Hu SL, Mu SD, Bai J, et al. Japanese encephalitis, Tibet, China. Emerg Infect Dis. 2011;17:934–6.
152. Zheng Y, Li M, Wang H, Liang G. Japanese encephalitis and Japanese encephalitis virus in Mainland China. Rev Med Virol. 2012;22:301–22.
153. Wang Y, Ge J, Liang X. The surveillance of Japanese encephalitis in China. Chin J Med. 1985;65:648–52.
154. Gao X, Li X, Li M, Fu S, Wang H, Lu Z, Cao Y, He Y, Zhu W, Zhang T, et al. Vaccine strategies for the control and prevention of Japanese encephalitis in Mainland China, 1951-2011. PLoS Negl Trop Dis. 2014;e3015:8.
155. Wang H, Li Y, Liang X, Liang G. Japanese encephalitis in Mainland China. Jpn J Infect Dis. 2009;62:331–6.
156. Feng Y, Fu S, Zhang H, Petersen LR, Zhang B, Gao X, Yang W, Zhang Y, Dao B, Li K, et al. High incidence of Japanese encephalitis, Southern China. Emerg Infect Dis. 2013;19:672–3.
157. Hu Q, Chen B, Zhu Z, Tian J, Zhou Y, Zhang X, Zheng X. Recurrence of Japanese encephalitis epidemic in Wuhan, China, 2009-2010. PLoS One. 2013;8:e52687.
158. China N. China's National Climate Change Programme. People's Republic of China: National Development and Reform Commission; 2007.
159. Bai L, Morton LC, Liu Q. Climate change and mosquito-borne diseases in China: a review. Glob Health. 2013;9:10.
160. Bi P, Tong S, Donald K, Parton KA, Ni J. Climate variability and transmission of Japanese encephalitis in Eastern China. Vector Borne Zoonotic Dis. 2003;3:111–5.
161. Bi P, Zhang Y, Parton KA. Weather variables and Japanese encephalitis in the metropolitan area of Jinan city, China. J Infect. 2007;55:551–6.
162. Huo A-M, Zhao D-S, Fang L-Q, Cao W-C. Association between infectious diseases with natural foci and meteorological factors in North China. J Pathog Biol. 2011;1:003.
163. Lian X, Zhengzhi Y, Li R. Multiple stepwise regression analysis of relationship between disease occurred and meteorological factors. Guangming J Chin Med. 2009;6
164. Liu Z, Du A. Grey correlation analysis of meteorological factors and Japanese encephalitis incidences. J Math Med. 2008;22:64–6.
165. Baolin L. Environmental management for the control of rice-field breeding mosquitoes in China. Vector-borne disease control in humans through rice agroecosystem management: Proceedings. IRRI; 1988. p. 111–121.
166. Wu ZM, Chu HL, Wang G, Zhu XJ, Guo XX, Zhang YM, Xing D, Yan T, Zhao MH, Dong YD, et al. Multiple-insecticide resistance and classic gene mutations to Japanese Encephalitis Vector Culex tritaeniorhynchus from China. J Am Mosq Control Assoc. 2016;32:144–51.
167. Yuan L, Wu R, Liu H, Wen X, Huang X, Wen Y, Ma X, Yan Q, Huang Y, Zhao Q, et al. Tissue tropism and molecular characterization of a Japanese encephalitis virus strain isolated from pigs in Southwest China. Virus Res. 2016;215:55–64.

168. Teng M, Luo J, Fan JM, Chen L, Wang XT, Yao W, Wang CQ, Zhang GP. Molecular characterization of Japanese encephalitis viruses circulating in pigs and mosquitoes on pig farms in the Chinese province of Henan. Virus Genes. 2013;46:170–4.
169. Wang SP. Japanese encephalitis in Taiwan: a review of recent studies. Bull World Health Organ. 1964;30:279–84.
170. Wu YC, Huang YS, Chien LJ, Lin TL, Yueh YY, Tseng WL, Chang KJ, Wang GR. The epidemiology of Japanese encephalitis on Taiwan during 1966-1997. Am J Trop Med Hyg. 1999;61:78–84.
171. Hsu LC, Chen YJ, Hsu FK, Huang JH, Chang CM, Chou P, Lin IF, Chang FY. The incidence of Japanese encephalitis in Taiwan—a population-based study. PLoS Negl Trop Dis. 2014;8: e3030.
172. Su CL, Yang CF, Teng HJ, LC L, Lin C, Tsai KH, Chen YY, Chen LY, Chang SF, Shu PY. Molecular epidemiology of Japanese encephalitis virus in mosquitoes in Taiwan during 2005-2012. PLoS Negl Trop Dis. 2014;8:e3122.
173. Kabilan L. Control of Japanese encephalitis in India: a reality. Indian J Pediatr. 2004;71:707–12.
174. Smithburn KC, Kerr JA, Gatne PB. Neutralizing antibodies against certain viruses in the sera of residents of India. J Immunol. 1954;72:248–57.
175. Webb JKG, Pereira SM. Clinical diagnosis of an arthropod borne type virus encephalitis in children in North Arcot District, Madras state, India. Indian J Med Sci. 1956;10:573–80.
176. Carey DE, Myers RM, Pavri KM. Japanese encephalitis studies in Vellore, South India. II. Antibody response of patients. Indian J Med Res. 1968;56:1319–29.
177. Gajanana A, Thenmozhi V, Samuel PP, Reuben R. A community-based study of subclinical flavivirus infections in children in an area of Tamil Nadu, India, where Japanese encephalitis is endemic. Bull World Health Organ. 1995;73:237–44.
178. Carey DE, Myers RM, Webb JK, Reuben R. Japanese encephalitis in South India. A summary of recent knowledge. J Indian Med Assoc. 1969;52:10–5.
179. Chakraborty SK, Sarkar JK, Chakraborty MS, Mukherjee KK, Mitra AC, Mukherjee MK, Das BC, Chaudhuri SK, Sett GS, Chakraborty B. Outbreak of encephalitis in the coal mine area near Asansol—a preliminary note. Bull Calcutta Sch Trop Med. 1973;21:1–2.
180. Mukherjee MK, Sarkar JK, Chakraborty SK, Sen Gupta SK, Mukherjee K, Chakraborty MS, Chatterjee A, Das BC, Nandy K. Outbreak of encephalitis due to Japanese encephalitis in Bankura—a preliminary report. Bull Calcutta Sch Trop Med. 1973;21:2–3.
181. Rajagopalan PK, Panicker KN. A note on the 1976 epidemic of Japanese encephalitis in Burdwan district, West Bengal. Indian J Med Res. 1978;68:3938.
182. Dhillon GP, Raina VK. Epidemiology of Japanese encephalitis in context with Indian scenario. J Indian Med Assoc. 2008;106:660–3.
183. Khare J. Current status of Japanese encephalitis in Uttar Pradesh; Proceedings of the National Conference on Japanese encephalitis. New Delhi: ICMR, 1982; 22–4.
184. Tiwari S, Singh RK, Tiwari R, Dhole TN. Japanese encephalitis: a review of the Indian perspective. Braz J Infect Dis. 2012;16:564–73.
185. Kumari R, Joshi PL. A review of Japanese encephalitis in Uttar Pradesh, India. WHO South-East Asia J Public Health. 2012;1:374–95.
186. Parida M, Dash PK, Tripathi NK, Ambuj SS, Saxena P, Agarwal S, Sahni AK, Singh SP, Rathi AK, et al. Japanese encephalitis outbreak, India, 2005. Emerg Infect Dis. 2006;12:1427–30.
187. Chakraborty AK, Chakravarti SK, Chakravarty MS. Outbreak of Japanese encephalitis in two districts of Assam during 1980: some epidemiological features. Indian J Public Health. 1987;31:5–11.
188. Vajpayee A, Dey PN, Chakraborty AK, Chakraborty MS. Study of the outbreak of Japanese encephalitis in Lakhimpur district of Assam in 1989. J Indian Med Assoc. 1992;90:114–5.
189. Phukan AC, Borah PK, Mahanta J. Japanese encephalitis in Assam, Northeast India. South-east Asian J Trop Med Public Health. 2004;35:618–22.

190. Borthakur A, Das N, Bora B. Data from the World Health Organization (WHO) National Network Laboratory for Japanese Encephalitis. J Glob Infect Dis. 2013;5:76–9.
191. Patgiri SJ, Borthakur AK, Borkakoty B, Saikia L, Dutta R, Phukan SK. An appraisal of clinicopathological parameters in Japanese encephalitis and changing epidemiological trends in upper Assam, India. Indian J Pathol Microbiol. 2014;57:400–6.
192. Vajpayee A, Mukherjee MK, Chakraborty AK, Chakraborty MS. Investigation of an outbreak of Japanese encephalitis in Rourkela City (Orissa) during 1989. J Commun Dis. 1991;23:18–21.
193. Borah J, Dutta P, Khan SA, Mahanta J. Epidemiological concordance of Japanese encephalitis virus infection among mosquito vectors, amplifying hosts and humans in India. Epidemiol Infect. 2013;141:74–80.
194. Hotta S, Aoki H, Samoto S, Yasui T, Kawabe M. Virologic-epidemiological studies on Indonesia. II. Measurement of anti-arbovirus antibodies in sera from residents of Lombok, South Sumatera and West Java, in comparison with results concerning sera from residents of Japanese main islands. Kobe J Med Sci. 1970;16:215–34.
195. Hotta S, Aoki H, Samoto S, Yasui T, Noerjasin B. Virologic-epidemiological studies on Indonesia. 3. HI antibodies against selected arboviruses (groups A and B) in human and animal sera collected in Surabaja, East Java, in 1968. Kobe J Med Sci. 1970;16:235–50.
196. Mackenzie JS, Johansen CA, Ritchie SA, van den Hurk AF, Hall RA. Japanese encephalitis as an emerging virus: the emergence and spread of Japanese encephalitis virus in Australasia. Curr Top Microbiol Immunol. 2002;267:49–73.
197. Van Peenen PF, Irsiana R, Sulianti Saroso J, Joseph SW, Shope RE, Joseph PL. First isolation of Japanese encephalitis virus from Java. Mil Med. 1974;139:821–3.
198. Olson JG, Ksiazek TG, Lee VH, Tan R, Shope RE. Isolation of Japanese encephalitis virus from Anopheles annularis and Anopheles vagus in Lombok, Indonesia. Trans R Soc Trop Med Hyg. 1985;79:845–7.
199. Van Peenen PF, Joseph PL, Atmosoedjono S, Irsiana R, Saroso JS. Isolation of Japanese encephalitis virus from mosquitoes near Bogor, West Java, Indonesia. J Med Entomol. 1975;12:573–4.
200. Dirk Van Peenen PF, Joseph PL, Atmosoedjono S, Irsiana R, Saroso JS. Japanese encephalitis virus from pigs and mosquitoes in Jakarta, Indonesia. Trans R Soc Trop Med Hyg. 1975;69:477–9.
201. Olson JG, Ksiazek TG, Tan R, Atmosoedjono S, Lee VH, Converse JD. Correlation of population indices of female Culex tritaeniorhynchus with Japanese encephalitis viral activity in Kapuk, Indonesia. Southeast Asian J Trop Med Public Health. 1985;16:337–42.
202. Yamanaka A, Mulyatno KC, Susilowati H, Hendrianto E, Utsumi T, Amin M, Lusida MI, Soegijanto S, Konishi E. Prevalence of antibodies to Japanese encephalitis virus among pigs in Bali and East Java, Indonesia, 2008. Jpn J Infect Dis. 2010;63:58–60.
203. Kanamitsu M, Taniguchi K, Urasawa S, Ogata T, Wada Y, Saroso JS. Geographic distribution of arbovirus antibodies in indigenous human populations in the Indo-Australian archipelago. Am J Trop Med Hyg. 1979;28:351–63.
204. Olson JG, Ksiazek TG, Gubler DJ, Lubis SI, Simanjuntak G, Lee VH, Nalim S, Juslis K, See R. A survey for arboviral antibodies in sera of humans and animals in Lombok, Republic of Indonesia. Ann Trop Med Parasitol. 1983;77:131–7.
205. Spicer PE, Phillips D, Pike A, Johansen C, Melrose W, Hall RA. Antibodies to Japanese encephalitis virus in human sera collected from Irian Jaya. Follow-up of a previously reported case of Japanese encephalitis in that region. Trans R Soc Trop Med Hyg. 1999;93:511–4.
206. Widjaja S, Soekotjo W, Hartati S, Jennings GB, Corwin AL. Prevalence of hemagglutination-inhibition and neutralizing antibodies to arboviruses in horses of Java. Southeast Asian J Trop Med Public Health. 1995;26:109–13.
207. Ompusunggu S, Hills SL, Maha MS, Moniaga VA, Susilarini NK, Widjaya A, Sasmito A, Suwandono A, Sedyaningsih ER, Jacobson JA. Confirmation of Japanese encephalitis as an

endemic human disease through sentinel surveillance in Indonesia. Am J Trop Med Hyg. 2008;79:963–70.
208. Yoshida M, Igarashi A, Suwendra P, Inada K, Maha MS, Kari K, Suda H, Antonio MT, Arhana BN, Takikawa Y, et al. The first report on human cases serologically diagnosed as Japanese encephalitis in Indonesia. Southeast Asian J Trop Med Public Health. 1999;30:698–706.
209. Kari K, Liu W, Gautama K, Mammen MP Jr, Clemens JD, Nisalak A, Subrata K, Kim HK, Xu ZY. A hospital-based surveillance for Japanese encephalitis in Bali, Indonesia. BMC Med. 2006;4:8.
210. Liu W, Clemens JD, Kari K, Xu ZY. Cost-effectiveness of Japanese encephalitis (JE) immunization in Bali, Indonesia. Vaccine. 2008;26:4456–60.
211. Schuh AJ, Guzman H, Tesh RB, Barrett AD. Genetic diversity of Japanese encephalitis virus isolates obtained from the Indonesian archipelago between 1974 and 1987. Vector Borne Zoonotic Dis. 2013;13:479–88.
212. Lincoln AF, Sivertson SE. Acute phase of Japanese B encephalitis; two hundred and one cases in American soldiers, Korea, 1950. J Am Med Assoc. 1952;150:268–73.
213. Sabin AB, Schlesinger RW, et al. Japanese B encephalitis in American soldiers in Korea. Am J Hyg. 1947;46:356–75.
214. Hullinghorst RL, Burns KF, Choi YT, Whatley LR. Japanese B encephalitis in Korea; the epidemic of 1949. J Am Med Assoc. 1951;145:460–6.
215. Hong YJ. Japanese Encephalitis in South Korea. J Pediatr Infect Dis Immunol. 2014;26:257–62.
216. Self LS, Ree HI, Lofgren CS, Shim JC, Chow CY, Shin HK, Kim KH. Aerial applications of ultra-low-volume insecticides to control the vector of Japanese encephalitis in Korea. Bull World Health Organ. 1973;49:353–7.
217. Lee D-W, Choe YJ, Kim JH, Song KM, Cho H, Bae G-R, Lee J-K. Epidemiology of Japanese encephalitis in South Korea, 2007–2010. Int J Infect Dis. 2012;16:e448–52.
218. Sohn YM. Japanese encephalitis immunization in South Korea: past, present, and future. Emerg Infect Dis. 2000;6:17–24.
219. Sunwoo JS, Jung KH, Lee ST, Lee SK, Chu K. Reemergence of Japanese Encephalitis in South Korea, 2010-2015. Emerg Infect Dis. 2016;22:1841–3.
220. Lee EJ, Cha GW, YR J, Han MG, Lee WJ, Jeong YE. Prevalence of neutralizing antibodies to Japanese encephalitis virus among high-risk age groups in South Korea, 2010. PLoS One. 2016;11:e0147841.
221. Hammon WM, Schrack WD Jr, Sather GE. Serological survey for a arthropod-borne virus infections in the Philippines. Am J Trop Med Hyg. 1958;7:323–8.
222. Ksiazek TG, Trosper JH, Cross JH, Basaca-Sevilla V. Additional isolations of Japanese encephalitis virus from the Philippines. Southeast Asian J Trop Med Public Health. 1980;11:507–9.
223. Santiago ATA, Claveria FG. Medically important mosquitoes (Diptera: Culicidae) identified in rural Barangay Binubusan, Lian, Batangas Province, Philippines. Philippine J Sci. 2012;141:e10.
224. Lopez AL, Aldaba JG, Roque VG Jr, Tandoc AO 3rd, Sy AK, Espino FE, DeQuiroz-Castro M, Jee Y, Ducusin MJ, Fox KK. Epidemiology of Japanese encephalitis in the Philippines: a systematic review. PLoS Negl Trop Dis. 2015;9:e0003630.
225. Chan YC, Loh TF. Isolation of Japanese encephalitis virus from the blood of a child in Singapore. Am J Trop Med Hyg. 1966;15:567–72.
226. Paul FM. Japanese B encephalitis in Singapore children. J Singapore Paediatr Soc. 1978;20:13–7.
227. Paul FM. Morbidity and mortality of Japanese B encephalitis in Singapore. J Singapore Paediatr Soc. 1987;29(Suppl 1):87–93.
228. Phoon WO. Japanese encephalitis in Singapore children. Singapore Med J. 1963;3:11–7.

229. Yoshida M. Immunological study of Japanese encephalitis virus—serological analysis of strains isolated from Thailand, India, Singapore and Taiwan. Kansenshogaku Zasshi. 1994;68:529–35.
230. Koh YL, Tan BH, Loh JJ, Ooi EE, SY S, Hsu LY. Japanese encephalitis, Singapore. Emerg Infect Dis. 2006;12:525–6.
231. See E, Tan HC, Wang D, Ooi EE, Lee MA. Presence of hemagglutination inhibition and neutralization antibodies to Japanese encephalitis virus in wild pigs on an offshore island in Singapore. Acta Trop. 2002;81:233–6.
232. Ting SH, Tan HC, Wong WK, Ng ML, Chan SH, Ooi EE. Seroepidemiology of neutralizing antibodies to Japanese encephalitis virus in Singapore: continued transmission despite abolishment of pig farming? Acta Trop. 2004;92:187–91.
233. Prevot M, Puyuelo R, Philpot VB Jr, Guicheney A. First identification of the virus of Japanese B encephalitis in Viet Nam. Bull Soc Pathol Exot Filiales. 1954;47:490–2.
234. Nguyen H, Nguyen T. Japanese encephalitis in Vietnam 1985–1993. Southeast Asian J Trop Med Public Health. 1995;26:47–50.
235. Yen NT, Duffy MR, Hong NM, Hien NT, Fischer M, Hills SL. Surveillance for Japanese encephalitis in Vietnam, 1998-2007. Am J Trop Med Hyg. 2010;83:816–9.
236. Do QH, Vu TQH, Huynh TKL, Dinh QT, Deubel V. Current situation of Japanese encephalitis in the south of Vietnam, 1976-1992. 熱帯医学 Trop Med. 1995;36:202–14.
237. Ohba SY, Van Soai N, Van Anh DT, Nguyen YT, Takagi M. Study of mosquito fauna in rice ecosystems around Hanoi, northern Vietnam. Acta Trop. 2015;142:89–95.
238. Hai LT, Nguyen NH. Outlines of pig production in Vietnam. Pig News and Information (United Kingdom). 1997.
239. Lindahl JF, Stahl K, Chirico J, Boqvist S, Thu HT, Magnusson U. Circulation of Japanese encephalitis virus in pigs and mosquito vectors within Can Tho city, Vietnam. PLoS Negl Trop Dis. 2013;7:e2153.
240. Yen NT, Liu W, Hanh HD, Chang NY, Duong TN, Gibbons RV, Marks F, Thu NA, Hong NM, Park JK, et al. A model immunization programme to control Japanese encephalitis in Viet Nam. J Health Popul Nutr. 2015;33:207–13.
241. Erlanger TE, Weiss S, Keiser J, Utzinger J, Wiedenmayer K. Past, present, and future of Japanese encephalitis. Emerg Infect Dis. 2009;15:1–7.
242. Elyan DS, Moustafa L, Noormal B, Jacobs JS, Aziz MA, Hassan KS, Wasfy MO, Monestersky JH, Oyofo BA. Serological evidence of flaviviruses infection among acute febrile illness patients in Afghanistan. J Infect Dev Ctries. 2014;8:1176–80.
243. Kadamov DS. The bloodsucking mosquitoes (Culicidae) of North Afghanistan. Med Parazitol (Mosk). 2006:Jan-Mar;(1):59–60.
244. Rueda LM, Pecor JE, Lowen RG, Carder M. New record and updated checklists of the mosquitoes of Afghanistan and Iraq. J Vector Ecol. 2008;33:397–402.
245. Kim H, Marks F, Novakovic U, Hotez PJ, Black RE. Multistakeholder partnerships with the Democratic Peoples' Republic of Korea to improve childhood immunisation: a perspective from global health equity and political determinants of health equity. Trop Med Int Health. 2016;21:965–72.
246. Savini H, Gautret P, Gaudart J, Field V, Castelli F, Lopez-Velez R, Lim PL, Shaw M, von Sonnenburg F, Loutan L, et al. Travel-associated diseases, Indian Ocean Islands, 1997-2010. Emerg Infect Dis. 2013;19:1297–301.
247. Cao-Lormeau VM. Tropical islands as new hubs for emerging arboviruses. Emerg Infect Dis. 2016;22(5):913.
248. Japanese encephalitis in a U.S. traveler returning from Thailand, 2004. MMWR Morb Mortal Wkly Rep. 2005;54:123–5.
249. Doti P, Castro P, Martinez MJ, Zboromyrska Y, Aldasoro E, Inciarte A, Requena-Mendez A, Milisenda J, Fernandez S, Nicolas JM, et al. A case of Japanese encephalitis in a 20 year-old Spanish sportsman, February 2013. Euro Surveill. 2013;18:20573.

250. Hills SL, Stoltey J, Martinez D, Kim PY, Sheriff H, Zangeneh A, Eilerman SR, Fischer M. A case series of three US adults with Japanese encephalitis, 2010-2012. J Travel Med. 2014;21:310–3.
251. Jeurissen A, Strauven T. A case of aseptic meningitis due to Japanese encephalitis virus in a traveller returning from the Philippines. Acta Neurol Belg. 2011;111:143–5.
252. Langevin S, Libman M, Drebot MA, Laverdiere M. A case of Japanese encephalitis virus infection acquired during a trip in Thailand. J Travel Med. 2012;19:127–9.
253. Tappe D, Nemecek A, Zipp F, Emmerich P, Gabriel M, Gunther S, Dobler G, Schmidt-Chanasit J, Stich A. Two laboratory-confirmed cases of Japanese encephalitis imported to Germany by travelers returning from Southeast Asia. J Clin Virol. 2012;54:282–5.
254. Werlinrud AM, Christiansen CB, Koch A. Japanese encephalitis in a Danish short-term traveler to Cambodia. J Travel Med. 2011;18:411–3.
255. Berge TO, Shuey HE. Isolation of Japanese B encephalitis virus; report of a fatal case in an American soldier returned from Korea. J Am Med Assoc. 1956;162:564–7.
256. Perez-Pina F, Merikangas UR. Japanese B encephalitis in an American soldier returning from Korea. N Engl J Med. 1953;249:531–2.
257. Eick-Cost AA, Hu Z, Klein TA, Putnak RJ, Jarman RG. Seroconversion to Japanese encephalitis virus among U.S. infantry forces in Korea. Am J Trop Med Hyg. 2015;93:1052–4.
258. Ravanini P, Huhtamo E, Ilaria V, Crobu MG, Nicosia AM, Servino L, Rivasi F, Allegrini S, Miglio U, Magri A et al. Japanese encephalitis virus RNA detected in Culex pipiens mosquitoes in Italy. Euro Surveill. 2012;17:pii: 20221.
259. Garcia-Bocanegra I, Busquets N, Napp S, Alba A, Zorrilla I, Villalba R, Arenas A. Serosurvey of West Nile virus and other flaviviruses of the Japanese encephalitis antigenic complex in birds from Andalusia, southern Spain. Vector Borne Zoonotic Dis. 2011;11:1107–13.
260. Platonov A, Rossi G, Karan L, Mironov K, Busani L, Rezza G. Does the Japanese encephalitis virus (JEV) represent a threat for human health in Europe? Detection of JEV RNA sequences in birds collected in Italy. Euro Surveill 2012;17:pii: 20241.
261. Nemeth N, Bosco-Lauth A, Oesterle P, Kohler D, Bowen R. North American birds as potential amplifying hosts of Japanese encephalitis virus. Am J Trop Med Hyg. 2012;87:760–7.
262. Nemeth NM, Bosco-Lauth AM, Sciulli RH, Gose RB, Nagata MT, Bowen RA. Serosurveillance for Japanese encephalitis and West Nile viruses in resident birds in Hawai'i. J Wildl Dis. 2010;46:659–64.
263. Pfeffer M, Dobler G. Emergence of zoonotic arboviruses by animal trade and migration. Parasit Vectors. 2010;3:35.
264. Uchil PD, Satchidanandam V. Phylogenetic analysis of Japanese encephalitis virus: envelope gene based analysis reveals a fifth genotype, geographic clustering, and multiple introductions of the virus into the Indian subcontinent. Am J Trop Med Hyg. 2001;65:242–51.
265. Do LP, Bui TM, Phan NT. Mechanism of Japanese encephalitis virus genotypes replacement based on human, porcine and mosquito-originated cell lines model. Asian Pac J Trop Med. 2016;9:333–6.
266. Williams DT, Wang LF, Daniels PW, Mackenzie JS. Molecular characterization of the first Australian isolate of Japanese encephalitis virus, the FU strain. J Gen Virol. 2000;81:2471–80.
267. Schuh AJ, Li L, Tesh RB, Innis BL, Barrett AD. Genetic characterization of early isolates of Japanese encephalitis virus: genotype II has been circulating since at least 1951. J Gen Virol. 2010;91:95–102.
268. Mohammed MA, Galbraith SE, Radford AD, Dove W, Takasaki T, Kurane I, Solomon T. Molecular phylogenetic and evolutionary analyses of Muar strain of Japanese encephalitis virus reveal it is the missing fifth genotype. Infect Genet Evol. 2011;11:855–62.

269. Nitatpattana N, Dubot-Peres A, Gouilh MA, Souris M, Barbazan P, Yoksan S, de Lamballerie X, Gonzalez JP. Change in Japanese encephalitis virus distribution, Thailand. Emerg Infect Dis. 2008;14:1762–5.
270. Ma SP, Yoshida Y, Makino Y, Tadano M, Ono T, Ogawa M. Short report: a major genotype of Japanese encephalitis virus currently circulating in Japan. Am J Trop Med Hyg. 2003;69:151–4.
271. Yoshida Y, Tabei Y, Hasegawa M, Nagashima M, Morozumi S. Genotypic analysis of Japanese encephalitis virus strains isolated from swine in Tokyo, Japan. Jpn J Infect Dis. 2005;58:259–61.
272. Yun SM, Cho JE, YR J, Kim SY, Ryou J, Han MG, Choi WY, Jeong YE. Molecular epidemiology of Japanese encephalitis virus circulating in South Korea, 1983-2005. Virol J. 2010;7:127.
273. Do LP, Bui TM, Hasebe F, Morita K, Phan NT. Molecular epidemiology of Japanese encephalitis in northern Vietnam, 1964-2011: genotype replacement. Virol J. 2015;12:51.
274. Nga PT, del Carmen Parquet M, Cuong VD, Ma SP, Hasebe F, Inoue S, Makino Y, Takagi M, Nam VS, Morita K. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. J Gen Virol. 2004;85:1625–31.
275. Fulmali PV, Sapkal GN, Athawale S, Gore MM, Mishra AC, Bondre VP. Introduction of Japanese encephalitis virus genotype I, India. Emerg Infect Dis. 2011;17:319–21.
276. Pan XL, Liu H, Wang HY, Fu SH, Liu HZ, Zhang HL, Li MH, Gao XY, Wang JL, Sun XH, et al. Emergence of genotype I of Japanese encephalitis virus as the dominant genotype in Asia. J Virol. 2011;85:9847–53.
277. Gao X, Liu H, Wang H, Fu S, Guo Z, Liang G. Southernmost Asia is the source of Japanese encephalitis virus (genotype 1) diversity from which the viruses disperse and evolve throughout Asia. PLoS Negl Trop Dis. 2013;7:e2459.
278. Schuh AJ, Ward MJ, Leigh Brown AJ, Barrett AD. Dynamics of the emergence and establishment of a newly dominant genotype of Japanese encephalitis virus throughout Asia. J Virol. 2014;88:4522–32.
279. Kang BK, Hwang JM, Moon H, Han SY, Kim JM, Yang DK, Park BK, Song D. Comparison of the antigenic relationship between Japanese encephalitis virus genotypes 1 and 3. Clin Exp Vaccine Res. 2016;5:26–30.
280. Pyke AT, Williams DT, Nisbet DJ, van den Hurk AF, Taylor CT, Johansen CA, Macdonald J, Hall RA, Simmons RJ, Mason RJ, et al. The appearance of a second genotype of Japanese encephalitis virus in the Australasian region. Am J Trop Med Hyg. 2001;65:747–53.
281. Chen W-R, Rico-Hesse R, Tesh RB. A new genotype of Japanese encephalitis virus from Indonesia. Am J Trop Med Hyg. 1992;47:61–9.
282. Chen W-R, Tesh RB, Rico-Hesse R. Genetic variation of Japanese encephalitis virus in nature. J Gen Virol. 1990;71:2915–22.
283. Schuh AJ, Ward MJ, Brown AJ, Barrett AD. Phylogeography of Japanese encephalitis virus: genotype is associated with climate. PLoS Negl Trop Dis. 2013;7:e2411.
284. McMichael AJ, Woodruff RE, Hales S. Climate change and human health: present and future risks. Lancet. 2006;367:859–69.
285. Cao L, Fu S, Gao X, Li M, Cui S, Li X, Cao Y, Lei W, Lu Z, He Y, et al. Low protective efficacy of the current Japanese encephalitis vaccine against the emerging genotype 5 Japanese encephalitis virus. PLoS Negl Trop Dis. 2016;e0004686:10.
286. Tajima S, Yagasaki K, Kotaki A, Tomikawa T, Nakayama E, Moi ML, Lim CK, Saijo M, Kurane I, Takasaki T. In vitro growth, pathogenicity and serological characteristics of the Japanese encephalitis virus genotype V Muar strain. J Gen Virol. 2015;96(9):2661.
287. Oya A. Japanese Encephalitis vaccine. Pediatr Int. 1988;30:175–84.
288. Tauber E, Kollaritsch H, Korinek M, Rendi-Wagner P, Jilma B, Firbas C, Schranz S, Jong E, Klingler A, Dewasthaly S, et al. Safety and immunogenicity of a Vero-cell-derived,

inactivated Japanese encephalitis vaccine: a non-inferiority, phase III, randomised controlled trial. Lancet. 2007;370:1847–53.
289. Hennessy S, Liu Z, Tsai TF, Strom BL, Wan CM, Liu HL, Wu TX, Yu HJ, Liu QM, Karabatsos N, et al. Effectiveness of live-attenuated Japanese encephalitis vaccine (SA14-14-2): a case-control study. Lancet. 1996;347:1583–6.
290. Tsai TF, YX Y, Jia LL, Putvatana R, Zhang R, Wang S, Halstead SB. Immunogenicity of live attenuated SA14-14-2 Japanese encephalitis vaccine—a comparison of 1- and 3-month immunization schedules. J Infect Dis. 1998;177:221–3.
291. Guirakhoo F, Zhang ZX, Chambers TJ, Delagrave S, Arroyo J, Barrett AD, Monath TP. Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. Virology. 1999;257:363–72.
292. Monath TP, Soike K, Levenbook I, Zhang ZX, Arroyo J, Delagrave S, Myers G, Barrett AD, Shope RE, Ratterree M, et al. Recombinant, chimaeric live, attenuated vaccine (ChimeriVax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic and protective in non-human primates. Vaccine. 1999;17:1869–82.
293. Chotpitayasunondh T, Pruekprasert P, Puthanakit T, Pancharoen C, Tangsathapornpong A, Oberdorfer P, Kosalaraksa P, Prommalikit O, Tangkittithaworn S, Kerdpanich P, et al. Post-licensure, phase IV, safety study of a live attenuated Japanese encephalitis recombinant vaccine in children in Thailand. Vaccine. 2017;35:299–304.
294. Chokephaibulkit K, Sirivichayakul C, Thisyakorn U, Sabchareon A, Pancharoen C, Bouckenooghe A, Gailhardou S, Boaz M, Feroldi E. Safety and immunogenicity of a single administration of live-attenuated Japanese encephalitis vaccine in previously primed 2- to 5-year-olds and naive 12- to 24-month-olds: multicenter randomized controlled trial. Pediatr Infect Dis J. 2010;29:1111–7.
295. Erra EO, Askling HH, Rombo L, Riutta J, Vene S, Yoksan S, Lindquist L, Pakkanen SH, Huhtamo E, Vapalahti O, et al. A single dose of vero cell-derived Japanese encephalitis (JE) vaccine (Ixiaro) effectively boosts immunity in travelers primed with mouse brain-derived JE vaccines. Clin Infect Dis. 2012;55:825–34.
296. Erra EO, Askling HH, Yoksan S, Rombo L, Riutta J, Vene S, Lindquist L, Vapalahti O, Kantele A. Cross-protection elicited by primary and booster vaccinations against Japanese encephalitis: a two-year follow-up study. Vaccine. 2013;32:119–23.
297. Monath TP, Guirakhoo F, Nichols R, Yoksan S, Schrader R, Murphy C, Blum P, Woodward S, McCarthy K, Mathis D, et al. Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen. J Infect Dis. 2003;188:1213–30.
298. Erra EO, Askling HH, Yoksan S, Rombo L, Riutta J, Vene S, Lindquist L, Vapalahti O, Kantele A. Cross-protective capacity of Japanese encephalitis (JE) vaccines against circulating heterologous JE virus genotypes. Clin Infect Dis. 2013;56:267–70.
299. Baig S, Fox K, Jee Y, O'Connor P, Hombach J, Wang S, Hyde T, Fischer M, Hills S. Japanese encephalitis: status of surveillance and immunization in Asia and the Western Pacific, 2012. Wkly Epidemiol Rec. 2013;88:357–64.
300. Basu A, Dutta K. Recent advances in Japanese encephalitis. F1000Res. 2017;6:259.
301. Hoke CH Jr, Vaughn DW, Nisalak A, Intralawan P, Poolsuppasit S, Jongsawas V, Titsyakorn U, Johnson RT. Effect of high-dose dexamethasone on the outcome of acute encephalitis due to Japanese encephalitis virus. J Infect Dis. 1992;165:631–7.
302. Harinasuta C, Nimmanitya S, Titsyakorn U. The effect of interferon-alpha A on two cases of Japanese encephalitis in Thailand. Southeast Asian J Trop Med Public Health. 1985;16:332–6.

303. Solomon T, Dung NM, Wills B, Kneen R, Gainsborough M, Diet TV, Thuy TT, Loan HT, Khanh VC, Vaughn DW, et al. Interferon alfa-2a in Japanese encephalitis: a randomised double-blind placebo-controlled trial. Lancet. 2003;361:821–6.
304. Kumar R, Tripathi P, Baranwal M, Singh S, Tripathi S, Banerjee G. Randomized, controlled trial of oral ribavirin for Japanese encephalitis in children in Uttar Pradesh, India. Clin Infect Dis. 2009;48:400–6.
305. Dutta K, Basu A. Use of minocycline in viral infections. Indian J Med Res. 2011;133:467–70.
306. Kumar R, Basu A, Sinha S, Das M, Tripathi P, Jain A, Kumar C, Atam V, Khan S, Singh AS. Role of oral minocycline in acute encephalitis syndrome in India—a randomized controlled trial. BMC Infect Dis. 2016;16:67.
307. Singh AK, Mehta A, Kushwaha K, Pandey AK, Mittal M, Sharma B, Pandey J. Minocycline trial in Japanese encephalitis: a double blind, randomized placebo study. Pediatr Rev Int J Pediatr Res. 2016;3:372–7.
308. Rayamajhi A, Nightingale S, Bhatta NK, Singh R, Kneen R, Ledger E, Bista KP, Lewthwaite P, Mahaseth C, Turtle L, et al. A preliminary randomized double blind placebo-controlled trial of intravenous immunoglobulin for Japanese encephalitis in Nepal. PLoS One. 2015;e0122608:10.
309. Rayamajhi A, Nightingale S, Bhatta NK, Singh R, Ledger E, Bista KP, Lewthwaite P, Mahaseth C, Turtle L, Robinson JS, et al. Correction: A preliminary randomized double blind placebo-controlled trial of intravenous immunoglobulin for Japanese encephalitis in Nepal. PLoS One. 2015;e0136008:10.
310. Anderson KB, Gibbons RV, Thomas SJ, Rothman AL, Nisalak A, Berkelman RL, Libraty DH, Endy TP. Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. PLoS Negl Trop Dis. 2011;5:e1311.
311. Caramello P, Canta F, Balbiano R, Lipani F, Ariaudo S, De Agostini M, Calleri G, Boglione L, Di Caro A. Role of intravenous immunoglobulin administration in Japanese encephalitis. Clin Infect Dis. 2006;43:1620–1.
312. Ben-Nathan D, Lustig S, Tam G, Robinzon S, Segal S, Rager-Zisman B. Prophylactic and therapeutic efficacy of human intravenous immunoglobulin in treating West Nile virus infection in mice. J Infect Dis. 2003;188:5–12.
313. Walid MS, Mahmoud FA. Successful treatment with intravenous immunoglobulin of acute flaccid paralysis caused by West Nile virus. Perm J. 2009;13:43–6.
314. Dalakas MC. Mechanisms of action of IVIg and therapeutic considerations in the treatment of acute and chronic demyelinating neuropathies. Neurology. 2002;59:S13–21.
315. Luzina IG, Keegan AD, Heller NM, Rook GA, Shea-Donohue T, Atamas SP. Regulation of inflammation by interleukin-4: a review of “alternatives”. J Leukoc Biol. 2012;92:753–64.
316. Winter PM, Dung NM, Loan HT, Kneen R, Wills B, Thu le T, House D, White NJ, Farrar JJ, Hart CA, et al. Proinflammatory cytokines and chemokines in humans with Japanese encephalitis. J Infect Dis. 2004;190:1618–26.
317. Chirac P, Torreele E. Global framework on essential health R&D. Lancet. 2006;367:1560–1.

Dengue

Terapong Tantawichien and Usa Thisayakorn

Contents

1 Introduction

Dengue is one of the most important mosquito-borne viral infections caused by single-stranded RNA virus that are transmitted by the *Aedes aegypti* and *Aedes albopictus* mosquito species. Dengue is endemic in over 140 countries in Asia, the USA, the Eastern Mediterranean, and Africa. The World Health Organization (WHO) estimated that there are more than 2.5 billion people—mainly occurs in children living in tropical and subtropical countries—at risk of dengue infection with one or more dengue viruses. There are estimated nearly 100 million symptomatic dengue infections occurring worldwide annually, nearly 75% in Asia and the Western Pacific region [1]. During the past decades, the outbreaks of dengue

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infection have been reported throughout the world with increased severity. Ecologic and demographic changes are considered to be the contributing factors to the emergence of dengue infection in the past decades. Dengue has expanded into new countries and into urban settings associated with increased distribution of *A. aegypti*, population growth, urbanization, development of slums, migration of population, movement of dengue virus by infected travelers, trade development, and improved diagnostic capabilities in medical practice [2, 3]. Increased transmission of dengue virus in tropical urban areas has been created by substandard housing and crowding as well as deterioration in water, sewer, and waste management systems, all of which are intimately associated with unplanned urbanization [4–7]. So it is likely that dengue will expand its geographic reach and become an increasing burden on health resources in affected areas during the next decade. An effective vector-control management is the only means to reduce dengue infection in endemic areas. Because vector control has achieved only limited success so far in reducing the transmission of dengue, the usage of effective dengue vaccine in target population along with the preventive measures already used such as raising public awareness may be the means to effectively control of this disease in endemic area [8].

Hyperendemic dengue with a general increase in the number of dengue cases over time is a major public health problem in many countries in South and Southeast Asia where *A. aegypti* and *A. albopictus* are widespread in both urban and rural areas, where multiple virus serotypes are circulating, and where dengue is a leading cause of hospitalization and death in children. The extent of hyperendemicity and time between serotype introductions are key determinants in a population's serotype-specific immunity and, consequently, in the age distribution of clinically apparent dengue infection. In the past, the age distribution of indigenous dengue cases in South and Southeast Asia is different from that of the Americas, where these syndromes occur in all age groups included elderly. Recently several countries in Asia have reported an epidemic shift of dengue from mainly affecting children to affecting adolescents and young adults with increased severity [9–12]. In Thailand, the affected adults aged over 15 years old are reported to comprise 30–40% of dengue virus infected cases [13]. There are some differences in clinical presentations, laboratory findings, and severe complications between children and adult with dengue [14–16]. Increasing incidence of dengue among travelers (1.0–6.7%) has been recognized as a potential hazard to tourists as various reports in international travelers returning from endemic areas [17–20]. Recent reports revealed that adult dengue appears to occur more frequently than malaria among travelers returning from Asia, so the healthcare providers in Western countries are more likely to be confronted with travel-acquired dengue infections [21–24]. In returning travelers with fever, clinical manifestations of dengue infection are comparable with observations in endemic area where dengue may go unnoticed. We emphasize the need for continued dengue surveillance in non-endemic countries with careful evaluation and follow-up of febrile travelers after visiting countries where are dengue-endemic areas [25, 26]. Travelers should be encouraged to protect themselves from mosquito

bites, in order to avoid infections and onward transmission of dengue in new areas where *A. aegypti* is established.

2 Pathogen and Pathogenesis

Dengue virus, single-stranded RNA virus member of genus *Flavivirus* in the family *Flaviviridae*, is the etiologic agent of dengue infection. There are four serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) that are transmitted by the *Aedes aegypti* and *A. albopictus* mosquito species. Peak transmission occurs in rainfall season and high temperature in hyperendemic and endemic areas. Although dengue virus is transmitted by mosquitoes, unusual dengue transmissions through needlestick, receipt of infected blood component, tissue or organ transplantation, and transplacental infection have been reported [27–34]. After an incubation period of 4–8 days, infection by any dengue virus can produce a wide spectrum of illnesses ranging from asymptomatic or subclinical infection to undifferentiated fever, dengue fever (DF), and severe forms of the disease associated with plasma leakage (dengue hemorrhage fever: DHF), dengue shock syndrome (DSS), severe bleeding, encephalopathy, and multi-organ failure [35]. DHF is characterized by rapid onset of capillary leakage accompanied by thrombocytopenia, hemoconcentration, vascular collapse, abdominal pain, and hemorrhagic manifestations [36]. Despite the clinical classification of DF and DHF as distinct entities, they are likely to be a continuum of the same disease process with divergent outcomes with regard to the perturbation of vascular integrity. In case of dengue infection, asymptomatic cases are more frequent than the symptomatic cases with the variable ratio of asymptomatic to symptomatic dengue infections of 0.9:1 to 18:1, dependent on the geographical areas, the epidemiological contexts, and individual immunological attributes [37, 38]. However, patients with asymptomatic infection may act as reservoir for dengue virus to transmit to mosquitoes and subsequently to humans and should be considered in estimation of disease burden. Recovery from dengue infection with one serotype confers lifelong homologous immunity to that particular serotype but short-term protection against other serotypes, so secondary infection can occur with other dengue serotypes. Previous epidemiologic data reveals that secondary heterotypic dengue virus infection is a risk factor to develop severe DHF/DSS, mediated most likely by antibody-dependent enhancement (ADE) of infection. Pre-existing homotypic antibodies bind to heterotypic dengue virions (virus-antibody complexes) and enable Fcγ receptor-mediated uptake by target Fcγ receptor-bearing cells (e.g., monocyte/macrophage) resulting in increased viral replication and viremia [38]. Changing of inflammatory cytokine production (such as TNF∞, interleukin-1, interleukin-2, interleukin-6, interleukin-12, macrophage migration inhibitory factor, HMGB1, MCP-1) produced by T-lymphocytes, monocytes/macrophages, and endothelial cell is observed in dengue patients who have increased vascular permeability, thrombocytopenia, and activation of coagulation and fibrinolysis [39, 40]. In addition, secreted NS1 protein, anti-NS1

antibodies, and increased complement activation (C3a, C5a) might be involved in increased production of inflammatory cytokines, triggering local and systemic effects implicated in intravascular coagulopathy and virus-induced vascular leakage. Thrombocytopenia caused by bone marrow suppression, shortened platelet survival, and increased platelet consumption due to platelet adhesion occurs during the dengue infection and reaches nadir during the day of defervescence (toxic stage) [40, 41]. Although secondary dengue infection remains the strongest known risk factor for DHF/DSS, viral genetics, serotype sequence, host factors, and time interval between primary and secondary infections can modulate severity of illness [40, 42–45].

3 Clinical Manifestation

Dengue infection should be suspected if patients in dengue-epidemic or dengue-endemic area have a fever of 10 days or less with myalgia, headache, flushing, anorexia, nausea or vomiting, arthralgia, bone pain, periorbital pain with no obvious respiratory tract symptoms or signs, and no organ-specific symptoms of other infectious diseases. The clinical spectrum of dengue infection ranges from mild illness (undifferentiated fever, non-severe DF) to the life-threatening severe forms of the disease with plasma leakage (DHF/DSS), severe bleeding, or multi-organ failure, which may be fatal. Dengue fever in its classical form is nonfatal febrile illness with about 5–7 days associated with sudden onset, anorexia, myalgia, headache, and occasional rash. DHF is characterized by high continuous fever of 2–7 days and rapid onset of capillary leakage accompanied by thrombocytopenia, hemoconcentration, vascular collapse, abdominal pain, and hemorrhagic manifestations. Shock (DSS) occurs as a consequence of severe plasma volume loss into serous spaces (e.g., pleural space or peritoneum cavity) or severe internal hemorrhage. During the acute febrile phase, usually lasting 3–8 days, the clinical symptoms resemble those of DF and severe dengue (DHF), including fever, nausea or vomiting, headache, rash, and myalgia; nonetheless, abdominal pain and severe or widespread bleeding are less frequent in DF. Minor hemorrhagic manifestations such as petechiae, epistaxis, gingival bleeding, and menorrhagia do sometimes occur in patient with dengue, although DF is rarely associated with severe hemorrhage leading to shock. Age-related differences in dengue severity are poorly understood; however, there are some differences in clinical courses between children and adults. Plasma leakage (DHF) and DSS appear to be more frequent in children than adults, possibly reflecting age-dependent differences in intrinsic vascular permeability, but some reports suggest that bleeding manifestations, especially severe internal hemorrhage and hepatic dysfunction, are both more common in adults and older age groups [14, 16, 41, 43, 44, 46–50]. The symptoms generally last for 3–7 days before the fever subsides and symptoms remit. During convalescent stage, the patients with dengue infection, even in DSS, may have rapidly increasing appetite, convalescent rash on lower extremities (a confluent rash

with characteristic, scattered, round areas on pale skin), and sinus bradycardia. Most patients with dengue infection recover spontaneously, and abnormal hemostasis normalizes during the convalescent stage or within 1–2 weeks after defervescence. The emergence of severe bleeding, fulminant hepatic failure, and encephalopathy in DF and DHF have been the causes of an apparent increase in the complications of dengue in the adolescent, adult, and elderly [47–49, 51–54]. High mortality rate has previously been reported in elderly patients with dengue infection because of medical comorbidity and waning of host immunity [55–59].

The prognosis of dengue infection depends on early diagnosis, recognition of plasma leakage, and treatment with immediate replacement of fluid and intensive supportive care. The classification of severity has a high potential for being of physician's practice as to where and how intensively the patient should be observed and treated with intravenous fluids, blood, or plasma transfusion and medicines. WHO released a new classification in 2009, which is dengue with or without warning signs and severe dengue because WHO 1997 classification (DF, DHF, DSS) was poorly related to disease severity, difficult to use in clinical setting, and unhelpful in triage in outbreaks [35, 36, 60, 61]. These warning signs (persistent or severe vomiting, abdominal pain or tenderness, liver enlargement, drowsy or alteration of consciousness, fluid accumulation with respiratory distress, epitaxis, gum bleeding, gastrointestinal bleeding, retinal hemorrhage, oliguria, and hemoconcentration with severe thrombocytopenia) are used to alert the clinicians to monitor dengue infection progress. Physicians should be aware of these warning signs in patients with dengue infections before they develop severe dengue [62–64]. Severe dengue is defined by one or more of the following: plasma leakage (DHF) that may lead to shock (DSS), severe bleeding, and severe organ impairment such as hepatic failure, acute renal failure, and encephalopathy) as Fig. 1 [14, 35, 39–47]. If untreated, mortality can be as high as 20%, whereas appropriate case management and intravenous rehydration can reduce mortality to less than 1%. Virus factors (serotypes, structural and nonstructural proteins of dengue virus, and viral load) and host factors (age, gender differences, genetic, nutritional status, immune reaction, and coexisting medical conditions) might be involved in the severity of dengue infection.

3.1 Dengue Hemorrhagic Fever and Dengue Shock Syndrome

Typically DHF resembles DF in many clinical respects, but it is characterized by high continuous fever of 2–7 days, hemorrhagic diathesis, hepatomegaly, and circulatory disturbance (DSS). The critical stage associated with plasma leakage (20% increase in hematocrit over baseline) and marked thrombocytopenia ($<100 \times 10^9$/L) associated with bleeding frequently occur at the end of febrile phase of

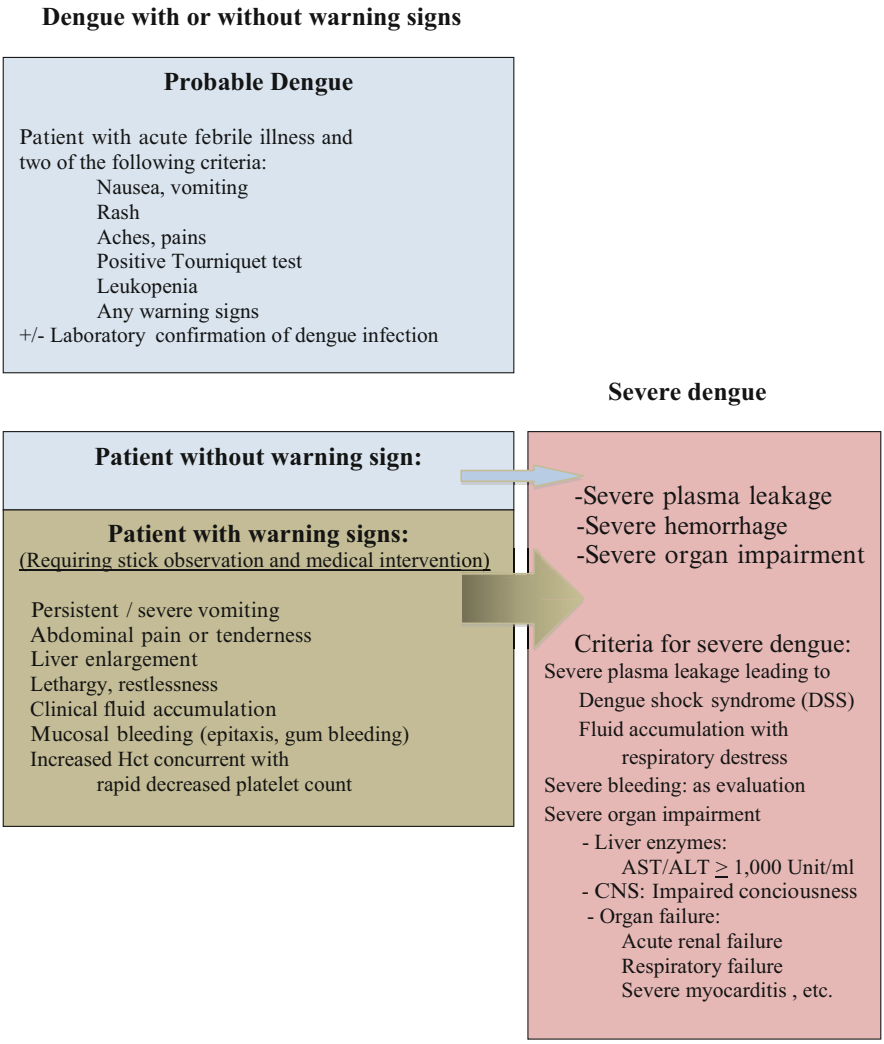

Fig. 1 Dengue case classification: dengue with or without warning signs and severe dengue (modified from WHO guideline 2009) [35]

illness [36]. Right side pleural fluid detected by chest roentgenogram or free fluid in the peritoneal cavity and thickening of gall bladder wall detected by ultrasonography has been interpreted as evidence of plasma leakage, which is usually only clinically detectable after intravenous fluid therapy unless plasma leakage is significant [65–67]. The right side or bilateral pleural effusion is generally not prominent but becomes increasingly more after excessive intravenous fluid administration. In mild DHF cases, the changes in blood pressure and pulse may be minimal and transient. Patients recover shortly after treatment. In more severe

DHF cases, a rapid, weak pulse, narrowing of the pulse pressure to less than 20 mm Hg, or an unobtainable blood pressure establishes DSS [36]. Clinical indicators of impending DSS include severe abdominal pain, change from fever to hypothermia, restlessness, sweating, prostration, and tender hepatomegaly. If plasma loss continues and becomes excessive, the patient's situation can progress rapidly into profound shock. Prolonged shock often complicates metabolic acidosis, severe gastrointestinal bleeding, and disseminated intravascular coagulopathy (DIC). DSS was an independent risk factor (odds ratio 220) for development of acute renal failure in adult patients with DHF [68]. Cardiac involvement was observed in few patients ranging from abnormality of electrocardiogram, mild elevation of cardiac biomarkers to myocarditis and/or pericarditis and death [69]. Acute respiratory failure is a rare complication but has a high mortality rate [70]. Although children are more likely to develop hypovolemic shock than adults in DHF characterized by increased microvascular permeability, a high mortality rate is seen in the adults and elderly with dengue infection [42, 49, 51, 55, 57, 62]. High fatality rate of dengue in adults was significantly associated with pre-existing comorbid medical illnesses such as cardiac diseases and renal diseases [49, 56–59, 62]. Because the altered vascular permeability is short-lived and spontaneously converts to normal level, the period of clinically significant plasma leakage usually lasts 24–48 h after defervescence. Diuresis ensues as plasma leakage terminates. Convalescent rash, transient hypertension, and sinus bradycardia were described during convalescence in patients with DHF/DSS.

3.2 Hemorrhage Associated with Dengue Infection

Hemorrhage contributes to morbidity and mortality, especially during the severe thrombocytopenia, usually occurring in 5 to 8 days after onset of illness [41]. The pathogenesis of abnormal bleeding in dengue is multifactorial and encompasses severe thrombocytopenia, platelet dysfunction, blood coagulation defects, and vasculopathy. There are typical coagulopathies of increased APTT and low fibrinogen levels in most patients, but severe thrombocytopenia and platelet dysfunction are probably the major cause of clinical bleeding. Variable degree of hemorrhage may occur at any sites, most commonly petechiae, epistaxis, gingival bleeding, or menorrhagia, and usually occurs on days 5–8 of the illness. Bleeding from the nose, gums, and upper gastrointestinal tract are not uncommon in patients with dengue infection. Vaginal bleeding (menorrhagia) is a common site of bleeding (24.6% in adults with dengue infection), and hormonal therapy such as Premarin and Primolut N is suggested for patients exhibiting excessive vaginal bleeding [48]. Of the dengue patients with plasma leakage (DHF), severity of bleeding varied markedly with spontaneous petechiae, hematemesis, melena, menorrhagia, and epistaxis. Risk factors of severe bleeding are platelets $\leq$20,000/mm^3 ($\leq 20 \times 10^9$/L), high aspartate aminotransferase (AST) or alanine aminotransferase (ALT) level, prolonged prothrombin time (PT), severe plasma leakage (DSS), DIC, or fulminant hepatic failure

[71]. Massive hematemesis may occur in adults with DF or DHF caused by pre-existing peptic ulcer or hemorrhagic gastritis, and it may be not associated with profound shock in adults as previously reported in children. In the few reports of endoscopic findings for dengue adults with upper gastrointestinal bleeding, hemorrhagic gastritis was the most common finding (40.9–58.5%), followed by gastric ulcer, and duodenal ulcer [53]. However, the role of endoscopic therapy in upper gastrointestinal bleeding of dengue patients is still unknown [72]. Life-threatening internal hemorrhage such as subcapsular splenic bleeding and ruptures is rare but can happen spontaneously or as a result of trauma, which may be unnoticed. Splenectomy is still the treatment of choice for splenic rupture, but numerous recent reports have documented favorable outcomes with conservative treatment [54, 73]. Early diagnosis, intensive supportive care, and replacement therapy are needed to avoid a fatal outcome in dengue patients who have severe hemorrhage.

There are pregnant women with DF or severe dengue reported in Asia, highlighting the concept that young women in hyperendemic and endemic area remain susceptible to dengue infection [74–76]. The obstetricians must be aware that dengue infection of pregnant women may occur and some history or laboratory results consistent with dengue infection must be identified. Dengue during pregnancy is also particularly important in pregnant travelers from non-endemic countries to countries where dengue is endemic [77]. Uterine hemorrhage resulting in spontaneous abortion and severe postpartum bleeding has also been reported in pregnant women [76]. Surgical procedures such as cesarean section performed on patients with dengue infection may unmask dengue-induced hemostatic defects, resulting in unexpected hemorrhage in postoperative period that is difficult to control [78]. It also has been reported that dengue infection was vertically transmitted to the fetus and led to a full-blown illness in the neonate similar to that seen in children and adults [74]. Although the effects of dengue infection on pregnant women and their fetuses or newborns are unclear, recent studies have demonstrated that this infection did not cause any infant abnormalities but may have been responsible for fetus deaths and morbidity in pregnant women [79–81].

3.3 Severe Organ Impairment and Unusual Manifestations

Hepatomegaly and increased levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were more commonly found in patients with dengue infection especially DHF [82–87]. So dengue infection should be included in the differential diagnosis of acute viral hepatitis in Asia. Unlike conventional viral hepatitis, patients with dengue have a level of AST that are greater than that of ALT which may be due to excessive release of AST from damaged myocytes during dengue infections, and these levels of liver enzymes increase to a maximal 7–9 days after onset of illness, then decrease to normal levels within 2 weeks [48, 49, 82–84]. Potential mechanisms of liver injury involve a variety of potential insults including direct effects of infected virus serotypes or an adverse consequence of

dysregulated host immune responses on liver cells; compromised circulation and/or hypoxia caused by hypotension or localized vascular leakage inside the liver capsule; hepatotoxic effects of drugs such as acetaminophen or traditional herbal remedies, coinfection with other viruses such as virus hepatitis A, B, and C; and pre-existing underlying diseases (e.g., hemoglobinopathies, alcoholic liver diseases) [88]. Attention must therefore be given to the use of hepatotoxic drugs such as acetaminophen, antibiotics, and antiemetic drugs, all of which have the potential to aggravate liver damage in patient with dengue. Previous reports have found evidence that acetaminophen overdose may play an important role in causing acute liver failure in dengue patients [89, 90]. It is likely that relatively more adult dengue patients have more liver impairment than children. Pre-existing liver diseases such as chronic infection with virus hepatitis B or C, alcoholic liver disease, and cirrhosis may aggravate the liver impairment of dengue. Abnormal liver enzyme levels have been associated with severity and a poor outcome in patients with vascular leakage and abnormal bleeding [86, 87]. Increased levels of bilirubin and alkaline phosphatase were observed in a few patients. Severe liver impairment occurred in late stage of disease may complicate the outcome of dengue infection by causing acute hepatic failure and contributing directly to severe bleeding, as well as potentiating the severity of DIC [89, 90]. Severe jaundice and high mortality are observed in dengue patients with fulminant hepatic failure. The management of fulminant hepatic failure in dengue is primarily intensive supportive care; however, therapy with *N*-acetylcysteine (NAC) or providing artificial liver support was previously described [87].

The unusual manifestations of dengue infection have been recognized including severe internal hemorrhage, fulminant hepatic failure, encephalopathy, cardiomyopathy, cardiac arrhythmia, adult respiratory distress syndrome (ARDS), rhabdomyolysis, pancreatitis, appendicitis, coinfection with other viruses or tropical infectious diseases, and neurological complications (e.g., altered consciousness, seizures, paresis, and coma resulting from encephalitis and encephalopathy) [91–98]. The neurological manifestations secondary to dengue infection including encephalopathy, encephalitis, myelitis, neuro-ophthalmic complications, polyradiculopathy, neuropathy, and neuromuscular complications were ascribed in 0.5–21% of hospitalized patients [92, 94, 95, 99]. Possible causes of encephalopathy in patient with dengue include hypotension, cerebral edema, focal hemorrhage, hyponatremia, fulminant hepatic failure, and the direct invasion of dengue virus in the central nervous system [100, 101]. Acute renal failure is an accompanying presentation in DSS or dengue-associated fulminant hepatic failure. Previous studies revealed that 5.5% of the patients with DHF/DSS also had dual infection (e.g., urinary tract infection, diarrhea, or bacteremia) [48, 102]. Dual infection should be suspected in patients who have atypical manifestations, for example, fever for more than 10 days, mucus diarrhea, jaundice, persistent abdominal pain, recurrent fever, WBC $>$ 10,000/mm^3 ($>10 \times 10^9$/L) with neutrophilia, or the presence of the band form of neutrophil and acute renal failure [103]. The patient with severe dengue infection may have secondary bacterial sepsis, e.g., bacteremia,

and UTI after hospitalization. Failure in making a diagnosis of concurrent infection in patients with dengue may lead to otherwise preventable mortality [104].

4 Diagnosis

Attempts to differentiate dengue infection clinically from other acute febrile illnesses are unlikely to be successful; although, the diagnosis is aided if laboratory examination indicates leukopenia, thrombocytopenia, or mildly elevated AST levels. Early definite diagnosis of dengue infection can help clinicians in initiation of early supportive care, adequate fluid administration, and identification of patients with severe dengue who should be closely monitored for signs of plasma leakage, bleeding, and organ damage. This information might promote early supportive therapies, prevent the use of potentially harmful drugs, encourage assessment of complications, ensure the adequate use of treatment guidelines, and lead to the effective control of dengue outbreaks. The tourniquet test considered by the WHO has been used as a clue for probable dengue infection for a long time [35]. Unfortunately, the sensitivity and specificity of tourniquet test were not excellent, ranging between 34 and 56% and 68 and 94%, respectively, and a negative test does not exclude the disease [105–107].

Laboratory diagnosis of dengue infection is established either directly by isolation or detection of viral components in serum or tissue or indirectly by detection of virus-specific antibodies in serum [108]. The sensitivity of each approach is influenced by the duration and severity of the patient's illness. Within the first 2–3 days of illness, only reverse transcription polymerase chain reaction (RT-PCR) or dengue virus NS1 Ag assay can reliably confirm the diagnosis of dengue. Determination of dengue virus by RT-PCR in serum, tissues, saliva, or urine is definitely the most satisfactory test that might detect dengue viruses up to the 7th day after the onset of the symptoms, especially in severe cases [109–111]. A high circulating level of dengue virus NS1 was demonstrated in the early stage of dengue infection by different ELISAs in the plasma and/or sera of dengue patients [112]. Until now, ELISA used to detect acute phase (IgM), and convalescent phase (IgG) antibodies have been considered the most useful test for dengue diagnosis due to its high sensitivity and ease of use. Because dengue antibodies are better detected around the 5th day after the onset of the illness, antibody detections are unfeasible for rapid diagnosis. There are several commercial kits of rapid tests of IgM and IgG detection, but the sensitivity, specificity, and accuracy vary among these tests [113, 114]. Various combination tests for elevated levels of NS1 and dengue IgM/IgG in serum have yielded sensitivities between 75.5 and 92.9% with specificities ranging from 75.0 to 100% and are a pragmatic diagnostic approach in a patient in whom dengue infection is suspected. It should be stressed that in dengue-endemic areas, while early accurate laboratory tests are not widely available, dengue infection should be considered in every children and adults presenting with an acute undifferentiated febrile illness.

5 Management

No specific therapeutic agent to treat dengue infection is available, so treatment remains supportive care with particular emphasis on careful fluid administration. The early recognition of warning signs, plasma leakage, abnormal bleeding, signs of circulatory collapse, and other serious complications would reduce morbidity/mortality rates in patients with dengue infection. Dengue patient without warning signs may be treated at home with oral hydration and antipyretics with instructions to follow up at out-patient care and return to the hospital immediately if bleeding or warning signs suggestive of severe dengue develop. Oral rehydration is indicated to replace losses from vomiting and high fever. Acetaminophen, salicylates, nonsteroidal anti-inflammatory drugs (NSAIDs), and traditional medicines are commonly prescribed to febrile patients that these medications may cause severe bleeding or hepatic injury in dengue patients. Development of any warning signs indicates the need for close observation and hospitalization with appropriate use of intravenous fluids in patients with inadequate oral intake or a rapidly increasing hematocrit [35]. Monitoring all these patients for the development of warning signs of severity as recommended by WHO may impose a great burden on healthcare services in limited-resource countries. However, the patients with dengue infection should be hospitalized immediately if any of the followings are observed: severe nausea/vomiting, restlessness or lethargy, severe hemorrhage (e.g., hematemesis or hematochezia), narrowing of pulse pressure ($\leq$20 mmHg) or hypotension, sudden rise in hematocrit or continuous elevated hematocrit despite the administration of fluid, a platelet count of $\leq$20,000/mm^3 ($\leq 20 \times 10^9$/L), AST or ALT >500 U/mL, oliguria or acute renal failure, liver failure, heart failure, severe hypoxemia, pregnancy, and no opportunity to be followed up in an out-patient setting [115, 136].

Attentive clinical monitoring of patients with severe dengue or suspected DHF/DSS and intensively supportive treatment are lifesaving and have reduced fatality rates. The critical activities for hospitalized dengue patients are monitoring of abnormal bleeding, circulation, and vascular leakage by serial clinical assessments of hypovolemia/shock and rising of hematocrit to trigger intravenous fluid or blood component transfusion. Close monitoring measurements (e.g., vital signs, urine output, and serial hematocrit levels) to assess the severity of plasma leakage and promptly effective intravenous fluid resuscitation with crystalloid to counteract massive plasma leakage are required in critical stage of DHF to reduce morbidity and mortality. Patients with DHF need to be monitored closely for signs of shock until at least 24–48 h after defervescence. For patients suffering from plasma leakage with shock (DSS), the mainstay of therapy is early and effective replacement of plasma loss. The WHO recommends immediate volume replacement with Ringer's lactate, or physiologically normal saline solution, followed by plasma expander such as fresh frozen plasma or colloid solutions (albumin and dextran) in the event that shock persists [116]. Therapeutic responses to colloid and crystalloid solutions from two randomized controlled studies revealed that Ringer's lactate performed the least well and that the more severely ill patients identified by a narrow pulse pressure would benefit more from initial resuscitation with colloid solution than with

crystalloid solution [117–119]. In order to assess adequate volume replacement, the rate of intravenous fluid should be adjusted throughout the period of plasma leakage by frequent assessments of vital signs, hematocrit, and urine output and be kept to the minimum required to maintain cardiovascular stability until permeability reverts to a normal level. In cases with persistent shock despite a declining hematocrit after fluid resuscitation with crystalloid or colloid solutions, internal or concealed bleeding should be suspected. Transfusion therapy with packed red cell, concentrated platelets, and fresh frozen plasma to correct the bleeding tendency, anemia, coagulopathy, and hypovolemia is still the mainstay of treatment of severe bleeding in dengue patients. The prophylactic blood or platelet transfusions for severe thrombocytopenia may be harmful and should be avoided in uncomplicated cases [120]. The invasive procedures should be minimized to avoid hemorrhagic complications. Metabolic acidosis and hyponatremia occur more commonly in DSS, so sodium bicarbonate infusion should be considered along with early adequate fluid replacement. Medical comorbidities in adult and elderly patients, for example, coronary artery disease, peptic ulcer, hypertension, diabetes mellitus, cirrhosis, or chronic kidney disease, which should be considered for proper management, may contribute to the severity of dengue infection [55, 97, 121–123]. There is no evidence to support the use of chloroquine, corticosteroid, interferon, immune globulin, desmopressin, or carbazochrome sodium sulfonate (AC-17) for severe dengue infection [124–127]. With intensive support through the critical period of illness, spontaneous resolution of vasculopathy and circulatory failure usually can be expected within 2–3 days with complete recovery afterward. In the recovery period, the patients usually have more appetite, bradycardia, and convalescent rash and may have fatigue or mood disturbance for several weeks.

6 Prevention

To reduce burden of dengue, the WHO has set out specific objectives in global dengue control strategy: estimation of the true burden of dengue by 2015 and a reduction of dengue morbidity and mortality by 2020 by at least 25 and 50%, respectively (using 2010 as the baseline) [128]. It seems clear that implementations of effectively sustainable vector control and effective dengue vaccines are keys to success for this disease control. The WHO has recommended integrated vector management (IVM), an evidence-based approach which encourages optimal use of resources by examining local in-country evidence. Challenges of vector management remain in the development and deployment of vector-control strategies that effectively minimize dengue replication and transmission Dengue prevention currently relies on public health and community-based *A. aegypti* control programs with chemical or biological methods to remove and destroy mosquito-breeding sites [129]. Because this IVM approach has not succeeded in most of the Asian countries especially dengue-endemic regions, the new tools are needed to prevent and control dengue, including the development of a safe and efficacious dengue vaccine. The potential dengue vaccine is a vaccine consisting of a tetravalent combination of attenuated dengue strains, which

simultaneously induce protective and durable immune responses against all four dengue serotypes. Recent studies in Asia and Latin America show that recombinant live-attenuated tetravalent dengue vaccine (CYD-TDV) was safe and moderately efficacious when given three injections at months 0, 6, and 12 to children and adolescents [130, 131]. Overall vaccine efficacy of CYD-TDV was estimated to be 60% against virologically confirmed dengue (VCD) infection, with high levels of protection offered against hospitalization (80%) in subjects aged 2–16 years. However, variations of vaccine efficacy against VCD were observed in endemic settings, dengue serotype, and individual's pre-existing dengue antibody [130, 131]. Recent pooled analyses of the first 2–3 years of long-term follow-up provided further supportive evidence of efficacy against hospitalized dengue in children 9 years of age or older [132]. This vaccine has recently obtained licensure for use in children 9 years of age or older (9–45 years old) in Mexico, the Philippines, Brazil, Thailand, Singapore, and several other endemic countries. In view of the high disease burden in endemic countries, this vaccine, despite moderate overall efficacy, could have a substantial effect on public health [133–135]. The implementation of an efficacious dengue vaccine will shift the burden of disease; the age-related differences in clinical manifestations and prognoses described here indicate the importance of comparing a wide range of ages in future clinical studies of dengue.

7 Conclusion

Increase in the number of dengue cases is a major public health problem in many countries in South and Southeast Asia where *A. aegypti* and *A. albopictus* are widespread in both urban and rural areas. Several countries in Asia have reported an epidemic shift of dengue from mainly affecting children to affecting adolescents and young adults with increased severity. The clinical spectrum of dengue ranges from mild illness (undifferentiated fever and DF) to the life-threatening infection (DHF/DSS, severe bleeding, and multi-organ failure), which may be fatal. The early recognition of warning signs, plasma leakage, abnormal bleeding, circulatory collapse, and other serious complications would reduce mortality rates in patients with dengue infection. The implementations of effectively integrated vector management and efficacious dengue vaccines are keys to success for disease control in hyperendemic/endemic areas.

Conflicts of Interest Both authors have no conflicts of interest to declare.

References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496:504–7.
2. Kyle J, Harris E. Global spread and persistence of dengue. Annu Rev Microbiol. 2008;62:71–92.

3. Cummings DA, Iamsirithaworn S, Lessler JT, McDermott A, Prasanthong R, Nisalak A, et al. The impact of the demographic transition on dengue in Thailand: insights from a statistical analysis and mathematical modeling. PLoS Med. 2009;6(9):e1000139.
4. Guzman MG, Kouri G. Dengue and dengue hemorrhagic fever in the Americas. J Clin Virol. 2003;27:1–13.
5. Barbazan P, Yoksan S, Gonzalez JP. Dengue hemorrhagic fever epidemiology in Thailand: description and forecasting of epidemics. Microb Infect. 2002;4:699–705.
6. Nakhapakorn K, Tripathi NK. An information value based analysis of physical and climatic factors affecting dengue fever and dengue haemorrhagic fever incidence. Int J Health Geogr. 2005;4:13.
7. Anyamba A, Chretien JP, Small J, Tucker CJ, Linthicum KJ. Developing global climate anomalies suggest potential disease risks for 2006 – 2007. Int J Health Geogr. 2006;5:60.
8. Pang T, Thiam DGY, Tantawichien T, Ismail Z, Yoksan S. Dengue vaccine-time to act now. Lancet (letter). 2015;385:1725–6.
9. Charoensook O, Foy HM, Teeraratkul A, Silarug N. Changing epidemiology of dengue hemorrhagic fever in Thailand. Epidemiol Infect. 1999;122:161–6.
10. Pongsumpun P, Yoksan S, Tang IMA. Comparison of the age distributions in the dengue hemorrhagic fever epidemic in Santiago de Cuba (1997) and Thailand (1998). Southeast Asian J Trop Med Public Health. 2002;33(2):255–8.
11. Pancharoen C, Kulwichit W, Tantawichien T, Thisyakorn U, Thisyakorn C. Dengue infection: a global concern. J Med Assoc Thai. 2002;85(suppl 1):S25–33.
12. Kulanatne SAR, Gawarammana IB, Kumarasiri PRV. Epidemiology, clinical features, laboratory investigations and early diagnosis of dengue fever in adults. Southeast Asian J Trop Med Public Health. 2005;36(3):686–92.
13. Patumanond J, Tawichasri C, Nopparat S. Dengue hemorrhagic fever, Uttaradit, Thailand. Emerg Infect Dis. 2003;9:1348–50.
14. Kittigul L, Pitakarnjanakul P, Sujirarat D, Siripanichgon K. The differences of clinical manifestations and laboratory findings in children and adults with dengue virus infection. J Clin Virol. 2007;39:76–81.
15. Hanafusa S, Chanyasanha C, Sujirarat D, Khuankhunsathid I, Yaguchi A, Suzuki T. Clinical features and differences between child and adult dengue infections in Rayong Province, southeast Thailand. Southeast Asian J Trop Med Public Health. 2008;39:252–9.
16. Namvongsa V, Sirivichayakul C, Songsithichok S, Chanthavanich P, Chokejindachai W, Sitcharungsi R. Difference in clinical features between children and adult with dengue hemorrhagic shock syndrome. Southeast Asian J Trop Med Public Health. 2013;44(5):72–9.
17. Jelinek T. Dengue fever in international travelers. Clin Infect Dis. 2000;31(1):144–7.
18. Brien D, Tobin S, Brown GV, Torresi J. Fever in returned travelers. Clin Infect Dis. 2001;33:603–9.
19. Stephen C, Allwinn R, Brodt HR, Knupp B, Preiser W, Just-Nübling G. Travel-acquired dengue infection: clinical spectrum and diagnostic aspects. Infection. 2002;30:225–8.
20. Pongsumpun P, Patanarapelert K, Sriprom M, Varamit S, Tang IM. Infection risk to travelers going to dengue fever endemic regions. Southeast Asian J Trop Med Public Health. 2004;35 (1):155–9.
21. Schwartz E, Weld LH, Wilde-Smith A, von Sonnenburg F, Keystone JS, Kain KC, et al. Seasonality, annual trends, and characteristics of dengue among ill returned travelers, 1997–2006. Emerg Infect Dis. 2008;14(7):1081–8.
22. Burdino E, Milia MG, Sergi G, Gregori G, Allice T, Cazzato ML, et al. Diagnosis of dengue fever in North West Italy in travelers from endemic areas: a retrospective study. J Clin Virol. 2011;51:259–63.
23. Wilder-Smith A. Dengue infections in travelers. Pediatr Int Child Health. 2012;32:28–32.
24. Leder K, Torresi J, Brownstein JS, Wilson ME, Keystone JS, Barnett E, et al. Travel-associated illness trends and clusters, 2000–2010. Emerg Infect Dis. 2013;11(7):1049–57.

25. Freedman DO, Weld LH, Kozarsky PE, Fisk T, Robins R, von Sonnenburg F, et al. Spectrum of disease and relation to place of exposure among ill returned travelers. N Engl J Med. 2006;354:119–30.
26. Massed E, Wilde-Smith A. Risk estimates of dengue in travelers to dengue endemic areas using mathematical models. J Travel Med. 2009;16(3):191–3.
27. Chen LH, Wilson ME. Transmission of dengue virus without a mosquito vector: nosocomial mucocutaneous transmission and other routes of transmission. Clin Infect Dis. 2004;39: e56–60.
28. Wagner D, With K, Huzly D, et al. Nosocomial acquisition of dengue. Emerg Infect Dis. 2004;10(10):1872–3.
29. Tan F, Loh D, Prabhakaran K. Dengue haemorrhagic fever after living donor renal transplantation. Nephrol Dial Transplant. 2005;20:447–8.
30. Tambyah PA, Koay ESC, Poon MLM, Lin R, Ong BKC. Dengue hemorrhage fever transmitted by blood transfusion. N Engl J Med. 2008;359:1526–7.
31. Mohammed H, Linnen JM, Muñoz-Jordán JL, Tomashek K, Foster G, Broulik AS, et al. Dengue virus in blood donations, Puerto Rico, 2005. Transfusion. 2008;48:1348–54.
32. Wilder-Smith A, Chen LH, Massad E, Wilson ME. Threat of dengue to blood safety in dengue-endemic countries. Emerg Infect Dis. 2009;15(1):8–11.
33. Tangnararatchakit K, Tirapanich W, Tapaneya-Olarn W, Sumethkul V, Sirachainan N, Watcharananan S, et al. Severe nonfebrile dengue infection in an adolescent after postoperative kidney transplantation: a case report. Transpl Proc. 2012;44:303–6.
34. Costa SD, da Silva GB, Jacinto CN, Martiniano LVM, Amaral YS, Paes FJVM, et al. Dengue fever among renal transplant recipients: a series of 10 cases in a tropical country. Am J Trop Med Hyg. 2015;93(2):394–6.
35. World Health Organization. Dengue, guidelines for diagnosis, treatment, prevention and control. Geneva, Switzerland: WHO; 2009.
36. World Health Organization. Dengue hemorrhagic fever: diagnosis, treatment, prevention and control. Geneva: WHO; 1997.
37. Olivera-Botello G, Coudeville L, Fanouillere K, Guy B, Chambonneau L, Noriega F, Jackson N. Tetravalent dengue vaccine reduces symptomatic and asymptomatic dengue virus infections in healthy children and adolescents aged 2–16 years in Asia and Latin America. J Infect Dis. 2016;214:994–1000.
38. Hadinegoro R, Taurel AF, Capeding MR, Tran NH, Hadinegoro SR, Tawee Chotpitayasunondh T, et al. Symptomatic dengue disease in five Southeast Asian countries: epidemiological evidence from a dengue vaccine trial. PLoS Negl Trop Dis. 2016. https://doi.org/10.1371/journal.pntd.0004918.
39. Green S, Rothman A. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. Curr Opin Infect Dis. 2006;19:429–36.
40. Mg G, Harris E. Dengue. Lancet. 2015;385:453–65.
41. Chuansumrit A, Chaiyaratana W. Hemostatic derangement in dengue hemorrhagic fever. Thromb Res. 2014;133:10–6.
42. Gamble J, Bethell D, Day NP, Loc PP, Phu NH, Gartside IB, et al. Age-related changes in microvascular permeability: a significant factor in the susceptibility of children to shock? Clin Sci (London). 2000;98(2):211–6.
43. Guzman MG, Kouri G, Bravo J, Valdes L, Vazquez S, Halstead SB. Effect of age on outcome of secondary dengue 2 infections. Int J Infect Dis. 2002;6:118–24.
44. Hammond SN, Balmaseda A, Perez L, Tellez Y, Saborío SI, Mercado JC, et al. Differences in dengue severity in infants, children, and adults in a 3-year hospital-based study in Nicaragua. Am J Trop Med Hyg. 2005;73:1063–70.
45. Anderson KB, Gibbons RV, Cummings DAR, Nisalak A, Green S, Libraty DH, et al. A shorter time interval between first and second dengue infections is associated with protection from clinical illness in a school based cohort in Thailand. J Infect Dis. 2014;209:360–8.

46. Guilarde AO, Turchi MD, Siqueira JB, Feres VC, Rocha B, Levi JE, et al. Dengue and dengue hemorrhagic fever among adults: clinical outcomes related to viremia, serotypes, and antibody response. J Infect Dis. 2008;197:817–24.
47. Wichmann O, Hongsiriwan S, Bowonwatanuwang C, Chotivanich K, Sukthana Y, Pukrittayakamee S. Risk factors and clinical features associated with severe dengue infection in adults and children during the 2001 epidemic in Chonburi, Thailand. Trop Med Int Health. 2005;72(2):221–6.
48. Tantawichien T. Dengue fever and dengue haemorrhagic fever in adolescents and adults. Paediatr Int Child Health. 2012;32(S1):22–7.
49. Tantawichien T. Dengue fever and dengue hemorrhagic fever in adults. Southeast Asian J Trop Med Public Health. 2015;46(1):79–98.
50. Souza LJ, Pessanha LB, Mansur LC. Comparison of clinical and laboratory characteristics between children and adults with dengue. Braz J Infect Dis. 2013;17(1):27–31.
51. Agarwal R, Kapoor S, Nagar R, Misra A, Tandon R, Mathur A, et al. A clinical study of the patients with dengue hemorrhagic fever during the epidemic of 1996 at Lucknow, India. Southeast Asian J Trop Med Public Health. 1999;30(4):735–40.
52. Anuradha S, Singh NP, Rizvi SNA, Agarwal SK, Gur R, Mathur MD. The 1996 outbreak of dengue hemorrhagic fever in Delhi, India. Southeast Asian J Trop Med Public Health. 1998;29(3):503–6.
53. Tsai CJ, Kuo CH, Chen PC, Changcheng CS. Upper gastrointestinal bleeding in dengue fever. Am J Gastroenterol. 1991;86(1):33–5.
54. Pungjitprapai A, Tantawichien TA. fatal case of spontaneous rupture of the spleen due to dengue virus infection: case report and review. Southeast Asian J Trop Med Public Health. 2008;39:383–6.
55. Rigau-Perez JG, Laufer MK. Dengue-related deaths in Puerto Rico, 1992–1996: diagnosis and clinical alarm signals. Clin Infect Dis. 2006;42:1241–6.
56. Kuo MC, Chang JM, Lu PL, Chiu YW, Chen HC, Hwang HJ. Case report: difficulty in diagnosis and treatment of dengue hemorrhagic fever in patients with chronic renal failure. Am J Trop Med. 2007;76(4):752–6.
57. Lee IK, Liu JW, Yang KD. Clinical and laboratory characteristics and risk factors for fatality in elderly patients with dengue hemorrhagic fever. Am J Trop Med Hyg. 2008;79(2):149–53.
58. Gautret P, Gaudart J, Leder K, Schwartz E, Castelli F, Lim PL, et al. Travel-associated illness in older adults (>60 y). J Travel Med. 2012;19(3):169–77.
59. Pang J, Salim A, Lee VJ, Hibberd ML, Chia KS, Leo YS, et al. Diabetes with hypertension as risk factors for adult dengue hemorrhagic fever in a predominantly dengue serotype 2 epidemic: a case control study. PLoS Negl Trop Dis. 2012;6(5):e1641.
60. Srikiatkhachorn A, Gibbons RV, Green S, Libraty DH, Thomas SJ, Endy TP, et al. Dengue hemorrhagic fever: the sensitivity and specificity of the World Health Organization definition for identification of severe cases of dengue in Thailand, 1994-2005. Clin Infect Dis. 2010;50(8):1135–43.
61. Hadinegoro SR. The revised WHO dengue case classification: does the system need to be modified? Paediatr Int Child Health. 2012;32(Suppl. 1):33–8.
62. Leo YS, Thein TL, Fisher DA, Low JG, Oh HM, Narayanan RL, et al. Confirmed adult dengue deaths in Singapore: 5-year multi-center retrospective study. BMC Infect Dis. 2011;11:123.
63. Horstick O, Farrar J, Lum L, Martinez E, San Martin JL, Ehrenberg J, et al. Reviewing the development, evidence base, and application of the revised dengue case classification. Pathog Glob Health. 2012;106(2):94–101.
64. Prasad D, Kumar C, Jain A, Kumar R. Accuracy and applicability of the revised WHO classification (2009) of dengue in children seen at a tertiary healthcare facility in northern India. Infection. 2013;41:775–82.
65. Setcawan MW, Samsi TK, Pool TN, Sugianto D, Wulur H, et al. Gallbladder wall thickening in dengue hemorrhagic fever: an ultrasonographic study. J Clin Ultrasound. 1995;23:357–62.

66. Srikiatkhachorn A, Krautrachue A, Ratanaprakarn W, Wongtapradit L, Nithipanya N, Kalayanarooj S, et al. Natural history of plasma leakage in dengue hemorrhagic fever: a serial ultrasonographic study. Pediatr Infect Dis J. 2007;26:283–90.
67. Wang CC, CC W, Liu JW, Lin AS, Liu SF, Chung YH, et al. Chest radiographic presentation in patients with dengue hemorrhagic fever. Am J Trop Med Hyg. 2007;77(2):291–6.
68. Lee IK, Liu JW, Yang KD. Clinical characteristics, risk factors, and outcomes in adults experiencing dengue hemorrhagic fever complicated with acute renal failure. Am J Trop Med. 2009;80(4):651–5.
69. Miranda CH, Borges Mde C, Matsuno AK, Vilar FC, Gali LG, Volpe GJ, et al. Evaluation of cardiac involvement during dengue viral infection. Clin Infect Dis. 2013;57(6):812–9.
70. Wang CC, Liu SF, Liao SC, Lee IK, Liu JW, Lin AS, et al. Acute respiratory failure in adult patients with dengue virus infection. Am J Trop Med Hyg. 2007;77(1):151–8.
71. Chamnanchanunt S, Kanagaraj D, Thanachartwet V, Desakorn V, Rojnuckarin P. Early predictors of clinically significant bleeding in adults with dengue infection. Southeast Asian J Trop Med Public Health. 2012;12(4):890–8.
72. Wung JY, Tseng CC, Lee CS, Cheng KP. Clinical and upper gastroendoscopic features of patients with dengue virus infection. J Gastroenterol Hepatol. 1990;5(60):664–8.
73. Imbert P, Sordet D, Hovette P, Touze JE. Splenic rupture in a patient with dengue fever. Trop Med Parasitol. 1993;44(4):327–8.
74. Bunyavejchevin S, Tanawattenacharoen S, Taechakraichana N, Thisyakorn U, Tannirandorn Y, Limpaphayom K. Dengue hemorrhagic fever during pregnancy. J Obstet Gynaecol Res. 1997;23(5):445–8.
75. Corles G, Peiffer H, Talarmin A. Effects of dengue fever during pregnancy in French Guiana. Clin Infect Dis. 1999;28:637–40.
76. Thaithumyanon P, Thisyakorn U, Deerojnawong J, Innis BL. Dengue infection complicated by severe hemorrhage and vertical transmission in parturient woman. Clin Infect Dis. 1994;18:248–9.
77. Carroll D, Toovey S, Gompel AV. Dengue fever and pregnancy. Travel Med Infect Dis. 2007;5:183–8.
78. Adam I, Jumaa AM, Elbashir HM, Karsany MS. Maternal and perinatal outcomes of dengue in Port Sudan, Eastern Sudan. Virol J. 2010;7:153.
79. Basurko C, Carles G, Youssef M, Guindi WE. Maternal and fetal consequences of dengue fever during pregnancy. Eur J Obstet Gynecol Reprod Biol. 2009;147:29–32.
80. Chitra TV, Panicker S. Maternal and fetal outcome of dengue fever in pregnancy. J Vector Borne Dis. 2011;48:210–3.
81. Pouliot SH, Xiong X, Harville E, Paz-Soldan V, Tomashek KM, Breart G, et al. Maternal dengue and pregnancy outcomes: a systematic review. Obstet Gynecol Surv. 2010;65 (2):107–18.
82. Kuo CH, Tai DI, Chang-Chien CS, et al. Liver biochemical tests and dengue fever. Am J Trop Med Hyg. 1992;47(3):265–70.
83. Trung DT, Thao le TT, Hien TT, Hung NT, Vinh NN, Hien PT, et al. Liver involvement associated with dengue infection in adults in Vietnam. Am J Trop Med Hyg. 2010;83:774–80.
84. Kalayannarooj S, Vaughn DW, Nimmannitya S, Green S, Suntayakorn S, Kunentrasai N, et al. Early clinical and laboratory indicators of acute dengue illness. J Infect Dis. 1997;176:313–21.
85. Souza LJ, Alves JG, Nogueira RM, Gicovate Neto C, Bastos DA, Siqueira EW, et al. Aminotransferase changes and acute hepatitis in patients with dengue fever. Braz J Infect Dis. 2004;8(2):156–63.
86. Kittitrakul C, Silachamroon U, Phumratanaprapin W, Krudsood S, Wilairatana P, Treeprasertsuk S. Liver function tests abnormality and clinical severity of dengue infection in adult patients. J Med Assoc Thai. 2015;98(Suppl 1):S1–8.
87. Treeprasertsuk S, Kittitrakul C. Liver complications in adult dengue and current management. Southeast Asian J Trop Med Public Health. 2015;46(1):99–107.

88. Parkash O, Almas A, Jafri SM, Hamid S, Akhtar J, Alishah H. Severity of acute hepatitis and its outcome in patients with dengue fever in a tertiary care hospital, Karachi, Pakistan (South Asia). BMC Gastroenterol. 2010;10:43.
89. Kye Mon K, Nontprasert A, Kittitrakul C, Tangkijvanich P, Leowattana W, Poovorawan K. Incidence and clinical outcome of acute liver failure caused by dengue in a hospital for tropical diseases, Thailand. Am J Trop Med Hyg. 2016;95(6):1338–44.
90. Ling LM, Wilder-Smith A, Leo YS. Fulminant hepatitis in dengue haemorrhagic fever. J Clin Virol. 2007;38:265–8.
91. Thakane J, Walhelzar B, Banerjee K. Hemorrhagic manifestations and encephalopathy in case of dengue in India. Southeast Asian J Trop Med Public Health. 1996;27(3):471–5.
92. Solomon T, Dung NM, Vavghn DW, Kneen R, Thao LT, Raengsakulrach B, et al. Neurological manifestations of dengue infection. Lancet. 2000;335:1053–9.
93. Davis JS, Bourke P. Rhabdomyolysis associated with dengue virus infection. Clin Infect Dis. 2004;38:e109–11.
94. Garcia-Rivera EJ, Rigue-Perez JG. Encephalitis and dengue. Lancet. 2002;360:261.
95. Misra UK, Kalita J, Syam UK, Dhole TN. Neurological manifestations of dengue virus infection. J Neurol Sci. 2006;244:117–22.
96. Promphan W, Sopontamanarak S, Prvekprasert P, Kajornwattanakul W, Kongpattanayothin A. Dengue myocarditis. Southeast Asian J Trop Med Public Health. 2004;35(3):611–9.
97. Sam SS, Omar SFS, Teoh BT, Abd-Jamil J, AbuBakar S. Review of dengue hemorrhagic fever fatal cases seen among adults. PLoS Negl Trop Dis. 2013;7(5):e2194.
98. Premaratna R, Bailey S, Ratnasena GN, Silva HJ. Dengue fever mimicking acute appendicitis. Trans R Soc Trop Med Hyg. 2007;101:683–5.
99. Carod-Artal FJ, Wichmann O, Farrar J, Gasc J. Neurological complications of dengue virus infection. Lancet Neurol. 2013;16:906–19.
100. Lum LCS, Lam SK, Choy YS, et al. Dengue encephalitis: a true entity? Am J Trop Med Hyg. 1996;54(3):256–9.
101. Chokephaiblukit K, Kankirawatna P, Apintanapong S, Pongthapisit V, Yoksan S, Kositanont U, et al. Viral etiologies of encephalitis in Thai children. Pediatr Infect Dis J. 2001;20(2):216–8.
102. Pancharoen C, Thisyalorn U. Coinfection in dengue patients. Pediatr Infect Dis J. 1998;17(1):81–2.
103. Lee IK, Liu JW, Yang KD. Clinical characteristics and risk factors for concurrent bacteremia in adults with dengue hemorrhagic fever. Am J Trop Med Hyg. 2005;72(2):221–6.
104. Trunfio M, Savoldi A, Viganò O, Monforte AD. Bacterial coinfections in dengue virus disease: what we know and what is still obscure about an emerging concern. Infection. 2016. https://doi.org/10.1007/s15010-016-0927-6.
105. Gregory CJ, Lorenzi OD, Colón L, García AS, Santiago LM, Rivera RC, et al. Utility of the tourniquet test and the white blood cell count to differentiate dengue among acute febrile illnesses in the emergency room. PLoS Negl Trop Dis. 2011;5(12):e1400.
106. Halsey ES, Vilcarromero S, Forshey BM, Rocha C, Bazan I, Stoddard ST, et al. Performance of the tourniquet test for diagnosing dengue in Peru. Am J Trop Med Hyg. 2013;89(1):99–104.
107. Mayxay M, Phetsouvanh R, Moore CE, Chansamouth V, Vongsouvath M, Sisouphone S, et al. Predictive diagnostic value of the tourniquet test for the diagnosis of dengue infection in adults. Trop Med Int Health. 2011;16(1):127–33.
108. de Oliveira Poersch C, Pavoni DP, Queiroz MH, de Borba L, Goldenberg S, dos Santos CN, et al. Dengue virus infections: comparison of methods for diagnosing the acute disease. J Clin Virol. 2005;32:272–7.
109. Yamada KI, Takasaki T, Nawa M, Kurane I. Virus isolation as one of the diagnostic methods for dengue virus infection. J Clin Virol. 2002;24:203–9.
110. Lanciotti RS. Molecular amplification assays for detection of flaviviruses. Adv Virus Res. 2003;61:67–99.

111. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microl. 2002;40:376–81.
112. Vazqueza S, Ruiza D, Barreroa R. Kinetics of dengue virus NS1 protein in dengue 4-confirmed adult patients. Diagn Microbiol Infect Dis. 2010;68:46–9.
113. Kittigul L, Suankeow K. Use of a rapid immunochromatographic test for early diagnosis of dengue virus infection. Eur J Clin Microbiol Infect Dis. 2002;21:224–6.
114. Blacksell SD, Newton PN, Bell D, Kelley J, Mammen MP Jr, Vaughn DW, et al. The comparative accuracy of 8 commercial rapid immunochromatographic assays for the diagnosis of acute dengue virus Infection. Clin Infect Dis. 2006;42:1127–34.
115. Royal College Physician of Thailand. Practical guideline for management of dengue in adult. Southeast Asian J Trop Med Public Health. 2015;46(1):169–81.
116. Nimmannitaya S. Clinical spectrum and management of dengue haemorrhagic fever. Southeast Asian J Trop Med Public Health. 1987;20:325–30.
117. Dung NM, Day NP, Tam DT, Loan HT, Chau HT, Minh LN, et al. Fluid replacement in dengue shock syndrome: a randomized, double-blind comparison of four intravenous-fluid regimens. Clin Infect Dis. 1999;29:787–94.
118. Wills BA, Dung NM, Loan HT, et al. Comparison of three fluid solutions for resuscitation in dengue shock syndrome. N Engl J Med. 2005;353(9):877–89.
119. Akech S, Ledermann H, Maitland K. Choice of fluids for resuscitation in children with severe infection and shock: systematic review. Br Med J. 2011;341:c4416.
120. Lye DC, Lee VJ, Sun Y, Leo YS. Lack of efficacy of prophylactic platelet transfusion for severe thrombocytopenia in adults with acute uncomplicated dengue infection. Clin Infect Dis. 2009;48(9):1262–5.
121. Figueiredo MAA, Rodrigues LC, Barreto ML, Lima JWO, Costa MCN, Morato V, et al. Allergies and diabetes as risk factors for dengue hemorrhagic fever: results of a case control study. PLoS Negl Trop Dis. 2010;4(6):e699.
122. Lye DC, Lee VJ, Sun Y, Leo YS. The benign nature of acute dengue infection in hospitalized older adults in Singapore. Int J Infect Dis. 2010;14:e410–3.
123. Kuo MC, Chang JM, PL L, Chiu YW, Chen HC, Hwang SJ. Case report: difficulty in diagnosis and treatment of dengue hemorrhage fever in patients with chronic renal failure. Am J Trop Med Hyg. 2007;76(4):752–6.
124. Tassniyom S, Vasanawathana S, Chirawathul A, Rojanasuphot S. Failure of high-dose methylprednisolone in established dengue shock syndrome. Pediatrics. 1993;92(1):111–5.
125. Tassniyom S, Vasanawathana S, Dhiensiri T, Nisalak A, Chirawatkul A. Failure of carbazocrome sodium sulfonate (AC-17) to prevent dengue vascular permeability or shock. J Pediatr. 1997;131(4):525–8.
126. Tricou V, Minh NN, Van TP, Lee SJ, Farrar J, Wills B, et al. A randomized controlled trial of chloroquine for the treatment of dengue in Vietnamese adults. PLoS Negl Trop Dis. 2010;4(8):e785.
127. Kularatne SAM, Walathara C, Mahindawansa I, Wijesinghe S, Pathirage MKK, Kumarasiri PVR, et al. Efficacy of low dose dexamethasone in severe thrombocytopenia caused by dengue fever: a placebo controlled study. Postgrad Med J. 2009;85:525–9.
128. World Health Organization. Global strategy for dengue prevention and control 2012-2020. www.who.int
129. World Health Organization, Regional Office for South-East Asia. Comprehensive guidelines for prevention and control of dengue and dengue haemorrhagic fever. SEARO Technical Publication Series No. 60. 2011.
130. Capeding MR, Tran NH, Hadinegoro SRS, Ismail HIHJM, Chotpitayasunondh T, Chua MN, et al. Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. Lancet. 2014;384:1358–65.

131. Hadinegoro SR, Arredondo-García JL, Capeding MR, Deseda C, Chotpitayasunondh T, Dietze R, et al. Efficacy and long-term safety of a dengue vaccine in regions of endemic disease. N Engl J Med. 2015;373:1195–206.
132. Guya B, Briand O, Lang J, Saville M, Jackson N. Development of the Sanofi Pasteur tetravalent dengue vaccine: one more step forward. Vaccine. 2015;33:7100–11.
133. Endo IC, Ziegelmann PK, Patel A. The economic promise of developing and implementing dengue vaccines: evidence from a systematic review. Vaccine. 2016;34(50):6133–47.
134. López-Gatell H, Alpuche-Aranda CM, Santos-Preciado JI, Hernández-Ávila M. Dengue vaccine: local decisions, global consequences. Bull WHO. 2016;94:850–5.
135. Pang T. SAGE committee advice on dengue vaccine. Lancet Infect Dis. 2016:880–1.
136. Ngo NT, Cao XT, Kneen R, Wills B, Nguyen VM, Nguyen TQ, et al. Acute management of dengue shock syndrome: a randomized double-blind comparison of 4 intravenous fluid regimens in the first hour. Clin Infect Dis. 2001;32:204–12.

Human Rabies in South Asia

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Contents

1 Introduction

Rabies is an acute progressive encephalomyelitis caused by any of the viruses from the genus *Lyssavirus*, family *Rhabdoviridae*. One of the most feared diseases known to have plagued mankind since antiquity, rabies is the most lethal infection

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known to science. Despite the availability of prophylactic biologics since 1885, about 61,000 humans worldwide continue to die of this zoonotic disease every year, mostly in Asia and Africa [1]. It continues to be a neglected disease in most of the rabies-endemic countries of South Asia, which is the geographic epicenter of this serious public health problem.

2 Epidemiology

Currently, the International Committee on the Taxonomy of Viruses (ICTV) recognizes 14 species in the genus *Lyssavirus,* subdivided into two phylogroups based on genetic distances and serologic cross-reactivity [2]. Phylogroup I includes the species rabies virus (RABV); European bat lyssaviruses, type 1 (EBLV-1) and type 2 (EBLV-2); Duvenhage virus (DUVV), Australian bat lyssavirus (ABLV); Aravan virus (ARAV); Khujand virus (KHUV); Bokeloh bat lyssavirus (BBLV); and Irkut virus (IRKV). Phylogroup II includes Lagos bat virus (LBV), Mokola virus (MOKV), and Shimoni bat virus (SHIBV). The other viruses, West Caucasian bat virus (WCBV), Ikoma lyssavirus (IKOV), and an additional representative Lleida bat virus (LLEBV) which does not yet have a taxonomic status, are not included in either of these phylogroups. Dogs, carnivores, and bats are implicated in the maintenance of RABV; bats are also known reservoirs and vectors for the rest of the recognized lyssavirus species, except MOKV and IKOV where the reservoirs remain undetermined. In addition to RABV which is responsible for a majority of human rabies cases globally, six other lyssaviruses have been associated with human disease: MOKV, DUVV, ABLV, EBLV-1 and -2, and IRKV. Almost all human rabies in South Asia is caused by RABV, since there are no documented human cases caused by other lyssaviruses. Several phylogenetic clades of RABV notably of the arctic/arctic-like and the Indian subcontinent lineages circulate in South Asia [3].

Although evolutionary analyses indicate that lyssaviruses most likely originated in bats, there is limited information about RABV and other lyssaviruses in bats in South Asia due to lack of systematic surveillance. Isolation of RABV from fruit bats in Thailand and lyssaviruses in an Indian flying fox and from frugivorous bats in Sri Lanka was reported [4–6]. Serological evidence of lyssavirus infection in bats has been reported from Bangladesh and India [7, 8]. Human infection with uncharacterized lyssaviruses acquired through bat exposures has been reported from India [9].

2.1 Human Rabies in South Asia

The South Asian Association for Regional Cooperation (SAARC) is an economic and geopolitical organization of Afghanistan, Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan, and Sri Lanka. All countries are rabies endemic except Maldives and the islands of Andaman, Nicobar, and Lakshadweep in India. The SAARC region

Table 1. Rabies: annual disease burden in South Asian countries [13, 102]

Country	Estimated human rabies deaths	Estimated human rabies cases per 100,000 population	% of dog bites in total animal bite cases
India	18,000–20,000	3	>95%
Pakistan	2000–5000	1.3	>90%
Afghanistan	2000–3000	5.7	Not available
Bangladesh	1500–2000	1.5	95%
Nepal	100–150	0.21	98.5%
Sri Lanka	<50	0.26	95%
Bhutan	<5	0.07	99%
Maldives	0	0	0

is one of the most densely populated regions of the world, comprising about 3% of the world's area and 21% of the world's total population. SAARC countries contribute 45% of the global burden of human rabies, and more than 1.5 billion people in these countries are at potential risk of the disease. An estimated 4 million people receive rabies vaccination following exposure to suspected rabid animals every year, a considerable economic drain on SAARC economies [10].

India accounts for over 70% of the area and population of the SAARC region and has the highest burden of human rabies in the world. Based on the estimated number of human cases, Afghanistan is reported to have the highest incidence at 5.7 per 100,000 population, followed by India, Bangladesh, and Pakistan (Table 1). Rabies is not a notifiable disease in most countries. Moreover systematic surveillance for human and animal rabies is not done in most countries, masking the true magnitude of the problem. The World Health Organization reinitiated annual reporting of human rabies in 2015 [11]. South Asia is also a frequent tourist destination and a major source of guest workers globally and as such contributes human cases to other global regions [12].

3 Pathogenesis

Rabies is a rapidly progressive encephalomyelitis, usually transmitted by virus-laden saliva of animals through a bite or break in the skin or mucous membranes. The incubation period of rabies is prolonged, usually 2 weeks to 3 months, but with incubation periods that may extend to 8 years [14]. The incubation period for rabies is longer than the 14-day period required to vaccinate against rabies. This observation by Louis Pasteur in the 1880s rendered rabies the world's second vaccine-preventable disease (after smallpox).

In describing rabies pathogenesis, it is important to note that wild-type rabies virus (so-called street rabies virus) varies considerably in biology from laboratory-adapted strains (so-called fixed rabies virus). Street rabies viruses are highly neuronotropic and replicate at low levels with prolonged and highly variable incubation periods that

evolutionarily benefit a highly virulent infection. Wild-type rabies does not cause prominent cytopathic effects or generate a significant immune response before death [15, 16]. In contrast, fixed rabies viruses replicate promiscuously within neurons, glia, and immune cells, have a relatively constant (fixed) incubation period, and cause neuronal and lymphocyte death through apoptosis [17–19]. Fixed rabies viruses are highly inflammatory and therefore well suited as killed vaccines administered intradermally or intramuscularly to generate systemic immunity [1].

Rabies viruses vary phylogenetically, with clearly appreciable differences in species tropism and virulence [20]. RABV is exquisitely neuronotropic, with limited replication in the muscle (canine rabies), skin (bat rabies), and salivary and lacrimal glands (for transmission). Cellular tropism is dependent on the surface glycoprotein (G) of the enveloped virus [21]. Neutralization epitopes on the G protein are well defined; some are conformational [22]. Most protective immunity is humoral and directed at the G glycoprotein [23]. Several host cell receptors have been identified using fixed RABV. The nicotinic acetylcholine receptor (nAchR) on muscle end plates is best characterized, for canine rabies. The surface receptors and/or permissive cells in the skin, and the central nervous system (CNS) receptors, are defined by fixed virus strains [24]. The $p75^{NTF}$ receptor may couple rabies virions for retrograde axonal transport [25]. Infection proceeds by endocytosis, in a retrograde fashion from axon to cell body to dendrites to next axon. Wild-type RABV is propagated almost exclusively trans-synaptically [26]. For that reason, RABV is used by neuroanatomists to precisely map synaptic connections [27]. Pseudoviruses incorporating the RABV glycoprotein are sufficient to deliver recombinant nucleic acids and proteins to neurons [28]. Pseudoviruses may replace conventional neutralization assays of RABV because of their improved safety, enhancing prospects for more national rabies reference laboratories [23].

RABV is internalized in motor neurons primarily by clathrin-mediated endocytosis in acidic vesicles [29]. The virus envelope is transported by retrograde axonal transport at high velocities (8.0 cm/day) in $p75^{NTR}$-containing endosomal vesicles to the neuronal cell body [25]. RABV-encoded G glycoprotein has been associated with retrograde axonal transport of rabies virions [30]. The P phosphoprotein includes a LC8 dynein binding motif but is not involved in transport, rather replication [31]. Most rabies replication occurs in the neuronal body [32]. The ribonucleoprotein (RNP) consists of a phosphoprotein (P), a reverse polymerase (L), and a nucleoprotein (N) matrix. The RNP aggregates in virus factories that are microscopically apparent as Negri bodies [33].

The genetic components of the RABV that contribute to pathogenesis, other than the G protein, are incompletely known. The meta-genomic contribution of the host is undefined but clearly important, because the virus genome does not determine furious and paralytic forms of rabies [34]. Surface expression of the G glycoprotein is limited, thereby evading detection by the immune system and correlating with neurovirulence; overexpression of the G glycoprotein also induces apoptosis [35]. There are no virus genomic studies of the cardiac tropism common in dog but not bat rabies, but the RABV P phosphoprotein determines RABV replication in muscle [36]. The viral genetic basis of immunological differences in responses to

dog and bat rabies is unknown. The P protein has been well studied in fixed rabies models and inhibits type I interferon synthesis and signaling; wild-type strains remain interferon sensitive, and interferon pathways are induced in human brain homotypic cultures by wild-type RABV (unpublished data, RW) [37].

While mathematically improbable based on the salivary load of RABV and the abundance of nAChR, half of rabies bites do not result in rabies. This may be due to interferon induction or abortive rabies. Trauma to wounds induces interferons that render the traumatized tissues less permissive to RABV replication [38]. Type I interferons control RABV replication in muscle [36]. The inflammatory effects of bacterial superinfection of bite wounds on RABV replication is not known [39]. Suturing of the wound increases the rate of rabies infection, presumably by further inoculating infected saliva distally into non-inflamed tissue. This local non-neuronal phase of RABV replication, associated with a prolonged incubation period, highlights the extreme importance of wound care in preventing rabies. Abortive rabies is controlled by cellular immunity systemically or at the level of the spinal cord [40]. RABV causes an acute flaccid paralysis syndrome (AFP) that often mimics acute inflammatory demyelinating paralysis (AIDP, Guillain-Barre-Landry syndrome) in humans. In animals, the immune response to rabies is more prominent in paralytic rabies, and survivors (abortive rabies) show permanent motor neuron damage [41]. Lack of disease despite humoral evidence of RABV infection occurs rarely, both naturally and in animal models, and is also considered abortive rabies [40]. Based on serosurveys, this may also occur in humans [42, 43]. In most instances, however, with inefficient RABV replication in non-neural tissues and viral downregulation of the cell surface expression of G glycoprotein, no immune response develops following replication in the wound and the encephalitis proceeds unchecked [16].

At the level of the first synaptic transmission of RABV in the spinal cord, several propagation pathways ensue [26]. Virus is transported retrograde along first-order motor neurons to the motor cortex. In contradistinction, rabies is transported anterograde along interconnecting neurons to autonomic and sensory nerves within the spinal cord [26]. Infection of the dorsal root ganglion does not propagate proximally, but produces marked inflammation and the characteristic radicular pain and paresthesias referring to the bitten limb.

Within the CNS, rabies spreads rapidly and trans-synaptically to involve most of the brain. It is striking how widespread infection does not lead to clinical symptoms for several days. This speaks in part to the lack of cytopathic effect, apoptosis, and inflammation in wild-type RABV infection. The temporal disconnect between RABV infection and rabies symptoms must be explained. One author (RW), based on metabolic studies of human CSF during rabies [44, 45], posits that the delay is similar to that seen in genetic metabolic disorders, characterized by gradual loss of key metabolites or accumulation of toxic metabolites.

In order to propagate as a productive infection, the RABV must then emigrate to salivary and lacrimal glands. RABV in the CNS is transported anterograde through all nerves, including motor, sensory, and autonomic, resulting in global peripheral neuropathy [46]. Myoclonus corresponding to the bitten limb is followed by generalized paresis. Airway tone, pupillary responses, and diaphragm function

are less commonly affected. The patient becomes insensate except for visual afferents. Visceral organs including the heart, adrenals, kidneys, the GI tract, and bladder are also infected by RABV at their points of innervation [46]. Infection of the vagus nerve is postulated to cause asystoles through episodic overactivity during incipient failure; by day 7 of hospitalization, the vagus is rarely active and atropine produces paradoxical vasodilation without changes in heart rate. Failure of epinephrine production by the adrenal medulla may lead to initial surges in sympathetic tone followed by hypotension as production ceases [47]. Fecal and bladder retention are seen in rabies (distinguishing rabies from Guillain-Barre-Landry syndrome). Ileus often occurs in the second week of encephalitis.

Eventually, immune surveillance detects massive infection of the CNS by RABV, and influenza-like symptoms of the clinical prodrome appear. Clinical signs of fever and inflammation must be mediated by the innate immune system because most rabies patients with rapidly progressive neurological symptoms and signs die without evidence for adaptive immunity [16].

Without critical care, patients die within 2–3 days of admission. Dehydration and ketosis may contribute to clinical demise. Tachyarrhythmias followed by cardiac arrests (asystole) are common in first 7 days after admission. Cardiac stunning is also described [48, 49]. Approximately 5–7 days after first hospitalization (i.e., after objective, severe neurological signs are noted), hyponatremia and dehydration from salt wasting develop. Increased intracranial pressure may correlate with the hyponatremia, but also with elevated concentrations of *N*-acetylaspartate, a molecular water pump, in cerebrospinal fluid (CSF) [44]. The mechanism of salt wasting has not been characterized, but mineralocorticoids palliate the process, and thyroid hormones are consistent with sick euthyroid syndrome, excluding panhypopituitarism as the cause [50]. Increased sympathetic tone to the kidney may contribute to salt wasting; the role of natriuretic peptides is unknown. Hyponatremia is often followed a day later by generalized intracranial vasospasm of the conduit arteries [50, 51]. Vasospasm is transient and without anatomic correlate at autopsy, but corresponds to clinical dysautonomia, coma, and EEG and CSF metabolic changes [52]. At this point, care of many patients is discontinued by physicians unfamiliar with rabies, but electrical activity and brain perfusion persist [53]. There is then a clinically quiet interval characterized by metabolic perturbations, but no vascular events [44].

The humoral immune response usually develops within 5–16 days of hospitalization. Vaccine failure (usually from lack of RIG administration) and administration of rabies vaccine during rabies produce very high concentrations of serum and CSF-neutralizing antibodies that can be associated with cognitive impairment and/or spasticity in survivors [54]. White matter is affected radiologically in vaccinated rabies, suggesting extension of the immune response to glial elements. In contrast, rabies survivors without rabies vaccination are often intellectually normal and with normal brain imaging [55]. Antibodies can clear some CNS viruses non-cytolytically [56].

Clearance of RABV from saliva by PCR regularly follows development of serum-neutralizing antibody, progressing linearly over 2 weeks [57] (unpublished data). Serum-neutralizing antibody also correlates with loss of cultivable RABV in skin

biopsy, with an irregular appearance of rabies antigen and loss of detectable genome by PCR. With rabies-neutralizing antibody in CSF, cultivable virus disappears over days and heterogeneous patterns of rabies antigen and genome appear [58].

Immune responses vary between dog and bat RABV phylogenies (unpublished data, RW). In dog rabies, the systemic response is rapid and intense (asymptotic over a few days), while the concurrent development of neutralizing antibodies in CSF is limited in titer. In bat rabies, the systemic response is gradual (reaching asymptote over a week), with concurrent development of CSF antibodies in CSF that often exceed serum titers. There are two side effects temporally correlated with the humoral immune response to rabies. Third-degree heart block develops in 40% of patients with dog rabies. Patients with bat rabies often develop cerebral edema associated with CSF-neutralizing antibody titers greater than 10 IU/ml.

At 12–15 days after admission, an episode of systemic dysautonomia and high cerebral arteriolar resistance occurs, associated with a fall of cerebral blood velocities and intracranial pressure [52]. The crisis is accompanied by fixed dilated pupils and development of diabetes insipidus. The EEG amplitude becomes near isoelectric. CSF protein increases. Cerebral artery blood flow becomes chaotic, cerebral edema ensues, and the patient dies. No histological changes to the arteries are noted at autopsy. This catastrophe is usually predicted by prominent dysautonomia and vasospasm on days 6–8.

4 Clinical Manifestations

4.1 Variability in Presentation

Rabies is misdiagnosed because of the prolonged incubation that challenges association of the present encephalomyelitis with an animal exposure weeks to months ago. There is also no way of detecting RABV infection while it is incubating. The clinical presentation is also highly variable. Many authors classify canine rabies into furious and paralytic forms of rabies [59]. In one author's (RW) experience with over 70 cases, the distinction of furious and paralytic forms may actually be more of a continuum. The continuum may also include milder disease (see above).

The prodromal period lasts up to a week and is flu-like, along with insomnia, restlessness, and sore throat or dysphagia. Fever is always present but episodic. Pain and severe itching or paresthesias often refer to the bitten extremity before objective neurological signs occur. Abdominal pain may raise concerns about an acute abdomen. Hallucinations may occur, prompting referral to psychiatric care or poison control. Episodes of agitation, aggression, hallucinations, or emotional or sexual arousal appear episodically and increase in frequency over time. It is characteristic to have intervals of complete normalcy in behavior and cognition in between. Focal myoclonic jerks or paralysis may develop and usually map to the bitten limb; some authors note percussion myoclonus as characteristic of rabies [59]. Orofacial dyskinesias and myokymia are common and are often misdiagnosed

as seizures. Sympathetic dysautonomia is often prominent, including sweating, tachycardia, hypertension, hypersalivation, and priapism. Saliva is often viscid and can obstruct bronchi. Hydrophobia (pharyngeal and diaphragmatic spasms when drinking water), aerophobia (precipitated by drafts or oxygen cannulae), and pneumothoraces are unique to human rabies, helpful when present, but not specific. Pain, loss of deep tendon reflexes, and weakness may ascend, suggestive of Guillain-Barre-Landry syndrome, but with urinary or fecal retention.

4.2 Differential Diagnosis

There are many other diseases that mimic rabies. It is particularly important to inquire about a prior animal bite in preceding years. In one author's experience (RW), anti-*N*-methyl-D-aspartate receptor antibody encephalitis is the most common alternative diagnosis and was often associated with documented animal bite exposures [60]. Symptoms of NMDAR encephalitis include agitation, aggression, hypersalivation, hydrophobia, and orofacial dyskinesias. Seizures are common in NMDAR encephalitis but unusual in rabies. Many arthropod-borne encephalitides (e.g., Japanese encephalitis) may also mimic rabies. While movement disorders may occur in both, prominent hypertonia is unusual in rabies and suggests an alternative diagnosis such as West Nile encephalitis. CSF and MRI imaging in rabies are near normal, unlike most arbovirus encephalitides at time of presentation. In malaria-endemic areas, up to 11% of cerebral malaria cases may be rabies [61]. The semiology of tetanus and rabies may be confused. One author (RW) has encountered herpes simplex 1 encephalitis with hypersalivation and hydrophobia in two instances. Scorpion or elapid intoxications mimic rabies, which is perhaps not surprising when alpha-bungarotoxin and rabies G glycoprotein share sequence homology [62]. Ascending paresis can represent human rabies as well as AIDP, campylobacter-associated acute motor axonal neuropathy (AMAN), or other forms of acute flaccid paralysis [63]. These can be quite difficult to distinguish, even by nerve conduction velocity studies; an axonal neuropathy favors rabies, West Nile virus, or other cause of AFP and is a contraindication to the use of IVIG or corticosteroids.

4.3 Stages of Rabies

Classically, five stages are described, including incubation, prodrome, acute encephalitis, coma, and death. Further stages are evident with critical care, broken into week-long periods. Cardiac arrests are common in the first week of hospitalization, and hyponatremia and coma supervene 6–8 days after first hospitalization. The rabies patient is usually fully paralyzed and insensate by 10 days of hospitalization. The neurological examination is essentially worthless during the encephalitis and coma phases because of the prominent peripheral neuropathies of rabies. The examination is

often confused with brainstem death unless EEG or cerebral perfusion studies are performed [64]. Dysautonomia, loss of EEG activity, pupillary dilation, and diabetes insipidus occur 12–15 days after admission and, when present, suggest futility of further medical care. Rabies patients may survive for weeks with an isoelectric EEG and with good cerebral blood flow before succumbing. Alternative criteria for medical futility have been proposed (www.mcw.edu/rabies).

Survival has been associated with an early appearance of CSF-neutralizing antibody titers in the range of 1.0–10 IU/ml (unpublished, RW). Recovery is not clinically evident in the second week after admission, but can be detected by clearance of virus RNA in saliva, alterations in DFA appearance in skin biopsies, metabolic changes in CSF, and the advent of immunological sequelae (heart block in dog rabies) [44] (www.mcw.edu/rabies). With recovery, the patient again develops orofacial dyskinesias, cough, hypersalivation, and gastric or endobronchial hypersecretion. Oddly, distal deep tendon reflexes recover before corneal or vestibular reflexes, perhaps reflecting systemic before CNS clearance. Neurological recovery requires 1–2 weeks from first detection of neutralizing antibodies in CSF but then proceeds rapidly. Movement disorders are common during rehabilitation without sapropterin (tetrahydrobiopterin) or dopamine supplementation [45]. Profound weight loss may be a challenge during recovery despite vigorous caloric supplementation [65].

5 Laboratory Diagnosis

Antemortem diagnosis of rabies has always been encouraged [1]. Prompt laboratory confirmation can help avoid unnecessary costs and aid in prognostication, decisions regarding treatment or palliation, infection control, postexposure prophylaxis to close contacts of the patient, and closure and grief counseling of family members. In countries which are working toward rabies elimination (e.g., Sri Lanka, Thailand), continual surveillance and laboratory confirmation of clinically suspected cases of rabies is essential. Additionally, laboratory testing can aid in molecular epidemiological studies.

Given increasing reports of rabies survivors, antemortem diagnosis may increasingly alter treatment and provide the indication for broad-spectrum antivirals active against the RABV. Speed of laboratory transport and testing become key. Rabies reference labs are few in number and often logistically distant [66]. Bedside immunological and virological tests are needed for therapeutic monitoring. In the near future, pseudoviruses may permit serological testing outside high containment reference laboratories [67]. Genome-, proteome-, or metabolome-based testing promises syndrome-based diagnosis of myriad infections off one limited sample [68, 69]. “Omics” instrumentation is far more ubiquitous than rabies reference laboratories, and data may be transmitted by the internet for expert analysis.

The currently used common conventional and advanced laboratory techniques for diagnosis of human rabies and their advantages and limitations are summarized in Table 2. Postmortem diagnosis of rabies is often limited by cultural or physician

Table 2 Laboratory techniques for diagnosis of human rabies

Clinical specimen	Technique	Detection of	Performance	Advantages	Limitations	References
Post-mortem diagnosis						
Brain tissue Obtained at autopsy by craniotomy	Fluorescent Antibody technique (FAT)	Viral antigen	Nearly 100% sensitive and specific	Results available within a few hours	Ideal when smears made from fresh brain tissue	[72, 73]
Other minimally invasive methods for post-mortem brain sampling (without craniotomy) are: sub-occipital cisternal puncture, retro-orbital and trans-nasal route.				WHO recommended 'Gold-standard' for laboratory confirmation of rabies post-mortem.	Results on formalin-fixed or partially decomposed/ autolysed samples less reliable.	
					Need for expensive fluorescent microscope and skilled personnel to interpret test results limits its use in many developing countries.	
	Direct Rapid Immunohistochemical Test (dRIT)	Viral antigen	Nearly 100% sensitive and specific	Comparable to the 'Gold-standard' FAT	Reagents not indigenously manufactured or commercially available for use in rabies endemic developing countries	[74]
				Easy interpretation using ordinary light microscope; Does not require fluorescent microscope		
				Frozen or glycerol preserved brain tissues can be used		
				More suitable for use in field conditions		

	Histological Identification of 'Negri Bodies'	Inclusion bodies (aggregates of viral particles) specific to rabies encephalitis demonstrated by histological tests (Seller's technique) on smears from fresh brain tissue or staining (haematoxylin and eosin) sections of paraffin embedded brain tissues	Positive in 50–71% of human infections	Seller's method on fresh brain smears simple and rapid technique	Less sensitive than immunological methods, especially in autolysed specimens. No longer recommended for primary diagnosis, both in humans and animals	[73, 75]
	Reverse Transcriptase PCR (RT-PCR)	Viral RNA by nucleic acid amplification technique	100% sensitive and specific	Valuable in cases where brain tissue obtained postmortem by trans-nasal/retro-orbital/occipital route is scanty	Requirement of expensive infrastructure and trained manpower and stringent quality control	[76–78]
				More reliable than FAT in decomposed/autolysed/archived tissues	Conventional gel electrophoresis-based PCR carries risk of amplicon contamination	
					Development of real time TaqMan probe based PCR assay challenging due to viral genetic heterogeneity	

(continued)

Table 2 (continued)

Clinical specimen	Technique	Detection of	Performance	Advantages	Limitations	References
	Mice inoculation technique (MIT) and rapid tissue culture infection test (RTCT)	Virus isolation in suckling mice or cell lines (e.g. mouse neuroblastoma)	High sensitivity and specificity	Valuable for obtaining large quantity of virus for molecular characterization and other studies	Not feasible for use as routine diagnostic assay; requires specialized laboratory and infrastructure, adequate biocontainment	[73, 79, 80]
Ante-mortem diagnosis						
Nuchal skin Obtained by excision or punch biopsy	Direct Immunoflorescence (IF) on frozen sections of skin	Viral antigen	25–70% sensitive	Can also be used for post-mortem rabies diagnosis, when brain tissue cannot be obtained	Cumbersome technique, requires multiple sections with hair follicles to be examined	[81–83]
					Cryostat required to prepare frozen sections of skin- not practicable in all settings	
	Reverse Transcriptase PCR (RT-PCR)	Viral RNA by nucleic acid amplification technique	60–98% sensitive and 100% specific	Can also be used for post-mortem rabies diagnosis, when brain tissue cannot be obtained	Requires full thickness biopsy and adequate hair follicles for increased sensitivity	[76, 84]
					Nucleic acid extraction may be challenging	
Saliva	Reverse Transcriptase PCR (RT-PCR)	Viral RNA by nucleic acid amplification technique	85–100% sensitive after serial sampling and 100% specific	Easy, non-invasive specimen collection	Multiple samples need to be tested for increased sensitivity	[76, 84, 85]
Cerebrospinal fluid (CSF)	Reverse Transcriptase PCR (RT-PCR)	Viral RNA by nucleic acid amplification technique	Low sensitivity	CSF obtained by post-mortem lumbar puncture has higher sensitivity; can be used when brain tissue cannot be obtained	Low sensitivity for antemortem diagnosis; invasive technique for specimen collection	[76, 77]
					Inversely correlates with presence of neutralizing antibodies	

	Rapid Fluorescent Focus Inhibition Test (RFFIT), and Fluorescence Antibody Virus Neutralization Test (FAVN)	Neutralizing antibodies	Depends on duration of illness; Sensitivity rises with increased duration of survival (>90% after 2 weeks)	Useful to monitor the immune response within the central nervous system and thus a possible clearance of the rabies virus in patients who are treated with experimental therapy	Limited diagnostic value due to short survival and late seroconversion	[76, 77, 86–88]
					Labour intensive test; requirement of cell culture facility and fluorescent microscope	
Blood (Serum)	Rapid Fluorescent Focus Inhibition Test (RFFIT), and Fluorescence Antibody Virus Neutralization Test (FAVN)	Neutralizing antibodies	Depends on duration of illness; Sensitivity rises with increased duration of survival (>90% after 2 weeks)	Diagnostic in individuals never vaccinated previously	Has to be interpreted with caution in previously vaccinated individuals; four fold rise in paired sera to be demonstrated for confirmation of diagnosis	[76, 77, 86–88]
					Test is sensitive to cytotoxicity in poor-quality sera; nonspecific inhibitors of virus in sera may produce false positive results.	
Corneal smear	Fluorescent Antibody technique (FAT)	Viral antigen	Low sensitivity and specificity	Easy and rapid technique	Not recommended as a routine test because of the risk of corneal scarification	[1, 75, 89]

aversion to autopsies. Percutaneous needle autopsies can substitute, albeit with increased sampling error [70]. The dRIT test is a well-performing substitute to immunofluorescent testing of the brain [71].

6 Treatment

6.1 Isolation

There has never been a laboratory-confirmed case of rabies transmission during medical care or at autopsy of a rabies patient (other than by transplantation of organs) [90]. Some countries including the Centers for Disease Control (USA) recommend standard precautions as for any patient [91]. Standard precautions is understood to include barrier precautions when there is a risk of splash of rabies-infected secretions (saliva, respiratory specimens, tears; not blood, urine, stool, semen) onto mucosa or broken skin. With the advent of rabies survivors, criteria for discontinuation of isolation are needed.

6.2 Palliation

In situations where critical care is impossible logistically or financially, then palliative care is imperative. Rabies causes profound suffering by a patient who is intermittently fully conscious and communicative. There are only two studies addressing palliative care of rabies. Haloperidol was shown superior to other treatments of rabies delirium and is consistent with known deficiencies in tetrahydrobiopterin, a cofactor for dopamine synthesis [92]. Orally or rectally bioavailable medications permit palliation at home [93]. An interesting anecdotal observation is that patients with hydrophobia may tolerate ingesting preparations of clarified butter. If confirmed, this might permit an oral vehicle for many sedatives.

6.3 Vaccine Failures Now Survive

Survivors of human rabies are well documented with conventional ICU care, although functional outcomes are often poor [94]. These patients often received rabies vaccine without RIG and then progressed to rabies due to lack of bridging rabies antibody. Rabies-neutralizing titers among these survivors are remarkably high. Among them, a series of survivors have been reported in India, all neurologically damaged and several diagnosed after encephalitis resolved [94]. The author (RM) has speculated that recent rabies survivors have appeared in South Asia as the

direct result of an expanding middle and upper class with access to world-class medical care. The number of current survivors is small and, probabilistically, better outcomes will follow. Hospitals capable of treating tetanus or head trauma can treat rabies. The contraindications are societal, related to cost of prolonged care and rehabilitation in modern medical centers.

6.4 Milwaukee Protocol

There is currently no antiviral of proven efficacy in human rabies. The Milwaukee Protocol (MP) is a supportive critical care. It has been used over 80 times, producing 13 additional survivors by intention to treat (www.mcw.edu/rabies). The key concept of the protocol is to avoid fatal dysautonomia during the first week of hospitalization, while permitting immunologically unskewed development of neutralizing antibodies in the patient during the second week to clear the infection.

Over time (now in version 5), the MP has characterized and prevented regular complications of rabies during critical care (www.mcw.edu/rabies). Salt wasting is prevented by the use of exclusively normal saline solutions and mineralocorticoids. Interestingly, this approach minimized vasospasm [95]. Ketogenesis detected in CSF has been associated with imminent death from rabies [44]. Insulin with glucose resulted in reduced dysautonomia and longer average survival. The PR interval on electrocardiogram is monitored; heart block can be overcome by pacing or the use of xanthines [96]. Sapropterin (tetrahydrobiopterin) accelerates rehabilitation considerably by minimizing transient movement disorders [45].

6.5 Future Therapies

Several broad-spectrum antivirals targeting other pathogens are active against RABV and are being evaluated in clinical trials [97, 98]. Modern molecular genetic approaches (DNA vaccines, siRNA, recombinant virus vaccines) have shown promise in vitro but are challenged by delivery to the CNS. Among the monoclonal RIG products under development, the authors do not know of any that are capable of clearing RABV infection intracellularly [56]. Most antibodies penetrate the CNS with 0.5% efficiency. Camel and shark antibodies are much smaller, monomeric, and penetrate with greater efficiency [99, 100].

7 Control Measures

7.1 Regional Programs and One Health Approach

The World Health Organization (WHO) has identified rabies as one of the neglected tropical diseases, and the WHO Regional Office for Southeast Asia has been advocating for a regionally coordinated rabies elimination program in the SAARC region. There is a clear precedent for successful regional action [101]. Regional societies such as Rabies in the Americas (RITA) were likely crucial to the success of this endeavor (www.rabiesintheamericas.org).

To work toward human rabies elimination in South Asia, member countries should have a national strategy for rabies elimination following a stepwise approach. Currently a comprehensive rabies control program exists in only three countries. The "One Health" concept is technically supported by the WHO, Food and Agriculture Organization of the United Nations (FAO), World Organization for Animal Health (OIE), Global Alliance for Rabies Control (GARC), and World Animal Protection (WAP), among others. Rabies surveillance can be improved by making human and animal rabies a notifiable disease, strengthening laboratory capacity for diagnosis, and enhancing real-time reporting through coordination between the medical and veterinary sectors. Studies in Asia, Africa, and Latin America have revealed that investment in mass dog vaccination programs is a feasible and cost-effective approach to human rabies elimination and member countries can benefit from the OIE dog rabies vaccine bank for this purpose. The dog population in India alone, consisting of mainly free roaming dogs, is estimated to be as high as 25 million (2.8% of the global dog population) [102]. An estimated 17 million animal bites, mostly by dogs, are reported every year in India. However, canine population estimation has not been done using a standard methodology in most countries, and pet registration and vaccination are not mandatory [103, 104]. Dog population management should be undertaken using scientific, humane, and socioculturally acceptable methods in individual countries [13].

In recent years, a few countries have made a considerable progress toward realizing the goal of rabies elimination. Sri Lanka has registered a sharp decline in the number of human rabies deaths through mass dog vaccination campaigns, improved accessibility to human postexposure prophylaxis (PEP), and an effective vaccine delivery system [105]. Bhutan and Bangladesh have recorded significant reductions in human rabies incidence [13]. In India, considerable success in terms of decrease in human rabies deaths has been observed in areas where rabies control programs have been implemented, though confined to small urban areas in the states of Rajasthan, Tamil Nadu, Jharkhand, and Sikkim [106–110]. Along with public health agencies, the contribution of nongovernmental organizations and regional professional associations (Rabies in Asia foundation, http://www.rabiesinasia.org/; Association for Prevention and Control of Rabies in India, http://www.apcri.org/) has also been crucial in achieving these goals.

7.2 *Prophylaxis*

There are three critical components to postexposure prophylaxis [1]. Rabies is a lipid-enveloped virus, so soap and water or topical disinfectants are highly effective at preventing rabies. Cosmetic wounds should be only approximated, never closed, and then definitively repaired once bacterial infection is excluded and PEP underway. There are a variety of WHO-approved rabies vaccine schedules that make use of the vaccine-sparing intradermal route to minimize cost [1]. Vaccines require 5–10 days to generate neutralizing antibodies and so require passive immunization (rabies immune globulin) to bridge the gap. The lack of administration of RIG is the most frequent cause of failure of PEP. RIG is of little benefit once 7 days have lapsed from the first vaccine. Vaccine coverage in animal bite victims is limited, and use of rabies immunoglobulin is abysmally low due to unavailability and/or high costs leading to a large number of preventable rabies deaths in most countries. Many bite victims resort to traditional remedies, such as chili paste or turmeric. To address the shortage of vaccines and to increase the cost-effectiveness, intradermal rabies vaccination (IDRV) is being practiced in India, Sri Lanka, and Bangladesh; the World Health Organization (WHO) has also facilitated IDRV training in Afghanistan, Bhutan, Nepal, and Pakistan [13].

As of 2016, all countries use vaccines of tissue culture or embryonated egg origin, which are safe and efficacious. Sri Lanka was the first country in this region to stop production and use of nerve tissue vaccine (NTV) in 1995, followed by Bhutan (1996), India (2005), Nepal (2006), Bangladesh (2011), and Pakistan (2016) [13]. India is the only country in the South and Southeast Asian region producing cell-culture based human rabies vaccines which are also supplied to neighboring countries. The Global Alliance for Vaccines and Immunization (GAVI) currently does not support funding for rabies vaccines; however, feasibility studies of GAVI support for rabies vaccines are currently underway.

Because of the safety and almost perfect efficacy of modern postexposure prophylaxis, there is increasing interest in pre-exposure prophylaxis as a component of childhood vaccine schedules in rabies-endemic countries [111]. The Indian Academy of Pediatrics recommends rabies vaccine for most children [112]. Vaccination provides at least 21 years of longevity in the serum antibody titers and anamnestic response and precludes the need for expensive rabies immune globulin products during post-exposure prophylaxis [113]. The immunization schedules for current, killed rabies vaccines were developed with less potent and efficacious vaccines and likely can be further reduced. Next-generation vaccines, particularly live attenuated vaccines, might obviate the need for repeat dosing [114].

Reference

1. World Health Organization. WHO Expert Consultation on Rabies. Second report. World Health Organ Tech Rep Ser 2013;(982):1–139, back.
2. Badrane H, Bahloul C, Perrin P, Tordo N. Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. J Virol. 2001;75(7):3268–76.
3. Nadin-Davis SA. Molecular epidemiology. In: Jackson AC, editor. Rabies-scientific basis of disease and its management. 3rd ed. Oxford: Academic; 2013. p. 123–77.
4. Smith PC, Lawhaswasdi K, Vick WE, Stanton JS. Isolation of rabies virus from fruit bats in Thailand. Nature. 1967;216(5113):384.
5. Pal SR, Arora B, Chhuttani PN, Broor S, Choudhury S, Joshi RM, et al. Rabies virus infection of a flying fox bat, Pteropus poliocephalus in Chandigarh, Northern India. Trop Geogr Med. 1980;32(3):265–7.
6. Gunawardena PS, Marston DA, Ellis RJ, Wise EL, Karawita AC, Breed AC, et al. Lyssavirus in Indian Flying Foxes, Sri Lanka. Emerg Infect Dis. 2016;22(8):1456–9.
7. Kuzmin IV, Niezgoda M, Carroll DS, Keeler N, Hossain MJ, Breiman RF, et al. Lyssavirus surveillance in bats, Bangladesh. Emerg Infect Dis. 2006;12(3):486–8.
8. Mani RS, Dovih DP, Ashwini MA, Chattopadhyay B, Harsha PK, Garg KM, Sudarshan S, Puttaswamaiah R, Ramakrishnan U, Madhusudana SN. Serological evidence of lyssavirus infection among bats in Nagaland, a North-Eastern state in India. Epidemiol Infect. 2017;145(8):1635–41.
9. Veeraraghavan N. A case of hydrophobia following bat bite. Madras Diocesan Press; 1954.
10. Hampson K, Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M, et al. Estimating the global burden of endemic canine rabies. PLoS Negl Trop Dis. 2015;9(4):e0003709.
11. Fahrion AS, Mikhailov A, Abela-Ridder B, Giacinti J, Harries J. Human rabies transmitted by dogs: current status of global data, 2015. Wkly Epidemiol Rec. 2016;91(2):13–20.
12. Carrara P, Parola P, Brouqui P, Gautret P. Imported human rabies cases worldwide, 1990-2012. PLoS Negl Trop Dis. 2013;7(5):e2209.
13. WHO-SEARO. Prevention and control of rabies in SAARC countries. Report of the workshop, Colombo, Sri Lanka 11–13 August 2015 http://www.searo.who.int/entity/emerging_diseases/documents/sea_cd_316.pdf. 2015.
14. Boland TA, McGuone D, Jindal J, Rocha M, Cumming M, Rupprecht CE, et al. Phylogenetic and epidemiologic evidence of multiyear incubation in human rabies. Ann Neurol. 2014;75(1):155–60.
15. Jackson AC, Randle E, Lawrance G, Rossiter JP. Neuronal apoptosis does not play an important role in human rabies encephalitis. J Neurovirol. 2008;14(5):368–75.
16. Kasempimolporn S, Hemachudha T, Khawplod P, Manatsathit S. Human immune response to rabies nucleocapsid and glycoprotein antigens. Clin Exp Immunol. 1991;84(2):195–9.
17. Lytle AG, Norton JE Jr, Dorfmeier CL, Shen S, McGettigan JP. B cell infection and activation by rabies virus-based vaccines. J Virol. 2013;87(16):9097–110.
18. Wanjalla CN, Faul EJ, Gomme EA, Schnell MJ. Dendritic cells infected by recombinant rabies virus vaccine vector expressing HIV-1 Gag are immunogenic even in the presence of vector-specific immunity. Vaccine. 2010;29(1):130–40.
19. Nakamichi K, Saiki M, Sawada M, Takayama-Ito M, Yamamuro Y, Morimoto K, et al. Rabies virus-induced activation of mitogen-activated protein kinase and NF-kappaB signaling pathways regulates expression of CXC and CC chemokine ligands in microglia. J Virol. 2005;79(18):11801–12.
20. Wallace RM, Gilbert A, Slate D, Chipman R, Singh A, Cassie W, et al. Right place, wrong species: a 20-year review of rabies virus cross species transmission among terrestrial mammals in the United States. PLoS One. 2014;9(10):e107539.
21. Faber M, Pulmanausahakul R, Hodawadekar SS, Spitsin S, McGettigan JP, Schnell MJ, et al. Overexpression of the rabies virus glycoprotein results in enhancement of apoptosis and antiviral immune response. J Virol. 2002;76(7):3374–81.

22. Kramer RA, Marissen WE, Goudsmit J, Visser TJ, Clijsters-Van d, Horst M, Bakker AQ, et al. The human antibody repertoire specific for rabies virus glycoprotein as selected from immune libraries. Eur J Immunol. 2005;35(7):2131–45.
23. Wright E, Temperton NJ, Marston DA, McElhinney LM, Fooks AR, Weiss RA. Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison. J Gen Virol. 2008;89(Pt 9):2204–13.
24. Lafon M. Rabies virus receptors. J Neurovirol. 2005;11(1):82–7.
25. Gluska S, Zahavi EE, Chein M, Gradus T, Bauer A, Finke S, et al. Rabies virus hijacks and accelerates the p75NTR retrograde axonal transport machinery. PLoS Pathog. 2014;10(8): e1004348.
26. Hemachudha T, Ugolini G, Wacharapluesadee S, Sungkarat W, Shuangshoti S, Laothamatas J. Human rabies: neuropathogenesis, diagnosis, and management. Lancet Neurol. 2013;12 (5):498–513.
27. Wei Y, Gong K, Ao Q, Wang A, Gong Y, Zuo H, et al. Lentiviral vectors enveloped with rabies virus glycoprotein can be used as a novel retrograde tracer to assess nerve recovery in rat sciatic nerve injury models. Cell Tissue Res. 2014;355(2):255–66.
28. Kato S, Kobayashi K, Kobayashi K. Improved transduction efficiency of a lentiviral vector for neuron-specific retrograde gene transfer by optimizing the junction of fusion envelope glycoprotein. J Neurosci Methods. 2014;227:151–8.
29. Piccinotti S, Whelan SP. Rabies internalizes into primary peripheral neurons via clathrin coated pits and requires fusion at the cell body. PLoS Pathog. 2016;12(7):e1005753.
30. Klingen Y, Conzelmann KK, Finke S. Double-labeled rabies virus: live tracking of enveloped virus transport. J Virol. 2008;82(1):237–45.
31. Tan GS, Preuss MA, Williams JC, Schnell MJ. The dynein light chain 8 binding motif of rabies virus phosphoprotein promotes efficient viral transcription. Proc Natl Acad Sci USA. 2007;104(17):7229–34.
32. Shuangshoti S, Thorner PS, Teerapakpinyo C, Thepa N, Phukpattaranont P, Intarut N, et al. Intracellular spread of rabies virus is reduced in the paralytic form of canine rabies compared to the furious form. PLoS Negl Trop Dis. 2016;10(6):e0004748.
33. Lahaye X, Vidy A, Pomier C, Obiang L, Harper F, Gaudin Y, et al. Functional characterization of Negri bodies (NBs) in rabies virus-infected cells: evidence that NBs are sites of viral transcription and replication. J Virol. 2009;83(16):7948–58.
34. Hemachudha T, Wacharapluesadee S, Lumlertdaecha B, Orciari LA, Rupprecht CE, Laongpant M, et al. Sequence analysis of rabies virus in humans exhibiting encephalitic or paralytic rabies. J Infect Dis 2003;188(7):960-966.
35. Morimoto K, Hooper DC, Spitsin S, Koprowski H, Dietzschold B. Pathogenicity of different rabies virus variants inversely correlates with apoptosis and rabies virus glycoprotein expression in infected primary neuron cultures. J Virol. 1999;73(1):510–8.
36. Okada K, Ito N, Yamaoka S, Masatani T, Ebihara H, Goto H, et al. Roles of the rabies virus phosphoprotein isoforms in pathogenesis. J Virol. 2016;90(18):8226–37.
37. Masatani T, Ozawa M, Yamada K, Ito N, Horie M, Matsuu A, et al. Contribution of the interaction between the rabies virus P protein and I-kappa B kinase to the inhibition of type I IFN induction signalling. J Gen Virol. 2016;97(2):316–26.
38. Gregorio J, Meller S, Conrad C, Di NA, Homey B, Lauerma A, et al. Plasmacytoid dendritic cells sense skin injury and promote wound healing through type I interferons. J Exp Med. 2010;207(13):2921–30.
39. Mahoney E, Reichner J, Bostom LR, Mastrofrancesco B, Henry W, Albina J. Bacterial colonization and the expression of inducible nitric oxide synthase in murine wounds. Am J Pathol. 2002;161(6):2143–52.
40. Galelli A, Baloul L, Lafon M. Abortive rabies virus central nervous infection is controlled by T lymphocyte local recruitment and induction of apoptosis. J Neurovirol. 2000;6(5):359–72.

41. Shuangshoti S, Thepa N, Phukpattaranont P, Jittmittraphap A, Intarut N, Tepsumethanon V, et al. Reduced viral burden in paralytic compared to furious canine rabies is associated with prominent inflammation at the brainstem level. BMC Vet Res. 2013;9:31.
42. Gilbert AT, Petersen BW, Recuenco S, Niezgoda M, Gomez J, Laguna-Torres VA, et al. Evidence of rabies virus exposure among humans in the Peruvian Amazon. Am J Trop Med Hyg. 2012;87(2):206–15.
43. Holzmann-Pazgal G, Wanger A, Degaffe G, Rose C, Heresi G, Amaya R, et al. Presumptive abortive human rabies—Texas, 2009. MMWR Morb Mortal Wkly Rep. 2010;59(7):185–90.
44. O'Sullivan A, Willoughby RE, Mishchuk D, Alcarraz B, Cabezas-Sanchez C, Condori RE, et al. Metabolomics of cerebrospinal fluid from humans treated for rabies. J Proteome Res. 2013;12(1):481–90.
45. Willoughby RE Jr, Opladen T, Maier T, Rhead W, Schmiedel S, Hoyer J, et al. Tetrahydrobiopterin deficiency in human rabies. J Inherit Metab Dis. 2008;32(1):65–72.
46. Jogai S, Radotra BD, Banerjee AK. Rabies viral antigen in extracranial organs: a post-mortem study. Neuropathol Appl Neurobiol. 2002;28(4):334–8.
47. de Morais CF, de Assis RV. Cardiac involvement in human rabies. Case report. Rev Inst Med Trop Sao Paulo. 1985;27(3):145–9.
48. Pathak S, Horton DL, Lucas S, Brown D, Quaderi S, Polhill S, et al. Diagnosis, management and post-mortem findings of a human case of rabies imported into the United Kingdom from India: a case report. Virol J. 2014;11:63.
49. Hemachudha T, Sunsaneewitayakul B, Desudchit T, Suankratay C, Sittipunt C, Wacharapluesadee S, et al. Failure of therapeutic coma and ketamine for therapy of human rabies. J Neurovirol. 2006;12(5):407–9.
50. Mori T, Katayama Y, Kawamata T, Hirayama T. Improved efficiency of hypervolemic therapy with inhibition of natriuresis by fludrocortisone in patients with aneurysmal subarachnoid hemorrhage. J Neurosurg. 1999;91(6):947–52.
51. Nathan BR. Cerebral correlates of hyponatremia. Neurocrit Care. 2007;6(1):72–8.
52. Willoughby RE, Roy-Burman A, Martin KW, Christensen JC, Westenkirchner DF, Fleck JD, et al. Generalized cranial artery spasm in human rabies. Dev Biol. 2008;131:367–75.
53. Solomon T, Marston D, Mallewa M, Felton T, Shaw S, McElhinney LM, et al. Paralytic rabies after a two week holiday in India. BMJ. 2005;331(7515):501–3.
54. Netravathi M, Udani V, Mani R, Gadad V, Ashwini M, Bhat M, et al. Unique clinical and imaging findings in a first ever documented PCR positive rabies survival patient: A case report. J Clin Virol. 2015;70:83–8.
55. Wiedeman J, Plant J, Glaser C, Messenger S, Wadford D, Sheriff H, et al. Recovery of a patient from clinical rabies—California, 2011. MMWR Morb Mortal Wkly Rep. 2012;61(4):61–5.
56. Dietzschold B, Kao M, Zheng YM, Chen ZY, Maul G, ZF F, et al. Delineation of putative mechanisms involved in antibody-mediated clearance of rabies virus from the central nervous system. Proc Natl Acad Sci USA. 1992;89(15):7252–6.
57. Anderson LJ, Nicholson KG, Tauxe RV, Winkler WG. Human rabies in the United States, 1960 to 1979: epidemiology, diagnosis, and prevention. Ann Intern Med. 1984;100(5):728–35.
58. Caicedo Y, Paez A, Kuzmin I, Niezgoda M, Orciari LA, Yager PA, et al. Virology, immunology and pathology of human rabies during treatment. Pediatr Infect Dis J. 2015;34(5):520–8.
59. Hemachudha T, Laothamatas J, Rupprecht CE. Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges. Lancet Neurol. 2002;1(2):101–9.
60. Gable MS, Gavali S, Radner A, Tilley DH, Lee B, Dyner L, et al. Anti-NMDA receptor encephalitis: report of ten cases and comparison with viral encephalitis. Eur J Clin Microbiol Infect Dis. 2009;28(12):1421–9.
61. Mallewa M, Fooks AR, Banda D, Chikungwa P, Mankhambo L, Molyneux E, et al. Rabies encephalitis in malaria-endemic area, Malawi, Africa. Emerg Infect Dis. 2007;13(1):136–9.

62. Lentz TL, Wilson PT, Hawrot E, Speicher DW. Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. Science. 1984;226 (4676):847–8.
63. Sheikh KA, Ramos-Alvarez M, Jackson AC, Li CY, Asbury AK, Griffin JW. Overlap of pathology in paralytic rabies and axonal Guillain-Barre syndrome. Ann Neurol. 2005;57 (5):768–72.
64. Hantson P, Guerit JM, de Tourtchaninoff M, Deconinck B, Mahieu P, Dooms G, et al. Rabies encephalitis mimicking the electrophysiological pattern of brain death. A case report. Eur Neurol. 1993;33(3):212–7.
65. Kumar KV, Ahmad FM, Dutta V. Pituitary cachexia after rabies encephalitis. Neurol India. 2015;63(2):255–6.
66. Rubin J, David D, Willoughby RE Jr, Rupprecht CE, Garcia C, Guarda DC, et al. Applying the Milwaukee protocol to treat canine rabies in Equatorial Guinea. Scand J Infect Dis. 2009;41(5):372–5.
67. Wright E, McNabb S, Goddard T, Horton DL, Lembo T, Nel LH, et al. A robust lentiviral pseudotype neutralisation assay for in-field serosurveillance of rabies and lyssaviruses in Africa. Vaccine. 2009;27(51):7178–86.
68. Mehta S, Sreenivasamurthy S, Banerjee S, Mukherjee S, Prasad K, Chowdhary A. Pathway analysis of proteomics profiles in rabies infection: towards future biomarkers? OMICS. 2016;20(2):97–109.
69. French CD, Willoughby RE, Mwaikambo ED, Granger DL, Foley JF, Wong SJ, et al. Cerebrospinal fluid metabolomics rapidly discriminates rabies from other inflammatory brain diseases. Rabies in the Americas XXVI, Ft. Collins, 4–8 Oct 2015. 2015.
70. Cox JA, Lukande RL, Kalungi S, Van de Vijver K, Van Marck E, Nelson AM, et al. Practice of percutaneous needle autopsy; a descriptive study reporting experiences from Uganda. BMC Clin Pathol. 2014;14(1):44.
71. Madhusudana SN, Subha S, Thankappan U, Ashwin YB. Evaluation of a direct rapid immunohistochemical test (dRIT) for rapid diagnosis of rabies in animals and humans. Virol Sin. 2012;27(5):299–302.
72. Dean DJ, Abelseth MK, Atanasiu P. The fluorescent antibody test. In: Meslin FX, Koprowsky H, Kaplan MM, editors. WHO laboratory techniques in rabies. 4th ed. Geneva: WHO; 1996. p. 88–95.
73. Mani RS, Madhusudana SN. Laboratory diagnosis of human rabies: recent advances. Scientific World J. 2013;2013:569712.
74. Lembo T, Niezgoda M, Velasco-Villa A, Cleaveland S, Ernest E, Rupprecht CE. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. Emerg Infect Dis. 2006;12 (2):310–3.
75. Madhusudana SN. Rabies. Gurgaon: Macmillan Medical Communications; 2011.
76. Mani RS, Madhusudana SN, Mahadevan A, Reddy V, Belludi AY, Shankar SK. Utility of real-time Taqman PCR for antemortem and postmortem diagnosis of human rabies. J Med Virol. 2014;86:1804–12.
77. Mani RS, Anand AM, Madhusudana SN. Human rabies in India: an audit from a rabies diagnostic laboratory. Tropical Med Int Health. 2016;21(4):556–63.
78. Wacharapluesadee S, Sutipanya J, Damrongwatanapokin S, Phumesin P, Chamnanpood P, Leowijuk C, Hemachudha T. Development of a TaqMan real-time RT-PCR assay for the detection of rabies virus. J Virol Methods. 2008;151(2):317–20.
79. Koprowsky H. The mouse inoculation test. In: Meslin FX, Kaplan MM, Koprowsky H, editors. Laboratory techniques in rabies. 4th ed. Geneva, Switzerland: WHO; 1996. p. 80–7.
80. Webster WA, Casey GA. Virus isolation in neuroblastoma cell culture. In: Meslin FX, Kaplan MM, Koprowsky H, editors. Laboratory techniques in rabies. 4th ed. Geneva, Switzerland: WHO; 1996. p. 96–104.
81. Crepin P, Audry L, Rotivel Y, Gacoin A, Caroff C, Bourhy H. Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. J Clin Microbiol. 1998;36(4):1117–21.

82. Noah DL, Drenzek CL, Smith JS, et al. Epidemiology of human rabies in the United States, 1980 to 1996. Ann Intern Med. 1998;128:922–30.
83. Blenden DC, Creech W, Torres-Anjel MJ. Use of immunofluorescence examination to detect rabies virus antigen in the skin of humans with clinical encephalitis. J Infect Dis. 1986;154:698–701.
84. Dacheux L, Reynes JM, Buchy P, et al. A reliable diagnosis of human rabies based on analysis of skin biopsy samples. Clin Infect Dis. 2008;47:1410–7.
85. Nagaraj T, Vasanth JP, Desai A, Kamat A, Madhusudana SN, Ravi V. Ante mortem diagnosis of human rabies using saliva samples: comparison of real time and conventional RT-PCR techniques. J Clin Virol. 2006;36(1):17–23.
86. Smith JS, Yager PA, Baer GM. A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus neutralizing antibody. In: Meslin FX, Kaplan MM, Koprowsky H, editors. Laboratory techniques in rabies. 4th ed. Geneva, Switzerland: WHO; 1996. p. 181–91.
87. Cliquet F, Aubert M, Sagné L. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods. 1998;212(1):79–87.
88. Willoughby RE Jr. Rabies: rare human infection—common questions. Infect Dis Clin North Am. 2015;29(4):637–50.
89. Mathuranayagam D, Rao PV. Antemortem diagnosis of human rabies by corneal impression smears using immuno fluorescent technique. Indian J Med Res. 1984;79:463–7.
90. Human Rabies. Kentucky/Indiana, 2009. MMWR Morb Mortal Wkly Rep. 2010;59 (13):393–6.
91. Manning SE, Rupprecht CE, Fishbein D, Hanlon CA, Lumlertdacha B, Guerra M, et al. Human rabies prevention—United States, 2008: recommendations of the Advisory Committee on Immunization Practices. MMWR Recomm Rep. 2008;57(RR-3):1–28.
92. Marsden SC, Cabanban CR. Rabies: a significant palliative care issue. Progr Palliat Care. 2006;14(2):62–7.
93. Tarantola A, Crabol Y, Mahendra BJ, In S, Barennes H, Bourhy H, et al. Caring for patients with rabies in developing countries—the neglected importance of palliative care. Tropical Med Int Health. 2016;21(4):564–7.
94. Mani RS. Human rabies survivors in India: an emerging paradox? PLoS Negl Trop Dis. 2016;10(7):e0004774.
95. Hasan D, Lindsay KW, Wijdicks EF, Murray GD, Brouwers PJ, Bakker WH, et al. Effect of fludrocortisone acetate in patients with subarachnoid hemorrhage. Stroke. 1989;20 (9):1156–61.
96. Haught WH, Bertolet BD, Conti JB, Curtis AB, Mills RM Jr. Theophylline reverses high-grade atrioventricular block resulting from cardiac transplant rejection. Am Heart J. 1994;128 (6 Pt 1):1255–7.
97. Yamada K, Noguchi K, Komeno T, Furuta Y, Nishizono A. Efficacy of Favipiravir (T-705) in rabies postexposure prophylaxis. J Infect Dis. 2016;213(8):1253–61.
98. De Clercq E. Curious (old and new) antiviral nucleoside analogues with intriguing therapeutic potential. Curr Med Chem. 2015;22(34):3866–80.
99. Caljon G, Caveliers V, Lahoutte T, Stijlemans B, Ghassabeh GH, Van Den Abbeele J, et al. Using microdialysis to analyse the passage of monovalent nanobodies through the blood-brain barrier. Br J Pharmacol. 2012;165(7):2341–53.
100. Anderson GP, Teichler DD, Zabetakis D, Shriver-Lake LC, Liu JL, Lonsdale SG, et al. Importance of hypervariable region 2 for stability and affinity of a shark single-domain antibody specific for ebola virus nucleoprotein. PLoS One. 2016;11(8):e0160534.
101. Vigilato MA, Cosivi O, Knobl T, Clavijo A, Silva HM. Rabies update for Latin America and the Caribbean. Emerg Infect Dis. 2013;19(4):678–9.
102. Anonymous. India's ongoing war against rabies. Bull World Health Organ. 2009;87 (12):885–964.

103. Tenzin T, Ahmed R, Debnath NC, Ahmed G, Yamage M. Free-roaming dog population estimation and status of the dog population management and rabies control program in Dhaka City, Bangladesh. PLoS Negl Trop Dis. 2015;9(5):e0003784.
104. Rinzin K, Tenzin T, Robertson I. Size and demography pattern of the domestic dog population in Bhutan: Implications for dog population management and disease control. Prev Vet Med. 2016;126:39–47.
105. Karunanayake D, Matsumoto T, Wimalaratne O, Nanayakkara S, Perera D, Nishizono A, et al. Twelve years of rabies surveillance in Sri Lanka, 1999-2010. PLoS Negl Trop Dis. 2014;8(10):e3205.
106. Reece JF, Chawla SK. Control of rabies in Jaipur, India, by the sterilisation and vaccination of neighbourhood dogs. Vet Rec. 2006;159(12):379–83.
107. Totton SC, Wandeler AI, Zinsstag J, Bauch CT, Ribble CS, Rosatte RC, et al. Stray dog population demographics in Jodhpur, India following a population control/rabies vaccination program. Prev Vet Med. 2010;97(1):51–7.
108. Abbas SS, Venkataramanan V, Pathak G, Kakkar M. Rabies control initiative in Tamil Nadu, India: a test case for the 'One Health' approach. Int Health. 2011;3(4):231–9.
109. Gibson AD, Ohal P, Shervell K, Handel IG, Bronsvoort BM, Mellanby RJ, et al. Vaccinate-assess-move method of mass canine rabies vaccination utilising mobile technology data collection in Ranchi, India. BMC Infect Dis. 2015;15:589.
110. Anonymous. Sikkim hoping to become first rabies-free state. 14 May 2012.
111. Ravish HS, Srikanth J, Ashwath Narayana DH, Annadani R, Vijayashankar V, Undi M. - Pre-exposure prophylaxis against rabies in children: safety of purified chick embryo cell rabies vaccine (Vaxirab N) when administered by intradermal route. Hum Vaccin Immunother. 2013;9(9):1910–3.
112. Vashishtha VM, Choudhury P, Kalra A, Bose A, Thacker N, Yewale VN, et al. Indian Academy of Pediatrics (IAP) recommended immunization schedule for children aged 0 through 18 years—India, 2014 and updates on immunization. Indian Pediatr. 2014;51 (10):785–800.
113. Suwansrinon K, Wilde H, Benjavongkulchai M, Banjongkasaena U, Lertjarutorn S, Boonchang S, et al. Survival of neutralizing antibody in previously rabies vaccinated subjects: a prospective study showing long lasting immunity. Vaccine. 2006;24(18):3878–80.
114. Huang F, Ahmad W, Duan M, Liu Z, Guan Z, Zhang M, et al. Efficiency of live attenuated and inactivated rabies viruses in prophylactic and post exposure vaccination against the street virus strain. Acta Virol. 2015;59(2):117–24.

Kyasanur Forest Disease

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Contents

1 Introduction

Kyasanur forest disease virus (KFDV) belongs to the *Flaviviridae* family and genus *Flavivirus* that causes Kyasanur forest disease (KFD). It is a tick-borne viral infection which leads to either encephalitis or hemorrhage among infected individuals. The disease was first reported in 1957, from Shimoga district, state of Karnataka, with high mortality rate among nonhuman primates (NHPs) and human population living in the vicinity of Kyasanur forest, hence the name Kyasanur forest disease. In local language, KFD is known as "monkey fever" or "monkey disease" [1].

The infectious bite from tick nymph disseminates KFDV during the months of January to May. The clinical symptoms among infected individuals include acute febrile illness followed by hemorrhage and/or necrosis in organs and neurological manifestations but without any long-term neurological sequelae.

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Annually, more than 400 cases of KFDV are reported in India with the case fatality rate of 3.4%. The districts of Karnataka such as Shimoga, Uttara Kannada, Dakshina Kannada, Chikmaglur, and Udupi account for maximum numbers of KFDV cases and are therefore designated as endemic for KFDV. However, mortality rate is higher in NHPs (85%) than in human (10%) populations. It is recommended to use proper protective gears for serological examination of KFD cases, virus isolation, etc. [2–4].

The KFD vaccine has been developed in the 1970s through formalin-inactivated chick embryo fibroblast-tissue culture [5]. Other preventive measures include the protection from ticks by application of tick repellents dimethyl phthalate (DMP) oil, *N*,*N*-diethyl-meta-toluamide (DEET), and dibutyl phthalate (DBP) on exposed body parts. The carcasses of infected dead monkeys should be burned, and the forest floor must be sprinkled with insecticides like lindane or malathion in and around 50 m of radius [6].

2 Ecology and Epidemiology of KFDV

KFDV came into picture with a high mortality rate in two types of monkeys, *Semnopithecus entellus* (black-faced langur) and *Macaca radiata* (red-faced bonnet monkey), along with high prevalence of acute febrile illness in villagers living nearby the forest of Shimoga district of the state of Karnataka, India, in the year 1957. Earlier, KFDV was placed to Russian spring-summer encephalitis (RSSE) serocomplex, now known as tick-borne encephalitis (TBE) serocomplex of the *Flaviviridae* family. TBE manifests with neurological symptoms. However, the clinical features of KFDV reveal an atypical hemorrhagic symptom that correlate to KFDV genetic variant Omsk hemorrhagic fever virus (OHFV) and Alkhurma hemorrhagic fever virus (AHFV) [7, 8].

Many species of ticks belonging to different genera such as *Haemaphysalis*, *Hyalomma*, *Ornithodoros*, *Ixodes*, *Argas*, *Dermacentor*, *and Rhipicephalus* have been identified as major vectors for transmitting KFDV [9–14]. *Haemaphysalis* species are found in tropical evergreen and deciduous forests of southern and central India and in Sri Lanka [14, 15].

The emergence-reemergence of KFDV infections in Shimoga district is due to human intrusion into Sagar Taluk forest within the Shimoga district. This area is rich in flora and fauna and used for extensive land farming, timber practices, deforestation, irrigation, etc. Therefore, this leads to alternation in ecosystem along with close proximity among humans, animals, and ticks. In addition, an increase in temperature and change in seasonal pattern may further contribute to increase in tick populations transmitting KFDV among humans and NHPs. The hosts and vectors along with conducive environment might be the major factors for the localization and circulation of KFDV in Karnataka and nearby regions [16].

KFDV have also been reported in other areas of the Western Ghats like Uttara Kannada, Dakshina Kannada, Chikmaglur, Udupi, and Chamarajanagar,

Fig. 1 Areas affected with Kyasanur Forest Disease. The five districts of State of Karnataka are endemic for KFD while, neutralizing antibodies are reported from Gujarat and Goa. The recent KFDV activity is reported in humans and monkeys from Kerala and Tamil Nadu

Karnataka, in 2012; Nilgiri district, Tamil Nadu, in 2013; Wayanad and Malappuram district, Kerala, in 2014; and Pali village, Goa, in 2015 [2, 17]. In year 2006, KFD was reported in Gulbarga at the border of Karnataka and Maharashtra [3]. A recent outbreak of KFD in January 2016 has been reported from Sindhudurg, Maharashtra [18] (Fig. 1). Apart from India, KFDV infections were reported from Yunnan Province, China, in 1989 and named as Nanjianyin virus [19]. Genetic variants of KFDV like AHFV and OHFV were identified from Saudi Arabia, Makkah, Jeddah, and Jizan in 1994 and Egypt-Sudan border in 2010 and Western Siberia in 1943 and 1945, respectively [20–22].

There are about 400–500 cases of KFD reported in India every year [7, 23]. In the years 2003–2012, out of 3263 suspected KFD cases, 823 were confirmed with 28 deaths, a case fatality rate of 3.4% [24]. Report by Kasabi et al. (2013) has confirmed 61 KFD cases out of 215 suspected patients in Shimoga district [25].

In November 2012, 12 monkeys and 6 human deaths were reported in Bandipur National Park, Chamarajanagar district, Karnataka. Further, the samples collected from the suspected human, monkey, and tick population in Chamarajanagar, Tamil Nadu, and Kerala were found positive for KFDV, outside the endemic area. Therefore, the public health department of Karnataka initiates the vaccination program in the endemic areas and promotes awareness regarding KFD [26].

3 KFDV Genome

KFDV is about 10,774 nts in genome size, 45 nm in diameter, and an enveloped spherical virus [27]. A single positive RNA virus translates and is cleaved by the host as well as viral proteases as structural proteins (envelope (E), precursor membrane (prM), and capsid (C)) and non-structural proteins (NS1, NS2AB, NS3, NS4AB, NS5) [28, 29] along with non-coding regions (NCRs) flanking at 5′ and 3′ ends that aids in cyclization, replication, and translation of the viral genome [28, 30, 31].

The translation and cleavage of the viral polyprotein start with C protein (12–14 kDa) which is a basic protein forming homodimers on the ER membrane. It is involved in viral genome packaging and nucleocapsid formation. The next cleavage is mediated by signal peptidases that forms prM (18–19 kDa) and E (53–54 kDa) glycoproteins, possessing two transmembrane helices. The nascent prM protein helps in proper folding and assembling of E protein. The furin-like proteases cleave the prM and form M protein (~75aa) which help in virion maturation and budding from the infected cells. The E protein plays a major role in virion assembly, receptor binding, and membrane fusion and therefore, has been targeted for the production of neutralizing antibodies [32, 33].

NS1 (46–52 kDa), a highly conserved glycoprotein, exists in various forms, a monomer (site of replication complex), dimer (membrane bound), and hexamer (secreted) [34, 35]. NS1 has been implicated in viral replication, translation, and eliciting immune responses by activating TLRs and complement system [36–38]. NS1′ (53 kDa), an extended version of NS1, has been reported in mosquito-borne encephalitis group (WNV, JEV, and DENV) [39, 40]. NS2A (20–24 kDa) is a small, conserved hydrophobic protein that has been implicated in viral replication, assembly, and secretion. NS2A suppresses the antiviral response through the IFN signaling pathway [41]. NS3 (68–70 kDa) protein forms a heterodimer with NS2B and remains associated with ER membrane. This protein has been implicated in protease activity (NS3 proteases) which cleaves at NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions along with helicase and nucleotide triphosphatase activities that helps in viral replication and assembly

[42, 43]. NS4A/NS4B are small transmembrane proteins involved in viral replication and antagonizing the antiviral response via IFN signaling [44, 45]. NS5 (103–104 kDa), a multimeric viral protein, is the largest among all non-structural proteins. At N′ terminal, it has methyl-transferase activity, and at C′ terminus, it has RNA-dependent RNA polymerase activity. The protein has been implicated with antagonizing the IFN response via JAK/STAT pathway by suppressing Tyk2 tyrosine phosphorylation and activation of STAT genes [46, 47].

4 KFDV: Transmission and Life Cycle

KFDV belongs to tick-borne encephalitis group, a zoonotic arbovirus infection transmitted by the bite of infected ticks or encountering with sick or dead monkeys during forest visit. The virus maintains in small animal population, probably becoming a reservoir for infection and ticks as the major vectors for disseminating the virus [2]. The virus circulates efficiently in tick population via trans-stadial and/or transovarial route, where they take up the infection during their blood meals from viremic hosts and pass it in their further stages. Both the trans-stadial and trans-ovarial transmission are observed in *I. petauristae* ticks [48, 49].

The adult female tick has at least two to three hosts in their life cycle. The female adult tick is active during the month of June, where it lays eggs on the forest litter. Their numbers increase from July to August. The tick's larvae take their blood meals from small animals like birds and rodents in the months of October to December. However, the nymphs feed on cattles, monkeys, and humans in the months of January to May. The nymphal activity coincides with the sporadic outbreaks of KFDV infections in human and NHPs (Fig. 2). The ticks fall off after taking their blood meals and create a hot spot of infection. Humans acquire infection through the bite of infected nymphs during their forest visit for collecting woods or for recreational purposes. There are no reports of human-to-human transmission of KFDV infection because humans do not produce adequate viremia to infect ticks [50].

5 KFDV Replication

KFDV enters in the human body through the bite of an infectious nymph. KFDV enters into host cells either by receptor-mediated endocytosis or by binding to heparin sulfate proteoglycans [51]. Several putative receptors have also been reported for KFDV entry into host cells such as GRP78 (glucose-regulated protein 78), HSPs (heat shock proteins) 70 and 90, CD14, CD4, and cholesterol. Upon internalization, the acidic environment of endosome opens the nucleocapsid of the virus and releases the positive single-stranded RNA in the cytoplasm. The viral replication occurs in two phases. In the first phase, the RNA strand translates and

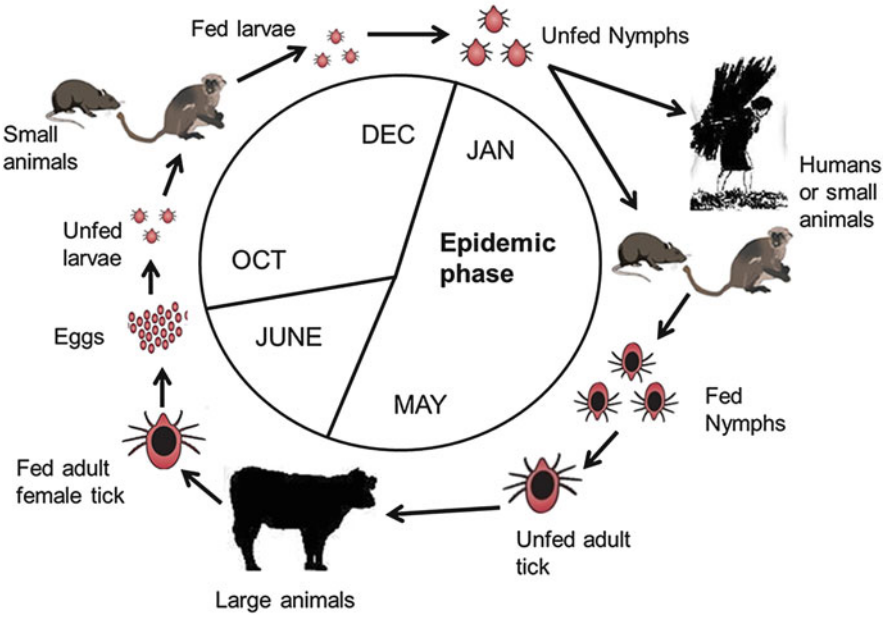

Fig. 2 Transmission and life cycle of Kyasanur Forest Disease Virus. KFDV belongs to tick-borne encephalitis group, where ticks are the major vector for disseminating virus among small animals, monkeys and humans. Each tick has at least two-three hosts in its life cycle. The adult tick lays eggs in the month of June. The unfed larvae take their blood meals from small animals or monkeys from October to December. The fed larvae then grew up to unfed nymphs, which feed on humans, small animals or monkeys in the month of January to May. The fed nymphs then grew up to unfed adult ticks, which feed on large animals like cattle. Again, the fed adult tick lays eggs in the next season

gets cleaved into viral components in the ER lumen both by hosts and viral proteases. In the second phase, the viral components interact with the host proteins and synthesizes a complementary negative RNA strand, which serves as an intermediate for synthesizing several copies of positive-strand RNA. Later on, the positive viral RNA genome gets enclosed into a specialized membrane-bound structure termed as vesicle packets. The vesicle packets are trafficked to Golgi for virion maturation and budding from the infected cells [52–55].

6 Host-KFDV Interaction and Molecular Pathogenesis

The molecular pathogenesis of KFDV has not been studied very well. The preliminary studies indicate the virulence of KFDV both in NHPs and humans. The KFDV and its genetic variants AHFV and OHFV belong to TBEV serocomplex, and both

leads to the hemorrhagic and neurological manifestations. The neurological manifestations start appearing in the later phases of infection [56].

Kenyon et al. (1992) reported the low levels of viremia with no febrile illness or biphasic symptoms in experimentally infected mouse with KFDV (P9605), which were reported in the *Bonnet macaques* [57]. Sawatsky et al. (2014) reported no viremia in mouse infected by KFDV (P6905). They further reported the infiltration of mononuclear cells and neutrophils and the formation of perivascular cuffs in the brain of KFDV (P9605)-infected mouse. KFDV (P9605) infection in mouse has been reported to show neurotropism with moderate meningitis and without tissue tropism, while AHFV (Zaki-1)-infected mouse demonstrated tissue tropism rather than neurotropic behavior. The neurotropic behavior of KFDV might be due to the neuro-adaptation of virus over several serial passages in culture. Pro-inflammatory cytokine profile of KFDV-infected mouse brain reveals an upregulation of IL-10, IFN-γ, and MCP-1 [58]. Tigabu et al. (2009) reported the severe neurological symptoms in mouse infected with OHFV (Guriev) [59]. Dodd et al. (2014) demonstrated the virulent behavior of KFDV (P9605) in three different mouse models C3H, C57BL, and A/J. The histopathological studies of infected mouse brain, lungs, and gut tissues reveal the high virus titer, while no lesions or antigens were found in the liver, spleen, and kidney. The increased expression of antiviral genes like Mx1 (myxovirus resistance 1), OAS (2,5 oligoisoadenylate synthetase 1), ISG15 (interferon-stimulated gene 15), IRF7 (interferon regulatory transcription factor), and STAT1 along with cellular helicases RIG-1 and MDA-5 has been reported in the mouse brain infected with KFDV [60]. Likewise, the activation of TLR3 was also reported after dsRNA replication [61]. In all these experiments, the neurological manifestations were observed late during infection. Recently, Basu et al. (2016) have reported a contradictory report in CD-1-infected mice, where neurological manifestations were first observed like gliosis, inflammation, loss of neurons, and syncytia formation due to infection in mid- and hindbrain, followed by hemorrhage and necrosis in organs like the liver and intestine [62].

Isaacs and Lindenmann (1957) demonstrated the antagonizing effects of type I interferon during viral replication, where interferon-stimulated genes (ISGs) were activated through the JAK/STAT pathway [63]. The type I interferon helps the infected cell in establishing an "antiviral state" and protecting the nearby uninfected cells from infection [64]. Cook et al. (2012) reported that KFDV replication was not suppressed by type I IFN (IFN-α2a) response in in vitro experiments. They also reported the role of NS5, a viral non-structural protein in inhibiting the IFN signaling [65]. However, the treatment of subtypes of type I interferon (IFN-αWA and IFN-αK) in A549 (human lung carcinoma) and VeroE6 (African green monkey kidney) cells infected with KFDV resulted into suppression in KFDV replication, but it still needs validation in vivo [66].

7 Symptoms of KFDV Infection

The incubation period of KFDV has been reported to range from 3 to 8 days. The most common and initial symptoms begin with sudden high fever, chills, myalgia, sore throat, decreased blood pressure and heart rate, pain in muscles, and frontal headache. Serological data demonstrated low count of WBC, RBC, and platelets. Secondary symptoms start appearing after 3–4 days, marked with neurological and hemorrhagic manifestations like photophobia, tremors, rigidity, mental disturbance, pneumonitis, and petechial hemorrhage, followed by bleeding from the nose, mouth, and intestinal tract in KFDV-infected patients. Other symptoms include vomiting, diarrhea, localized or generalized lymphadenopathy, bradycardia, conjunctival suffusion, bleeding from the retina and vitreous humor, opacity in lens, etc. [67, 68]. Lymphopenia and eosinopenia have been reported in the first and second week of disease, but few cases of lymphocytosis have also been reported between the third and fifth week of KFDV infection [8, 23]. After recovery, patients feel general lethargy for several weeks with a very rare case of neurological sequelae [69, 70].

Chaterjee et al. (1963) reported the cases of thrombocytopenia and neutropenia among patients infected with KFDV [71]. Pavri et al. (1989) reported increased IgE titer during KFDV infection [23]. The fatal human cases of KFDV have been reported with manifestations such as parenchymal degeneration of the kidney and expression of mononuclear phagocyte tissues in the liver, spleen, lungs, heart, and skeletal muscles followed by erythrophagocytosis [68]. Elevated levels of blood urea nitrogen (BUN) and liver transaminase and hypoalbuminemia have been reported in KFDV- and AHFV-infected patients [60].

8 Diagnosis and Treatment Regime

With the increase in the number of KFDV cases, the Directorate of Health and Family Welfare Department, Karnataka, declared KFD as a public health problem. Following the KFDV infection, the viremia reaches up to 3.0×10^6 CFU/ml within 3–8 days and remains elevated up to 14 days. High viremia coincides with the onset of disease symptoms. The initial symptom of KFD mimics with that of viral fever and/or GIT infections. The conventional methods such as HI (hemagglutinin inhibition) assay, CF (complement fixation), NT (neutralization tests) assays, and virus isolation have been used for the detection of KFDV [72, 73]. Therefore, robust, reliable, and rapid molecular diagnostic tools need to be developed for the diagnosis of KFD. Few diagnostic tools for the KFDV identification have been also developed by the National Institute of Virology, Pune [11]. The RT-PCR, real-time PCR, IgG, and IgM capture ELISA and immunofluorescence assays have been developed for the diagnosis of KFDV in acute-phase samples [74]. The symptomatic treatment regime has been adopted for patients suffering with KFDV infection.

The use of antipyretics, analgesics, and antibiotics, along with complete bedrest under the supervision of a qualified medical professional, has been suggested for KFD patients. The supportive therapy includes the administration of intravenous fluids like glucose-saline for counterbalancing the dehydration in patients. In cases of cerebral edema, dextrose (10%) I.V. or mannitol I.V. has been administered; however, anticonvulsants and corticosteroids have been recommended for neurological disorders [88].

9 Vaccines Against Kyasanur Forest Disease Virus

Since there are no antiviral drugs available for KFD, vaccines were designed to provide protection against KFDV infection. The Walter Reed Army Institute of Research Laboratory developed the first vaccine in the 1950s, which was formalin-inactivated mouse brain preparation of RSSEV serocomplex, administered subcutaneously. A weak hemagglutinin inhibition antibody response without any complement fixing antibodies in patients was observed after immunization [75–77, 89]. Following the first failure, KFDV-infected formalin-inactivated Swiss-albino mice brain preparation was tested in mice, which raised neutralizing antibodies with the life expectancy of 6 months in the refrigerator [78]. Thereafter, several other vaccines against KFDV infection were experimentally tested like live attenuated KFDV vaccine using the viral strain P9605 in langurs. It provided a transient protection by raising neutralizing antibodies for a few days [79]. Two doses of formalin-killed KFDV vaccine were tested in langurs subcutaneously, which nevertheless offered moderate protection by preventing the death of langurs but not from infection. This vaccine raised neutralizing antibodies even after 15 months of immunization [80]. Attenuated Langat E5 virus vaccine was tested against KFDV due to their cross-reactivity with KFDV [81]. The 17D yellow fever virus and inactivated TBEV vaccine were tested but without any success rate [82].

In the 1970s, formalin-inactivated chick embryo fibroblast-tissue culture vaccine against KFDV was prepared. The vaccine was immunogenic, stable, and safe, raised neutralizing antibodies against virus, and therefore was currently licensed and used in endemic areas [5, 78, 83]. Individuals are immunized with two doses of this vaccine, subcutaneously, at an interval of 1 month, followed by the booster dose after 6–9 months. Side effects of this vaccine are slight irritation for 2–8 min, followed by pain at the site of injection. Pregnant women, people suffering from jaundice or cardiac complaints, and those allergic to gentamicin, penicillin, and egg protein must not be administered [6, 84]. Efficacy and coverage of this vaccine are good; nonetheless, KFD cases are still reported annually. The change in the KFDV genome due to antigenic drift might be the reason that vaccines are proving to be inefficient with time. In addition, inappropriate storage conditions for the vaccine could be another reason for its ineffectiveness [2]. Shah et al. (2012) have designed the attenuated KFDV vaccine designated as MKTC (monkey kidney tissue culture-adapted KFDV P9605 strain), which was effective against KFD afflictions in

monkeys and can be prepared into a live attenuated vaccine, which noticeably decreased the KFD adversities in NHPs [85].

Few neutralizing antibodies have been reported in humans from areas which are non-endemic to KFD, including Kutch and Saurashtra of the state of Gujarat, Ramtek from Nagpur, and Kingaon and Parbatpur from West Bengal (Fig. 1). From serological data, hemagglutinin-inhibiting antibodies [33] and neutralizing antibodies were reported from Andaman and Nicobar Islands with the highest prevalence of antibodies in males than in females [86, 87].

10 Prevention and Control

In addition to vaccination program, the state health department should adopt rigorous surveillance programs in order to monitor the morbidity and mortality in NHPs and the human population. People should be strictly prohibited from going to the forest, if monkey deaths are reported. The carcass of monkeys should be burned, and insecticides like lindane or malathion should be sprinkled in and around 50 m of radius on the forest floor. Some professionals like hunters, herders, forest workers, farmers, and tourists, who visit the forest to collect wood or trek, are at higher risk of acquiring KFDV infection. People must cover themselves with thick clothes and wear boots before going into the forest. Application of tick repellents like dimethyl phthalate (DMP) oil, *N*,*N*-diethyl-meta-toluamide (DEET), and dibutyl phthalate (DBP) is also recommended on exposed body parts to repel insects [6, 84].

11 Conclusion

KFDV is designated as a BSL-4 handled pathogen, and after its identification in NHPs and human population, the Directorate of Health and Family Welfare Services, Karnataka, declared it as a public health concern.

The virus disseminates by ticks during the nymphal activity, in the month of November. The incubation period is about 3–8 days marked with acute febrile illness followed by hemorrhagic or neurological manifestations. Monkeys are the major amplifying hosts, small vertebrates are the reservoir, and humans are accidental dead-end hosts.

Formalin-inactivated chick embryo fibroblast-tissue culture vaccine against KFDV was prepared in the 1970s and currently is in use. Nevertheless, the cases are still reported from the endemic regions. Live attenuated virus vaccines or recombinant vaccines like subunit or chimeric vaccines are to be prepared against KFDV. More sensitive, rapid, and robust tools should be devised for KFDV surveillance, diagnosis, and prognosis.

References

1. Nichter M. Kyasanur forest disease: an ethnography of a disease of development. Med Anthropol Q. 1987;1(4):406–23.
2. Pattnaik P. Kyasanur forest disease: an epidemiological view in India. Rev Med Virol. 2006;16 (3):151–65.
3. Dobler G. Zoonotic tick-borne flaviviruses. Vet Microbiol. 2010;140(3–4):221–8.
4. Jesse L, Goodman DTD, Daniel E. Sonenshine tick-borne diseases of humans. Washington, DC: ASM Press; 2008.
5. Dandawate CN, Mansharamani HJ, Jhala HI. Experimental vaccine against Kyasanur forest disease (KFD) virus from embryonated eggs. II. Comparative immunogenicity of vaccines inactivated with formalin and beta-propio-lactone (BPL). Indian J Pathol Bacteriol. 1965;8 (4):261–70.
6. Banerjee K. Kyasanur forest disease. In: Monath TP, editor. Arboviruses: epidemiology and ecology. Boca Raton, FL: CRC Press; 1990. p. 93–116.
7. Work TH, Trapido H, Murthy DP, Rao RL, Bhatt PN, Kulkarni KG. Kyasanur forest disease. III. A preliminary report on the nature of the infection and clinical manifestations in human beings. Indian J Med Sci. 1957;11(8):619–45.
8. Work TH. Russian spring-summer virus in India: Kyasanur forest disease. Prog Med Virol. 1958;1:248–79.
9. Trapido H, Rajagopalan PK, Work TH, Varma MG. Kyasanur forest disease. VIII. Isolation of Kyasanur forest disease virus from naturally infected ticks of the genus Haemaphysalis. Indian J Med Res. 1959;47(2):133–8.
10. Singh KR, Pavri KM, Anderson CR. Transmission of Kyasanur forest disease virus by Haemaphysalis Turturis, Haemaphysalis Papuana Kinneari and Haemaphysalis Minuta. Indian J Med Res. 1964;52:566–73.
11. Bhat HR, Sreenivasan MA, Goverdhan MK, Naik SV. Transmission of Kyasanur forest disease virus by Haemaphysalis kyasanurensis trapido, Hoogstraal and Rajagopalan, 1964 (Acarina: Ixodidae). Indian J Med Res. 1975;63(6):879–87.
12. Singh KR, Bhatt PN. Transmission of Kyasanur forest disease virus by Hyalomma marginatum isaaci. Indian J Med Res. 1968;56(4):610–3.
13. Singh KR, Goverdhan MK, Rao TR. Experimental transmission of Kyasanur forest disease virus to small mammals by Ixodes petauristae, I. ceylonensis and Haemaphysalis spinigera. Indian J Med Res. 1968;56(4):594–609.
14. Bhat UK, Goverdhan MK. Transmission of Kyasanur forest disease virus by the soft tick, Ornithodoros crossi. Acta Virol. 1973;17(4):337–42.
15. Upadhyaya S, Murthy DP, Anderson CR. Kyasanur forest disease in the human population of Shimoga district, Mysore State, 1959-1966. Indian J Med Res. 1975;63(11):1556–63.
16. Boshell J. Kyasanur forest disease: ecologic considerations. Am J Trop Med Hyg. 1969;18 (1):67–80.
17. Murhekar MV, Kasabi GS, Mehendale SM, Mourya DT, Yadav PD, Tandale BV. On the transmission pattern of Kyasanur forest disease (KFD) in India. Infect Dis Poverty. 2015;4:37.
18. Awate P, Yadav P, Patil D, et al. Outbreak of Kyasanur forest disease (monkey fever) in Sindhudurg, Maharashtra State, India, 2016. J Infect. 2016;72(6):759–61.
19. Wang J, Zhang H, Fu S, et al. Isolation of kyasanur forest disease virus from febrile patient, yunnan, china. Emerg Infect Dis. 2009;15(2):326–8.
20. Zaki AM. Isolation of a flavivirus related to the tick-borne encephalitis complex from human cases in Saudi Arabia. Trans R Soc Trop Med Hyg. 1997;91(2):179–81.
21. Gritsun TS, Lashkevich VA, Gould EA. Tick-borne encephalitis. Antivir Res. 2003;57 (1–2):129–46.
22. Carletti F, Castilletti C, Di Caro A, et al. Alkhurma hemorrhagic fever in travelers returning from Egypt, 2010. Emerg Infect Dis. 2010;16(12):1979–82.
23. Pavri K. Clinical, clinicopathologic, and hematologic features of Kyasanur forest disease. Rev Infect Dis. 1989;11(Suppl 4):S854–9.

24. Holbrook MR. Kyasanur forest disease. Antivir Res. 2012;96(3):353–62.
25. Kasabi GS, Murhekar MV, Yadav PD, et al. Kyasanur forest disease, India, 2011-2012. Emerg Infect Dis. 2013;19(2):278–81.
26. Mourya DT, Yadav PD, Sandhya VK, Reddy S. Spread of Kyasanur forest disease, Bandipur Tiger Reserve, India, 2012-2013. Emerg Infect Dis. 2013;19(9):1540–1.
27. Banerjee K. Kyasanur Forest Disease. In: Manath TP, editor. The arboviruses: ecology and epidemiology. USA: CRC Press; 1988.
28. Harris E, Holden KL, Edgil D, Polacek C, Clyde K. Molecular biology of flaviviruses. Novartis Found Symp. 2006;277:23–39. discussion 40, 71-3, 251-3
29. Howley PK. DM fields virology. 5th ed. Philadelphia: Lippincott Williams and Wilkins; 2007.
30. Kofler RM, Hoenninger VM, Thurner C, Mandl CW. Functional analysis of the tick-borne encephalitis virus cyclization elements indicates major differences between mosquito-borne and tick-borne flaviviruses. J Virol. 2006;80(8):4099–113.
31. Villordo SM, Gamarnik AV. Genome cyclization as strategy for flavivirus RNA replication. Virus Res. 2009;139(2):230–9.
32. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol. 2005;3(1):13–22.
33. Ding D, Kilgore PE, Clemens JD, Wei L, Zhi-Yi X. Cost-effectiveness of routine immunization to control Japanese encephalitis in Shanghai, China. Bull World Health Organ. 2003;81(5):334–42.
34. Mackenzie JM, Jones MK, Young PR. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology. 1996;220(1):232–40.
35. Lindenbach BD, Rice CM. trans-Complementation of yellow fever virus NS1 reveals a role in early RNA replication. J Virol. 1997;71(12):9608–17.
36. Young PR, Hilditch PA, Bletchly C, Halloran W. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol. 2000;38(3):1053–7.
37. Macdonald J, Tonry J, Hall RA, et al. NS1 protein secretion during the acute phase of West Nile virus infection. J Virol. 2005;79(22):13924–33.
38. Smith GW, Wright PJ. Synthesis of proteins and glycoproteins in dengue type 2 virus-infected vero and Aedes albopictus cells. J Gen Virol. 1985;66(Pt 3):559–71.
39. Firth AE, Atkins JF. A conserved predicted pseudoknot in the NS2A-encoding sequence of West Nile and Japanese encephalitis flaviviruses suggests NS1′ may derive from ribosomal frameshifting. Virol J. 2009;6:14.
40. Mason PW. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. Virology. 1989;169(2):354–64.
41. Leung JY, Pijlman GP, Kondratieva N, Hyde J, Mackenzie JM, Khromykh AA. Role of nonstructural protein NS2A in flavivirus assembly. J Virol. 2008;82(10):4731–41.
42. Shiryaev SA, Ratnikov BI, Aleshin AE, et al. Switching the substrate specificity of the two-component NS2B-NS3 flavivirus proteinase by structure-based mutagenesis. J Virol. 2007;81(9):4501–9.
43. Bera AK, Kuhn RJ, Smith JL. Functional characterization of cis and trans activity of the Flavivirus NS2B-NS3 protease. J Biol Chem. 2007;282(17):12883–92.
44. Utama A, Shimizu H, Morikawa S, et al. Identification and characterization of the RNA helicase activity of Japanese encephalitis virus NS3 protein. FEBS Lett. 2000;465(1):74–8.
45. Luo D, Xu T, Watson RP, et al. Insights into RNA unwinding and ATP hydrolysis by the flavivirus NS3 protein. EMBO J. 2008;27(23):3209–19.
46. Lin CW, Cheng CW, Yang TC, et al. Interferon antagonist function of Japanese encephalitis virus NS4A and its interaction with DEAD-box RNA helicase DDX42. Virus Res. 2008;137(1):49–55.
47. Lin RJ, Chang BL, Yu HP, Liao CL, Lin YL. Blocking of interferon-induced Jak-Stat signaling by Japanese encephalitis virus NS5 through a protein tyrosine phosphatase-mediated mechanism. J Virol. 2006;80(12):5908–18.

48. Boshell J, Rajagopalan PK. Preliminary studies on experimental transmission of Kyasanur forest disease virus by nymphs of Ixodes petauristae Warburton, 1933, infected as larvae on Suncus murinus and Rattus blanfordi. Indian J Med Res. 1968;56(4):589–93.
49. Randolph SE. Transmission of tick-borne pathogens between co-feeding ticks: Milan Labuda's enduring paradigm. Ticks Tick Borne Dis. 2011;2(4):179–82.
50. Labuda M, Danielova V, Jones LD, Nuttall PA. Amplification of tick-borne encephalitis virus infection during co-feeding of ticks. Med Vet Entomol. 1993;7(4):339–42.
51. Avirutnan P, Zhang L, Punyadee N, et al. Secreted NS1 of dengue virus attaches to the surface of cells via interactions with heparan sulfate and chondroitin sulfate E. PLoS Pathog. 2007;3 (11):e183.
52. Lindenbach BD, Rice CM. Molecular biology of flaviviruses. Adv Virus Res. 2003;59:23–61.
53. Thongtan T, Wikan N, Wintachai P, et al. Characterization of putative Japanese encephalitis virus receptor molecules on microglial cells. J Med Virol. 2012;84(4):615–23.
54. Thurner C, Witwer C, Hofacker IL, Stadler PF. Conserved RNA secondary structures in Flaviviridae genomes. J Gen Virol. 2004;85(Pt 5):1113–24.
55. Khromykh AA, Meka H, Guyatt KJ, Westaway EG. Essential role of cyclization sequences in flavivirus RNA replication. J Virol. 2001;75(14):6719–28.
56. Dodd KA, Bird BH, Khristova ML, et al. Ancient ancestry of KFDV and AHFV revealed by complete genome analyses of viruses isolated from ticks and mammalian hosts. PLoS Negl Trop Dis. 2011;5(10):e1352.
57. Kenyon RH, Rippy MK, McKee KT Jr, Zack PM, Peters CJ. Infection of Macaca radiata with viruses of the tick-borne encephalitis group. Microb Pathog. 1992;13(5):399–409.
58. Sawatsky B, McAuley AJ, Holbrook MR, Bente DA. Comparative pathogenesis of Alkhurma hemorrhagic fever and Kyasanur forest disease viruses in a mouse model. PLoS Negl Trop Dis. 2014;8(6):e2934.
59. Tigabu B, Juelich T, Bertrand J, Holbrook MR. Clinical evaluation of highly pathogenic tick-borne flavivirus infection in the mouse model. J Med Virol. 2009;81(7):1261–9.
60. Dodd KA, Bird BH, Jones ME, Nichol ST, Spiropoulou CF. Kyasanur forest disease virus infection in mice is associated with higher morbidity and mortality than infection with the closely related Alkhurma hemorrhagic fever virus. PLoS One. 2014;9(6):e100301.
61. Munoz-Jordan JL, Fredericksen BL. How flaviviruses activate and suppress the interferon response. Virus. 2010;2(2):676–91.
62. Basu A, Yadav P, Prasad S, et al. An early passage human isolate of Kyasanur forest disease virus shows acute neuropathology in experimentally infected CD-1 mice. Vector Borne Zoonotic Dis. 2016;16(7):496–8.
63. Isaacs A, Lindenmann J, Valentine RC. Virus interference. II. Some properties of interferon. Proc R Soc Lond B Biol Sci. 1957;147(927):268–73.
64. Pestka S. The interferons: 50 years after their discovery, there is much more to learn. J Biol Chem. 2007;282(28):20047–51.
65. Cook BW, Cutts TA, Court DA, Theriault S. The generation of a reverse genetics system for Kyasanur forest disease virus and the ability to antagonize the induction of the antiviral state in vitro. Virus Res. 2012;163(2):431–8.
66. Cook BW, Ranadheera C, Nikiforuk AM, et al. Limited effects of Type I interferons on Kyasanur forest disease virus in cell culture. PLoS Negl Trop Dis. 2016;10(8):e0004871.
67. Grard G, Moureau G, Charrel RN, et al. Genetic characterization of tick-borne flaviviruses: new insights into evolution, pathogenetic determinants and taxonomy. Virology. 2007;361 (1):80–92.
68. Iyer CG, Laxmana Rao R, Work TH, Narasimha Murthy DP. Kyasanur forest disease VI. Pathological findings in three fatal human cases of Kyasanur forest disease. Indian J Med Sci. 1959;13:1011–22.
69. Madani TA, Azhar EI, Abuelzein el TM, et al. Alkhurma (Alkhurma) virus outbreak in Najran, Saudi Arabia: epidemiological, clinical, and laboratory characteristics. J Infect. 2011;62 (1):67–76.

70. Adhikari Prabha MR, Prabhu MG, Raghuveer CV, Bai M, Mala MA. Clinical study of 100 cases of Kyasanur forest disease with clinicopathological correlation. Indian J Med Sci. 1993;47(5):124–30.
71. Chatterjea JB, Swarup S, Pain SK, Laxmana RR. Haematological and biochemical studies in Dyasanur Forest disease. Indian J Med Res. 1963;51:419–35.
72. Bhatt PN, Work TH, Varma MG, Trapido H, Murthy DP, Rodrigues FM. Kyasanur forest diseases. IV. Isolation of Kyasanur forest disease virus from infected humans and monkeys of Shimoga district, Mysore state. Indian J Med Sci. 1966;20(5):316–20.
73. Pavri KM, Anderson CR. Serological response of man to Kyasanur forest disease. Indian J Med Res. 1970;58(11):1587–607.
74. Mourya DT, Yadav PD, Mehla R, et al. Diagnosis of Kyasanur forest disease by nested RT-PCR, real-time RT-PCR and IgM capture ELISA. J Virol Methods. 2012;186(1–2):49–54.
75. Bernard K, Wormser GP. Biodefense principles and pathogens Edited By M. S. Bronze and R. A. Greenfield Norfolk, United Kingdom: Horizon Bioscience, 2005. 838 pp. $380.00 (hardcover). Clin Infect Dis. 2006;42(5):735.
76. Pavri KM, Gokhale T, Shah KV. Serological response to Russian spring-summer encephalitis virus vaccine as measured with Kyasanur forest disease virus. Indian J Med Res. 1962;50:153–61.
77. Shah KV, Aniker SP, Murthy DP, Rodrigues FM, Jayadeviah MS, Prasanna HA. Evaluation of the field experience with formalin-inactivated mouse brain vaccine of Russian spring-summer encephalitis virus against Kyasanur forest disease. Indian J Med Res. 1962;50:162–74.
78. Mansharamani HJ, Dandawate CN, Krishnamurthy BG. Experimental vaccine against Kyasanur forest disease (KFD) virus from tissue culture source. I. Some data on the preparation and antigenicity tests of vaccines. Indian J Pathol Bacteriol. 1967;10(1):9–24.
79. Bhatt PN, Anderson CR. Attenuation of a strain of Kyasanur forest disease virus for mice. Indian J Med Res. 1971;59(2):199–205.
80. Bhatt PN, Dandawate CN. Studies on the antibody response of a formalin inactivated Kyasanur forest disease virus vaccine in langurs “Presbytis entellus”. Indian J Med Res. 1974;62 (6):820–6.
81. Thind IS. Attenuated Langat E5 virus as a live virus vaccine against Kyasanur forest disease virus. Indian J Med Res. 1981;73:141–9.
82. Kayser M, Klein H, Paasch I, Pilaski J, Blenk H, Heeg K. Human antibody response to immunization with 17D yellow fever and inactivated TBE vaccine. J Med Virol. 1985;17 (1):35–45.
83. Mansharamani HJ, Dandawate CN. Experimental vaccine against Kyasanur forest disease (KFD) virus from tissue culture source. II. Safety testing of the vaccine in cortisone sensitized Swiss albino mice. Indian J Pathol Bacteriol. 1967;10(1):25–32.
84. Mathew D. Circular on Kyasanur forest disease. 2015. http://www.ivakerala.com/?articles/circular-on-kyasanur-forest-disease/7.
85. Shah KV, Dandawate CN, Bhatt PN. Kyasanur forest disease virus: viremia and challenge studies in monkeys with evidence of cross-protection by Langat virus infection. F1000 Res. 2012;1:61.
86. Sarkar JK, Chatterjee SN. Survey of antibodies against arthropod-borne viruses in the human sera collected from Calcutta and other areas of West Bengal. Indian J Med Res. 1962;50:833–41.
87. Padbidri VS, Wairagkar NS, Joshi GD, et al. A serological survey of arboviral diseases among the human population of the Andaman and Nicobar Islands, India. Southeast Asian J Trop Med Public Health. 2002;33(4):794–800.
88. Borio L, Inglesby T, Peters CJ, et al. Hemorrhagic fever viruses as biological weapons: medical and public health management. JAMA. 2002;287(18):2391–405.
89. Aniker SP, Work TH, Chandrasekharaiya T, et al. The administration of formalin-inactivated RSSE virus vaccine in the Kyasanur forest disease area of Shimoga District, Mysore State. Indian J Med Res. 1962;50:147–52.

Challenges for Control of Arboviral Infections in South Asia

Tikki Pang, Tippi Mak, and Duane J. Gubler

Contents

1 Introduction

Arthropod-borne viruses (arboviruses) are viruses that are transmitted amongst vertebrate hosts by infected, bloodsucking arthropods such as mosquitoes, sandflies, ticks and fleas. Diseases in humans commonly caused by arboviruses include those characterized by encephalitis, febrile illness (sometimes with an

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Table 1 Selected arboviruses which cause human disease

Virus family	Examples
Flaviviridae	Dengue, yellow fever, Zika (ZKV), West Nile virus (WNV), tick-borne encephalitis (TBE), Japanese encephalitis (JE), Murray Valley encephalitis (MVE), Kyasanur Forest disease (KFD)
Togaviridae	Chikungunya, Ross River virus (RRV), Venezuelan equine encephalitis (VEE), Sindbis, Semliki Forest virus (SFV), Mayaro virus (MAYV)
Bunyaviridae	Rift Valley fever, Crimean-Congo haemorrhagic fever (CCHF), severe fever with thrombocytopenia syndrome virus (SFTSV), sandfly fever
Rhabdoviridae	Chandipura encephalitis virus

associated rash) and haemorrhagic fever (Table 1). Mosquitoes are the main vectors for arboviruses with *Aedes* and *Culex* species being the most important in transmission to humans. The major reasons for the global emergence and re-emergence of these diseases in recent decades have been attributed to environmental change, urbanization, movement of people, animals and pathogens via modern transportation, the emergence of these viruses from their sylvatic reservoirs, commercial transportation, insecticide resistance and land remediation [1].

Arthropod-borne viruses continue to present substantial public health challenges for many countries in the developing world, including those in South Asia. Driven by globalization, increased urbanization, deforestation and other environmental changes and fragile health systems, these diseases pose important challenges for governments in the region. Recent, and ongoing, outbreaks of dengue in India, Maldives, Pakistan and Sri Lanka, for example, illustrate the seriousness of the problems posed by these ubiquitous viruses. In addition to endemic viruses circulating in the region, there are also concerns about the potential spread to the region of yellow fever virus with potentially serious, and unknown, implications for South Asia. These infections are also likely to pose significant economic costs to countries in the South Asia region. For example, the annual cost of dealing with dengue in India is estimated at $173 million [2].

At a higher policy level, and as a recognition of the continued importance of preparedness for epidemics in South Asia, the World Health Organization (WHO), governments, financial institutions, development partners and health agencies from across the world have committed to accelerate strengthening and implementation of capacities required to cope with disease outbreaks and other health emergencies at a meeting in Bali, Indonesia, in June 2016 [3]. The commitment emphasized 'the critical importance of flexible preparedness planning, community strengthening and engagement, information sharing, strengthening of inter-sectoral collaboration of national and international partnerships, and the critical role that governments and technical partners play in financing and implementing them'. With regard to dengue, the global response complements the Asia-Pacific Strategic Plan for Dengue Prevention and Control (2008–2015) agreed upon by the Southeast Asia and Western Pacific regions of WHO.

This chapter will analyse and describe the challenges associated with the present situation of arboviral infections and disease in South Asia by addressing the 'what' (i.e. the nature and magnitude of the problem), the 'why' (i.e. the 'drivers' or factors behind the problem) and the 'how' (i.e. the strategies needed for prevention and control and mitigation of these infections).

2 The What: Epidemiology of Arboviral Infections in South Asia

Arboviral diseases such as dengue, Japanese encephalitis (JE), West Nile virus (WNV), Zika, chikungunya, Crimean-Congo haemorrhagic fever (CCHF), Kyasanur Forest disease (KFD), etc. are on the rise in Asia resulting in significant burden of disease [4]. While comprehensive, accurate and up-to-date data for South Asia for the various diseases are difficult to obtain, some important examples can be cited. India, in 2010, for example, reported a total of 28,292 cases and 110 deaths due to dengue, the highest number of cases and number deaths in a single year in the country in the previous two decades [5]. The year 2015 saw another major dengue outbreak with more than 11,000 confirmed cases and at least 41 deaths in the capital, New Delhi. Pakistan experienced a large dengue epidemic in Lahore in 2011 with cases of dengue haemorrhagic fever (DHF) and a mortality rate of 6% amongst DHF/DSS cases [6]. Between 2005 and 2015, WHO estimates indicate that India and Sri Lanka are amongst the top five countries in the world with recorded deaths due to dengue. At the global level, recent analysis and new data indicate that, in contrast to a fall in mortality rate from most infectious diseases, dengue deaths increased by 49% resulting in 18,400 deaths in 2015 as compared to 2005, yielding a pronounced upward trend [7]. The increasing geographic range of dengue and, in some areas (e.g. Latin America), increasing transmission intensity contribute to growing concerns about other viruses that are transmitted by *Aedes* mosquitoes, including chikungunya, yellow fever and Zika viruses. The dengue situation in some selected countries in South Asia is given in the Table 2.

Several countries in South Asia are also known to be high- or medium-incidence countries for disease caused by Japanese encephalitis (JE) virus (Fig. 1) which is transmitted by *Culex* spp. The WHO estimates that there are 67,900 cases each year, with only a small fraction (10%) reported, with most cases occurring in children [8]. JE remains a public health concern in over 20 Asian countries, given its epidemic potential and high fatality rate of 30%, with neuropsychiatric sequelae in up to half of the survivors [9]. For example, JE is a major public health problem in Northeast India with high rates of mortality of between 21 and 27% [10]. More recent data, derived from a systematic review of the literature, rather than national surveillance data, estimated JE incidences in several South Asian countries and showed that most cases occurred in the 0–14 years age group [8] (Table 3).

In 2011, India reported its first cases of CCHF with three deaths [11], and Chandipura virus was reported as a major cause of encephalitis in children in Andhra Pradesh in 2005–2006 with a case fatality rate of 54% [12]. Zika virus was first isolated in Asia in 1966 (Malaysia), and cases of febrile illness in Central Java were shown to be caused by Zika in 1977. Serologic evidence suggests ZIKV is widespread in the region [13]. Recent mathematical modelling studies, based on seasonal geographical suitability for Zika virus transmission and seasonal volume of airline travellers, indicate the strong possibility that Zika virus could be reintroduced from the Americas (Fig. 2) [14].

Table 2 Dengue situation in selected South Asian countries (2005–2014)

Average number dengue cases and (deaths) during 2005–2007, 2010–2012 and 2013–2014 including average case fatality rate during these periods in countries and the south asia region						
	Average of years			CFR		
Countries	2005–2007	2010–2012	2013–2014	2005–2007	2010–2012	2013–2014
Bangaladesh	1237 (5)	814 (2)	1062 (1)	0.88	0.53	0.06
Bhutan	82 (2)	662 (1)	13 (0)	1.4	0.23	0.00
India	9775 (134)	32,458 (173)	58,003 (161)	1.35	0.59	0.29
Pakistan[a]	4961 (41)	252,935 (219)	NA	0.8	0.1	NA
Maldives	1858 (4)	1636 (5)	710 (1.5)	0.16	0.24	0.21
Afghanistan	NA	NA	NA	–	–	–
Nepal	9 (4)	393 (1.5)	542 (0)	53.33	0.18	0.00
Sri Lanka	8429 (32)	35,678 (217)	39,655 (140)	0.39	0.62	0.39

Data provided by Dr. A.P. Dash, Central University of Tamil Nadu, India

NA not available

[a]Data for Pakistan for 2006 and 2011, respectively. Source: WHO Country Office, Pakistan

Fig. 1 JE encephalitis in Asia. Source: Tsai TR, Chang GW, Yu YX. Japanese encephalitis vaccines. In Plotkin SA and Orenstein WA, eds., Vaccines—3rd edition, WB Saunders, Inc., Philadelphia, PA, 1999;672–710

Table 3 Prevalence of JE disease in selected South Asian countries

Country	Incidence per 100,000 in 0–14 years age group	Incidence per 100,000 in ≥15 years age group
Nepal	5.1	1.8
Bangladesh Bhutan	2.5	0.3
India	4.7	0.3

Data from [8]

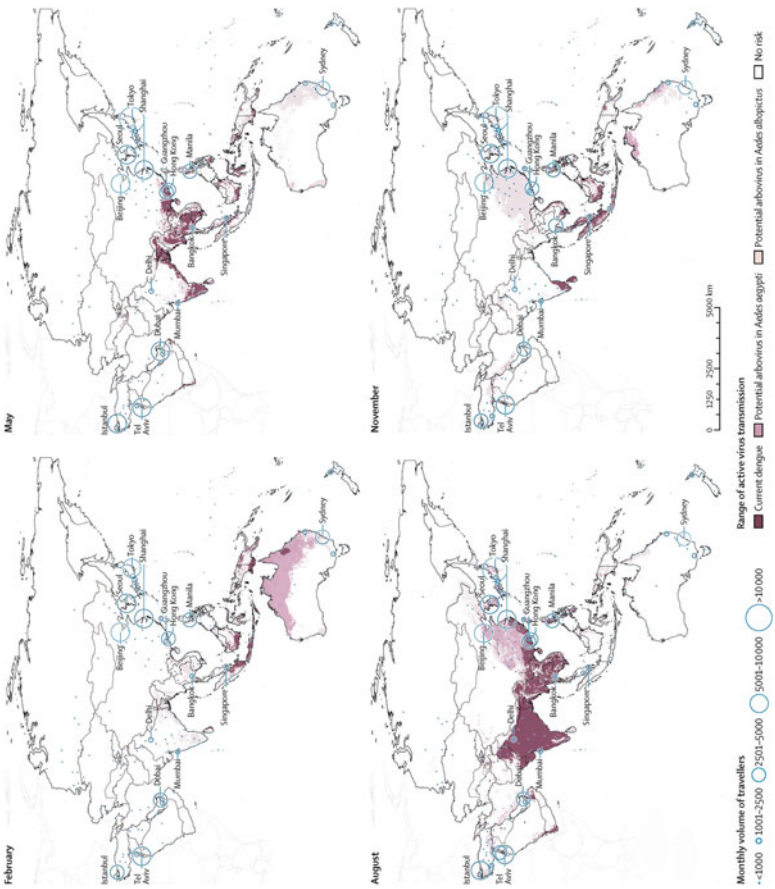

Fig. 2 Seasonal geographical suitability for Zika virus transmission in Asia-Pacific and seasonal volume of airline travellers arriving from the Americas. Source: From [14]

3 The Why: Factors Responsible for the Increase in Arboviral Infections

The principal 'drivers' of the increasing frequency and magnitude of epidemic arboviral diseases globally and in South Asia are the forces associated with population growth and globalization in their various dimensions [15], as well as other factors specific to the disease agents and their vectors. These will be described in more detail.

3.1 Increased Urbanization

High population density, overcrowding, clearing of forests, poor environmental cleanliness and haphazard urbanization in some South Asian cities favour increased transmission of arboviral infections which are largely associated with urban settings. Dhaka, the capital of Bangladesh, for example, has a population density nine times higher than Paris [16]. It has been estimated that in South Asia, 55% of people will be living in large cities by the year 2050, thus potentially aggravating the spread and prevalence of arboviral infections. At the same time, there is also evidence in South Asia of the rural spread of dengue, for example [17]. Linked to urbanization and increased prevalence of arboviral infections is the reality that many countries in South Asia lack access to a regular and clean water supply. In many cases, this leads to households needing to store water in various tanks/containers which, if not covered, cleaned regularly and/or treated with larvicides, can encourage the breeding of mosquito vectors.

3.2 Movement of People

The dramatic and unprecedented increase in air travel, facilitated by the proliferation of budget airlines in the region and beyond, has greatly increased the possibility of rapid spread of arboviral infections. Clearly, tourists are one of the main groups of people involved, but, importantly, migrant workers and refugees are also potentially important players in the spread of infections. The South Asia region—exemplified by countries like India, Nepal, Bangladesh and Sri Lanka—is the source of large numbers of migrant workers to various other regions, especially Southeast Asia. For example, over two million registered foreign workers are in Malaysia at the end of 2014 of which 24% are from Nepal and 14% from Bangladesh with a sharp increase of up to 1.5 million workers expected from the latter in the near future [18]. In more general terms, human movement has been shown to be an important factor in the transmission of vector-borne pathogens [19].

3.3 Trade Liberalization

In addition to movement of people, the spread of arboviral diseases has also been facilitated by trade liberalization and the movement of goods across borders, including those occurring between countries in South Asia. It has been shown, for example, that used tyres being transported on ships across borders can contain mosquito eggs and may be a contributor to the spread of *Aedes* spp. globally [20]. Trade and cross-border movements of agricultural products could also be another factor in the spread of arboviral infections.

3.4 Climate Change

Global warming and higher temperatures have important implications for arboviral infections, especially in temperate climates. Warmer temperatures, exemplified by what occurred during the El Nino phenomenon in 2015, for example, may favour both the virus and the vector and may increase mosquito populations by reducing breeding times and potentially drive the occurrence of outbreaks [21, 22]. However, the epidemiologic evidence suggests a more complicated, even conflicting, scenario. For dengue, for example, in every endemic country, the peak seasonal transmission period, as well as epidemics, has been observed to occur during the rainy season when temperatures are cooler by a few degrees. In those countries, the period of lowest dengue activity is during the hot dry period before the rains arrive. In addition, extreme weather events, such as flooding, cyclones and droughts, can have adverse effects on mosquito populations, thus potentially reducing transmission.

3.5 Fragile Health Systems

The ability of countries to prevent and control diseases caused by arboviruses is clearly dependent on the strength and preparedness of their healthcare delivery systems, especially in the face of outbreaks and a surge in demand for a robust response. Despite globalization's positive effect in improving economic development of the South Asian region, health systems remain fragile and largely unprepared for dealing with the large spectrum of health challenges of the future, including epidemics of infectious diseases. Weaknesses in the basic building block of health systems (e.g. in human resources, financing, information and surveillance systems, diagnostic capabilities, healthcare facilities, availability of medicines and vaccines, etc.), as a result of underinvestment in health systems by many governments in the region, have resulted in many countries in South Asia being ill-prepared to deal with arboviral infections. Though many countries in the region

have national infection prevention and control policies and operational plans, governments continue to struggle with their implementation and the need, and obligation, to implement the various articles of the International Health Regulations (IHR). In the future, there is also an additional challenge to integrate these prevention and control strategies with the achievement of universal health coverage, the foundation for achieving goal 3 of the Sustainable Development Goals (SDGs).

3.6 Underestimation of Disease Burden: Tip of the Iceberg

Beyond the forces of globalization, the magnitude of the problems faced by developing countries is also exacerbated by the likelihood that the true number of infections is probably grossly underestimated due to weak surveillance and diagnostic capabilities as well as the large number of asymptomatic infections. For dengue, of the 390 million infections estimated annually, only 96 million have clinical symptoms [23], representing a 30–40-fold underestimate, with as much as nearly a 300-fold underestimate suggested for India [2]. Other studies [23] also indicate that the under-reporting for dengue is probably also true for other countries in South Asia with wide variations in the estimated numbers of inapparent and apparent infections and the number of cases reported to the WHO (Table 4). For JE, as mentioned previously, it is estimated that only 10% of cases are reported [8].

Table 4 Model-based estimated numbers of apparent and inapparent dengue infections per year and estimated global burden rank for SEARO countries, 2010

Country	Annual apparent infections, mean	Annual inapparent infections, mean	WHO estimate	Absolute global rank[a]
India	32,541,392	99,692,319	12,484	1
Indonesia	7,590,213	23,009,108	130,575	2
Bangladesh	4,097,833	12,581,091	568	7
Pakistan Thailand	3,414,749 1,903,694	10,481,756 5,823,012	11,787 57,589	8 11
Myanmar	992,954	3,056,420	16,824	14
Sri Lanka	673,544	2,042,226	22,902	18
Nepal	571,773	1,769,014	13	26
Afghanistan	81,687	255,681	–	63
Timor-Leste	14,586	45,345	278	
Maldives	6372	19,735	933	103
Bhutan	4793	15,042	147	111

Adapted from Bhatt et al. [23] Supplementary Table T4, Apparent and inapparent mean and confidence (95%) burden estimates per country

SEARO South-East Asia Regional Office, WHO

[a]The global rank was determined by the rank index of the difference (upper limit minus the lower limit) in the 95% confidence interval of the mean annual apparent infections

3.7 Lack of Treatment Modalities

Despite numerous efforts over many decades, effective and practical treatment modalities for most arboviral infections are not available, and treatment is largely limited to treating and managing the symptoms of disease.

3.8 Insecticide Resistance

Insecticide resistance is a major threat to existing vector control methods. The heavy reliance on a single class of insecticides, the pyrethroids, and the previous widespread use of DDT have been largely responsible for the emergence of resistance [24]. To cite the example of dengue control, the insecticide temephos is widely used due to its low cost and community acceptance. Due to its widespread use, resistance to temephos in *A. aegypti* has, unfortunately, been reported in many parts of the world, including Latin American countries like Colombia [25]. It is also likely that the extent of temephos resistance is underestimated due to under-reporting and lack of surveillance. Resistance of vectors (*Aedes* and *Culex* spp.) to routinely used insecticides should also be closely monitored in endemic countries in the South Asia region.

4 The How: Control Strategies Needed to Mitigate the Problem

Underpinned by the need to have strong and resilient healthcare delivery systems, including strong surveillance and case management capabilities, the mainstay of effective prevention and control of arboviral infections is the development and implementation of comprehensive, integrated and coordinated strategies based on vector control, social mobilization and preventive vaccination.

4.1 Vector Control

Controlling mosquito vectors remains as the main strategy for dealing with most arboviral infections in endemic areas. While some impressive successes have been achieved through coordinated and intensive regional initiatives in eradicating *A. aegypti* infestations in past decades (e.g. during the 1950–1960s), for example, such efforts have been difficult to sustain. Even in countries with highly successful vector control efforts as a result of investments in technology and resources, coupled with strong and enforced legislation (e.g. Singapore), outbreaks of dengue and chikungunya, for example, continue to occur regularly (Fig. 3).

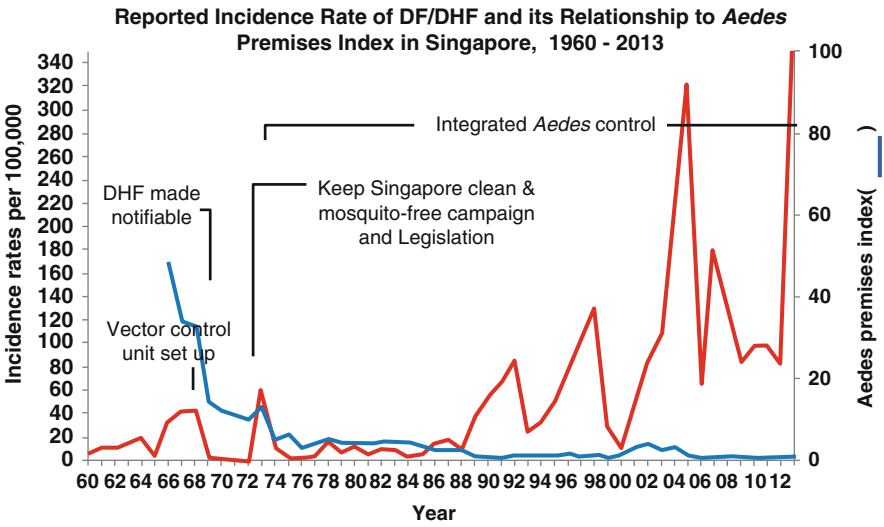

Fig. 3 Dengue in Singapore outbreaks despite low *Aedes* premises index. Source: National Environment Agency, Singapore

In many developing countries, including those in South Asia, however, vector control methods are often compromised by lack of resources and trained manpower, limited public awareness and by weak enforcement of regulations to reduce mosquito breeding in premises. While community involvement for vector control is being practiced in many endemic countries in the region, it is ineffective when used alone and needs to be combined with robust government partnership programmes. In light of less-than-optimal vector control strategies in many endemic countries, intensive efforts are under way to discover and evaluate novel methods of vector control. Foremost amongst these are the use of *Wolbachia* bacteria and sterile males created through genetic manipulation of *Aedes aegypti*. The *Wolbachia* bacterium decreases the ability of dengue, chikungunya and Zika virus infection and replication in the mosquito vectors, thus decreasing the ability of the mosquito to transmit the viruses to other persons. This method shows great promise, but it has not yet been fully validated, although it is in trials in several dengue-endemic countries, including Vietnam, Indonesia, Singapore, Brazil and Colombia. The success of the approach is also likely to be influenced by extensive differences in climate, field-release protocols, urbanization level and human density amongst the sites where this bacterium has been deployed [26].

The sterile male method of controlling insect pests has been successfully used in the USA and Mexico, for example, to control the screwworm fly using males sterilized by irradiation. The genetic manipulation approach sterilizes the male mosquito by driving a gene into the population that prevents the offspring of female mosquitoes that have mated with the sterile males from surviving to maturity [27]. This method also shows great promise and has been partially validated for

mosquitoes; it is currently in trials in several endemic countries, including Brazil and Panama though it has faced some public resistance due to concerns related to the potential and unknown hazards of releasing genetically modified organisms (GMOs) into the environment. Other new tools/approaches to mosquito control include spatial repellants, lethal ovitraps and new residual insecticides. Past and current tools have been recently reviewed [28].

4.2 Social Mobilization

The success of any prevention and control strategy strongly depends on social mobilization and the involvement of affected communities. Beyond standard approaches of education and raising awareness about these diseases through various media, communities should also be actively engaged and involved and actually have ownership of the various activities at the community level. To be effective, such activities should have the inclusive participation of all stakeholders, including teachers, community and religious leaders, and must be adapted to local contexts, culture and values. It should also be done in partnership with local governments. In Singapore, for example, the appointment of 'dengue fighters' amongst members of the community has been an effective tool of public engagement. WHO has been promoting a strategy called Communication for Behavioural Impact (COMBI), and countries like Maldives, Bhutan and Sri Lanka have organized training workshops.

4.3 Preventive Vaccination

Arguably, preventive vaccination against arboviral infections remains as one of the most important strategies for prevention and control. More broadly, vaccination not only provides protection for individuals, but it also provides indirect protection for the whole population due to reduced disease transmission. From a public health perspective, vaccination will ultimately help to decrease the overall incidence of disease and result in a reduction in frequency and magnitude of outbreaks. In this way it will alleviate the stress on already fragile health systems and reduce the economic and social impacts of arboviral infections and disease.

While ideally, vaccines should be developed against all of these infections, in practical terms, effective vaccines are currently available only for yellow fever, JE, TBE, KFD and, more recently, dengue. The yellow fever vaccine remains as one of the most successful vaccines ever developed with the ability to produce long-lasting, even lifelong, immunity against this serious disease.

Approximately 15 vaccines against JE are available and used in endemic countries (Fig. 4) [29]. There are four main types of JE vaccines in current use: the inactivated mouse brain-derived, live attenuated, live recombinant-chimeric and inactivated Vero cell-derived vaccines. WHO recommends that JE vaccination be

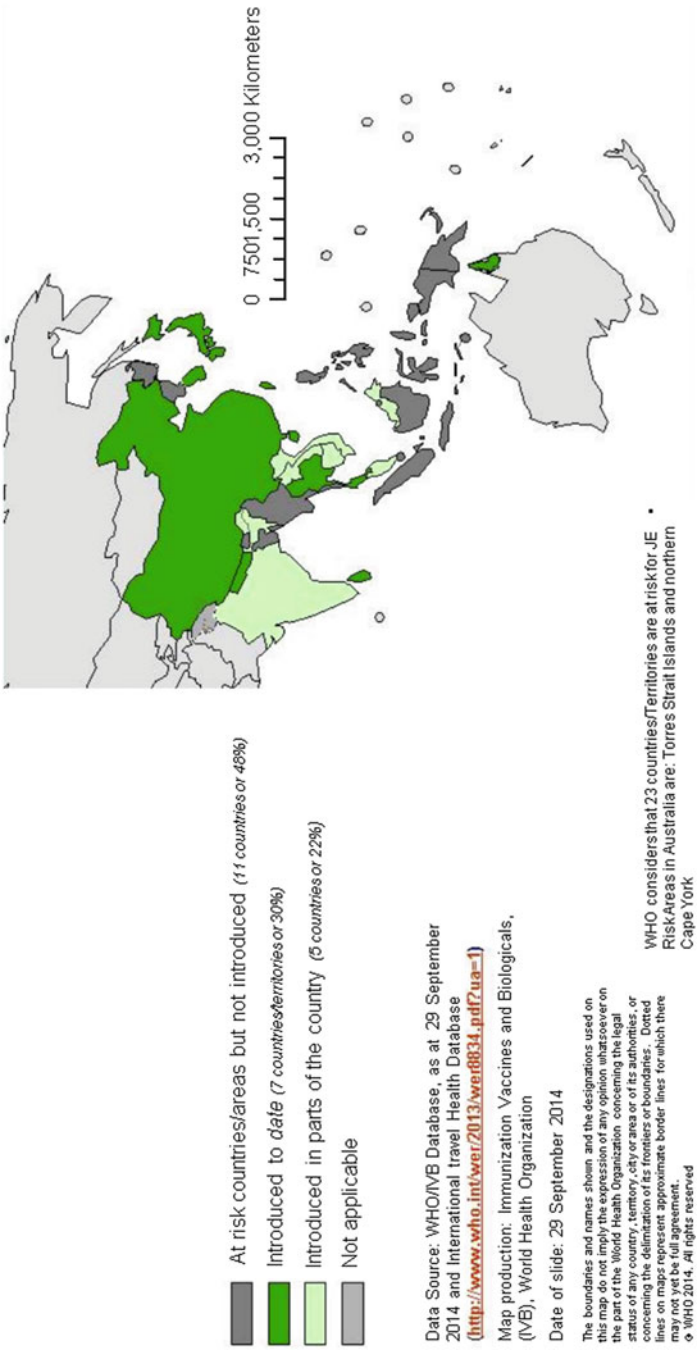

Fig. 4 Areas at risk of JE and use of JE vaccines, 2014. Source: From [29]

used as a mainstay for control and should be considered in national immunization schedules in areas where JE is recognized as a public health issue. The mouse brain-derived vaccines have been associated with hypersensitive reactions and, rarely, with acute encephalitis. WHO has thus encouraged gradual replacement of mouse brain-derived vaccines with the new generation of JE vaccines with improved safety profiles [30]. Live attenuated and recombinant vaccines tested in various countries have produced high seroprotection rates ranging from 80 to 99% [29] with long-lasting immunity of 3 years or more and an excellent safety profile.

Interestingly, the historical success of the yellow fever 17D vaccine provided a key platform for the development of a dengue vaccine by providing an effective backbone vector for protective dengue antigens. Following more than 20 years of development, a safe and effective live attenuated recombinant vaccine against all four dengue virus serotypes was recently developed by Sanofi Pasteur [31, 32]. The vaccine had an overall, average efficacy of 65%, reduced severe dengue disease by 93% and hospitalizations by 80%. The vaccine's safety has been demonstrated in many clinical trials [33]; it has so far been licenced and approved for use in 13 countries in Latin America and Southeast Asia (Brazil, Mexico, Costa Rica, El Salvador, Guatemala, Peru, Bolivia, Paraguay, the Philippines, Indonesia, Thailand, Singapore, Cambodia) with dossiers for approval submitted in at least 20 other endemic countries, including those in South Asia. The use of the vaccine has been endorsed and recommended by the WHO through recommendations from the Strategic Advisory Group of Experts (SAGE) on immunization and a position paper issued in July 2016 [34]. The use of the vaccine in large public health immunization programmes has been implemented in the Philippines and Brazil, in school children and in 9–45-year-old cohorts, respectively.

While concern has been expressed in some quarters about the vaccine's safety [35], further knowledge and information on the safety and efficacy of the Sanofi Pasteur vaccine is expected through robust post-licensure studies and monitoring in the implementing countries. Mathematical modelling has also been deployed to try and gain insights into the benefits and risks of the said vaccine in order to better inform its optimal deployment [36]. Such new approaches would be equally applicable to other vaccines in the future. Importantly, successful implementation also depends on public acceptance of the vaccine, sustainable financing of immunization programmes and strong political will and commitment from governments.

Reflecting the urgent need for a vaccine to prevent dengue, many other candidate vaccines, using a variety of approaches, are in preclinical testing, and two other promising candidate vaccines are in clinical development, one developed by the US CDC and licenced by Takeda and another by a consortium involving the US NIAID (National Institute of Allergy and Infectious Diseases) and Butantan Institute, Brazil [37, 38]. While some promising early results have been reported, these two vaccine candidates are at least 2–3 years away from licensure and approval in endemic countries (Fig. 5).

With the recent concern around the spread of Zika virus in Latin America and Asia, and potentially serious consequences to unborn children, there is also considerable interest in developing a vaccine against this virus, and several candidates are already in advanced stages of development [39].

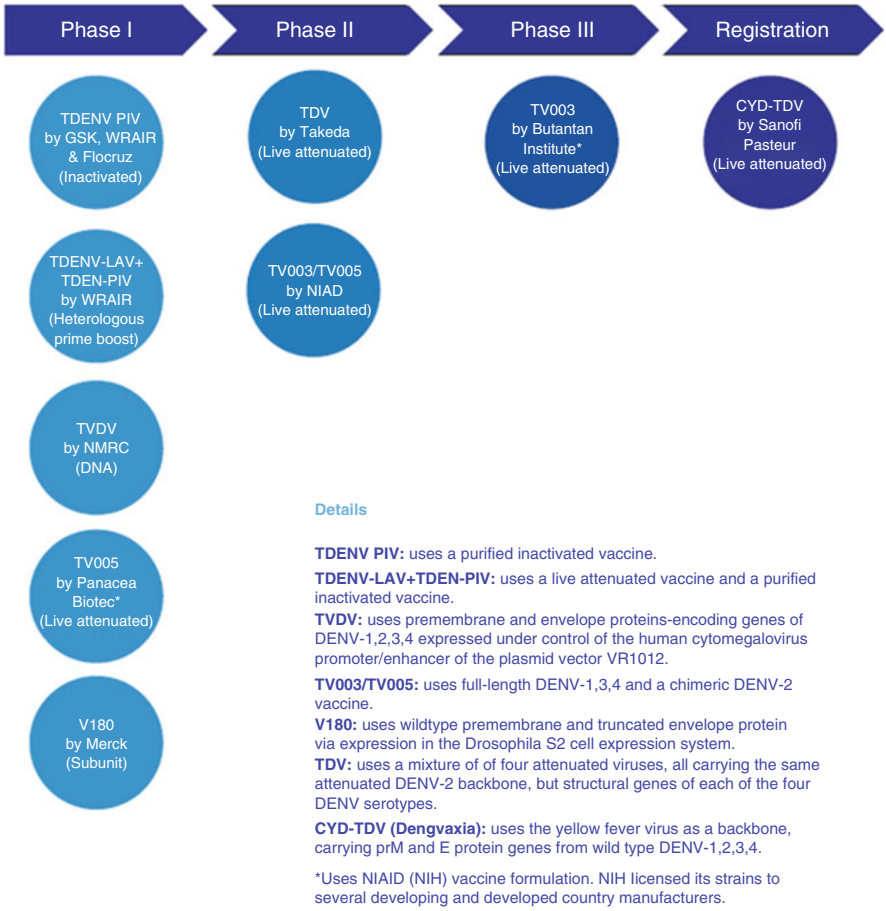

Fig. 5 Dengue vaccine candidates in clinical development as of March 2016. Source: Dengue Vaccine Initiative, 3 March 2016. http://www.denguevaccines.org/vaccine-development

Though an integrated and coordinated prevention and control strategy remains the ideal, the realization of effective integration is a complex challenge which is often hampered by lack of knowledge on how to best achieve such integration. More implementation research, including the application of mathematical modelling approaches, is needed in this important area. In the wider context of strategy implementation needs, two of these elements (i.e. vector control and preventive vaccination) are central technical elements of the WHO Global Strategy for Dengue Prevention and Control [40], while social mobilization is a key enabling factor supporting the technical elements. Wider needs for successful implementation are shown in Fig. 6.

While Fig. 6 was developed in relation to dengue prevention and control, its main dimensions are arguably applicable to other arboviral diseases. Some

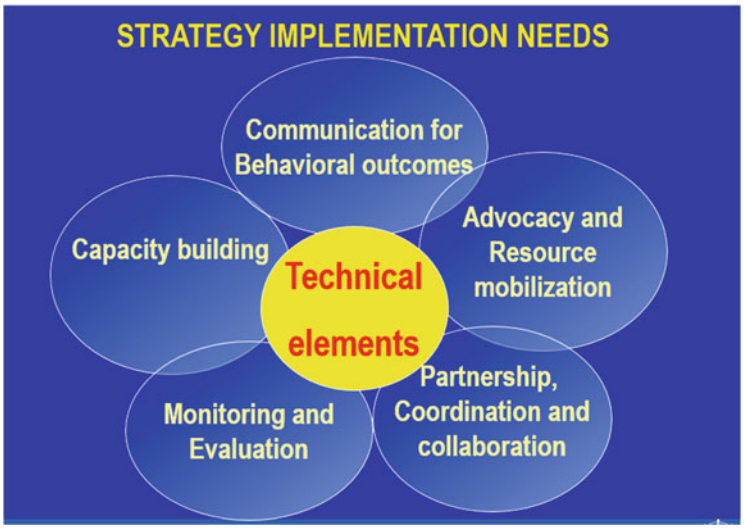

Fig. 6 Strategy implementation needs for dengue prevention and control. The *central circle* refers to the five technical elements of the WHO Global Strategy for Dengue Prevention and Control [40], i.e. diagnosis and case management, integrated surveillance and outbreak preparedness, sustainable vector control, future vaccine implementation and basic operational and implementation research. The *outer*, *inter-connected circles* represent important enabling and supporting factors for the integrated strategy to successfully implement the five technical elements. Source: Dr. Raman Velayudhan, WHO/SEARO

components, however, will need to take into account local context with regard to cultures, traditions, language and local capabilities and resources.

5 Conclusions

The present chapter has attempted to describe the current situation of arboviral infections and disease in South Asia, the factors which are responsible for the rise in the incidence and prevalence of these infections and strategies needed for more effective prevention and control. Driven by the forces of globalization, high population densities and fragile health systems, the South Asia region will continue to face significant public health challenges posed by arboviral infections. Governments in the region must demonstrate political will and commit sufficient resources to strengthen health systems and enhance national preparedness for outbreaks through integrated prevention and control strategies which are feasible and sustainable. Importantly, governments must also support collective and collaborative regional and international action and cooperation in order to mitigate the health, social and economic consequences of arboviral infection and disease in the future.

References

1. Liang GD, Gao XY, Gould EA. Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. Emerg Microbes Infect. 2015;4:e18. https://doi.org/10.1038/emi.2015.18.
2. Shepard DS, Halasa YA, Tyagi BK, Adhish SV, Nandan D, Karthiga KS, et al. Economic and disease burden of dengue illness in India. Am J Trop Med Hyg. 2014;91:1235–42. https://doi.org/10.4269/ajtmh.14-0002.
3. World Health Organization. Governments and health agencies commit to advancing global health security. http://www.searo.who.int/mediacentre/releases/2016/1628/en/. Accessed 2 Nov 2016.
4. Dash AP, Bhatia R, Sunyoto T, Mourya DT. Emerging and re-emerging arboviral diseases in Southeast Asia. J Vector Borne Dis. 2013;50:77–84.
5. Gupta E, Ballani N. Current perspectives on the spread of dengue in India. Infect Drug Resist. 2014;7:337–42.
6. Ahmed S, Mohammad WA, Hamid F, Akhter A, Afzal RK, Mahmood A. The 2011 dengue haemorrhagic fever outbreak in Lahore—an account of clinical parameters and pattern of haemorrhagic complications. J Coll Physicians Surg Pak. 2013;23:463–7.
7. GBD 2015 Mortality and Causes of Death Collaborators. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016;388:1459–544.
8. Campbell GL, Hills SL, Fischer M, Jacobson JA, Hoke CH, Hombach JM, et al. Estimated global incidence of Japanese encephalitis: a systematic review. Bull WHO. 2011;89:766–4E.
9. World Health Organization. Japanese encephalitis. Fact Sheet No 386. 2015. http://www.who.int/mediacentre/factsheets/fs386/en/. Accessed 4 Nov 2016.
10. Dev V, Sharma VP, Barman K. Mosquito-borne diseases in Assam, north-east India: current status and key challenges. WHO South East Asia J Public Health. 2015;4:20–9.
11. World Health Organization. First documented case of human Crimean-Congo Haemorrhagic Fever (CCHF) in India. http://www.searo.who.int/entity/emerging_diseases/topics/cchf/en/. Accessed 2 Nov 2016.
12. Tandale BV, Tikute SS, Arankalle VA, Sathe PS, Joshi MV, Ranadive SN, et al. Chandipura virus: a major cause of acute encephalitis in children in North Telangana, Andhra Pradesh, India. J Med Virol. 2008;80:118–24.
13. Musso D, Gubler DJ. Zika virus. Clin Microbiol Rev. 2016;29:487–524.
14. Bogoch II, Brady OJ, Kraemer MUG, German M, Creatore MI, Brent S, et al. Potential for Zika virus introduction and transmission in resource-limited countries in Africa and the Asia-Pacific region: a modelling study. Lancet Infect Dis. 2016;16:1237–45.
15. Kilpatrick AM, Randolph SE. Drivers, dynamics, and control of emerging vector-borne zoonotic diseases. Lancet. 2012;380:1946–55.
16. The Economist. The right kind of sprawl July 2, vol 420. http://www.economist.com/news/leaders/21701491-growing-cities-africa-and-asia-are-bound-spread-out-they-do-not-have-do-it-so. Accessed 5 Nov 2016.
17. Bhatia R, Dash AP, Sunyoto T. Changing epidemiology of dengue in South-East Asia. WHO South East Asia J Public Health. 2013;2:23–7.
18. http://www.forbes.com/sites/lorienholland/2016/03/22/malaysia-grapples-with-1-5-million-extra-migrant-workers/#9b6225a9cc7f
19. Stoddard S, Morrison AC, Vasquez-Prokopec GM, Soldan VP, Kochel TJ, Kitron U, et al. The role of human movement in the transmission of vector-borne pathogens. PLoS Negl Trop Dis. 2009;3:e481.
20. Tatem AJ, Rogers DJ, Hay SI. Global transport networks and infectious disease spread. Adv Parasitol. 2006;62:293–343. https://doi.org/10.1016/S0065-308X(05)62009-X.
21. Van Panhuis WG, Choisy M, Xiong X, Chok NS, Akarasewi P, Iamsirithaworn S, et al. Region-wide synchrony and travelling waves of dengue across eight countries in Southeast Asia. Proc Natl Acad Sci USA. 2015;112:13069–74. https://doi.org/10.1073/pnas.1501375112.

22. Colon-Gonzalez FJ, Fezzi C, Lake IR, Hunter PR. The effects of weather and climate change on dengue. PLoS Negl Trop Dis. 2013;7:e2503.
23. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496:504–7.
24. World Health Organization. A global brief on vector-borne diseases. 2014. http://apps.who.int/iris/bitstream/10665/111008/1/WHO_DCO_WHD_2014.1_eng.pdf. Accessed 6 Nov 2016.
25. Grisales N, Poupardin R, Gomez S, Fonseca-Gonzalez I, Ranson H, et al. Temephos resistance in Aedes aegypti in Colombia compromises dengue vector control. PLoS Negl Trop Dis. 2013;7:e2438. https://doi.org/10.1371/journal.pntd.0002438.
26. Dutra HLC, dos Santos LMB, Caragata EP, Silva JBL, Villela DAM, Maciel-de'Freitas R, et al. From lab to field: the influence of urban landscapes on the invasive potential of Wolbachia in Brazilian Aedes aegypti mosquitoes. PLoS Negl Trop Dis. 2015;9:e0003689. https://doi.org/10.1371/journal.pntd.0003689.
27. Azevedo Soares A. Promising new tools to fight Aedes mosquitoes. Bull WHO. 2016;94:562–3.
28. Achee NL, Gould F, Perkins TA, Reiner RC Jr, Morrison AC, Ritchie SA, et al. (2015) A critical assessment of vector control for dengue prevention. PLoS Negl Trop Dis 9(5): e0003655. doi:https://doi.org/10.1371/journal.pntd.0003655
29. World Health Organization. Background paper on Japanese encephalitis vaccines. SAGE Working Group on JE Vaccines. 2014. http://www.who.int/immunization/sage/meetings/2014/october/1_JE_Vaccine_Background_Paper.pdf. Accessed 2 Nov 2016.
30. World Health Organization. Japanese Encephalitis vaccines: WHO position paper. WHO Wkly Epidemiol Rec. 2015;90:69–88. http://www.who.int/wer/2015/wer9009.pdf?ua=1. Accessed 4 Nov 2016
31. Capeding MR, Tran NH, Hadinegoro SRS, Ismail HIHM, Chotpitayasunondh T, Chua MN, et al. Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. Lancet. 2014;384:1358–65. https://doi.org/10.1016/S0140-6736(14)61060-6.
32. Villar L, Dayan GH, Arredondo-García JL, Rivera DM, Cunha R, Deseda C, et al. Efficacy of a tetravalent dengue vaccine in children in Latin America. N Engl J Med. 2015;372:113–23. https://doi.org/10.1056/NEJMoa1411037.
33. Gailhardou S, Skipetrova A, Dayan GH, Jezorwski J, Saville M, Van der Vliet D, et al. Safety overview of a recombinant live-attenuated tetravalent dengue vaccine: pooled analysis of data from 18 clinical trials. PLoS Negl Trop Dis. 2016;10:e0004821. https://doi.org/10.1371/journal.pntd.000482.
34. World Health Organization. Wkly Epidemiol Rec. 2016;91:349–64. http://www.who.int/immunization/policy/position_papers/dengue/en/. Accessed 2 Nov 2016.
35. Halstead SB. Editor's choice: critique of WHO approval of a dengue vaccine. J Infect Dis. 2016. pii: jiw340. doi:10.1093/infdis/jiw340.
36. Ferguson NM, Rodriguez-Barraquer I, Dorigatti I, Mier-y-Teran-Romero L, Laydon DJ, Cummings DAT. Benefits and risks of the Sanofi-Pasteur dengue vaccine: modeling optimal deployment. Science. 2016;353:1033–6.
37. Schwartz LM, Halloran ME, Durbin AP, Longini IM. The dengue vaccine pipeline: Implications for the future of dengue control. Vaccine. 2015;33:3293–8. https://doi.org/10.1016/j.vaccine.
38. Vannice K, Durbin A, Hombach J. Status of vaccine research and development of vaccines for dengue. Vaccine. 2016;34:4461–5. https://doi.org/10.1016/j.vaccine.2016.06.082.
39. Marston HD, Lurie N, Borio LL, Fauci AS. Considerations for developing a Zika virus vaccine. N Engl J Med. 2016;375:1209–12. https://doi.org/10.1056/NEJMp1607762.
40. World Health Organization. Global strategy for dengue prevention and control 2012–2020. 2012. http://apps.who.int/iris/bitstream/10665/75303/1/9789241504034_eng.pdf?ua=1. Accessed 4 Nov 2016.