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Parasites and their vectors

A special focus on Southeast Asia

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Contents

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Chapter 1
Southeast Asia: Hotspot for Parasitic Infections

Yvonne A.L. Lim and Indra Vythilingam

1.1 Brief Overview

Southeast Asia (SEA) is a vibrant subregion of Asia located between the two mega Asian powers, India and China. This region is blessed with high diversity of flora and fauna, covering an area of approximately 4 million km^2, and is inhabited by an estimated 600 million people [1]. For the purposes of this book, we adopt the definition of SEA as the 11 member countries of the Association of Southeast Asian Nations (ASEAN) which was established in 1967 by founding member countries, namely Indonesia, Malaysia, Philippines, Singapore and Thailand. Besides these founding members, the current ASEAN countries also consist of neighbouring countries such as Brunei Darussalam, Cambodia, Timor-Leste (observer), Lao PDR, Myanmar and Vietnam. The pivotal aims of ASEAN are to promote regional economic growth, political stability, social progress and cultural developments (http://www.asean.org/asean/about-asean/overview).

Historically, this region was once plagued with political conflicts, uncertain economies and ethnic and social inequities. However, in recent times, this diverse cultural region is experiencing thriving economic, environmental and sociodemographic transformations. As a region with increasing geopolitical influence in view of Asia's global economic ascendancy, it is not surprising that the global focus is now on SEA as an emerging economic market.

The dynamic processes of rapid urbanisation, exponential population growth and mobility which SEA is undergoing have also led to the intensification of food production, agriculture, livestock and land use resulting in deforestation and inevitably climatic change. As the ecological balance is disturbed, new niches emerge encouraging infectious agents (e.g. parasites) to adapt and change. Evidences of these sometimes subtle adjustments between parasites and their ecologies are

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unfolding in SEA as reports on the emergence of zoonotic parasitic infections are appearing to be more common [2]. The problem of controlling parasitic infections is further augmented as drug resistance develops due to indiscriminate usage of antiparasitic agents enabling the parasites to thrive, thus compromising on the progress of malaria control programmes [3].

These interconnected driving forces have vital impact on human health and recent articles in *Lancet* (2011) alerted the global community of the significance of SEA region as an emerging hotspot for global health [4, 5]. Granted its rich biodiversity, SEA is at the focus of attention with regard to parasitic infections, in particular, zoonotic and vector-borne diseases (i.e. *Plasmodium knowlesi* infection) where the burden of these diseases can be substantial. Although many countries in this region are experiencing economic development, pockets of impoverished populations still exist, and these populations play significant roles in the propagation and transmission of neglected tropical diseases (e.g. soil-transmitted helminthiasis) [6].

Limited available financial resources and rapid urbanisation often results in insufficient clean water supply or proper waste disposal. These factors, coupled with the HIV/AIDS pandemic the region is facing and the conducive tropical or subtropical climate, facilitate the transmission of waterborne/foodborne and opportunistic parasites [7]. With advancing modes of transportation, increasing transboundary migrations and a burgeoning tourism trade, the potential for the spread of these infectious diseases will be borderless and immeasurable.

In 2015, the ASEAN Economic Community (AEC) with a goal of regional economic integration will be established. The AEC aspires to transform ASEAN into a region with borderless trade. There will be free movement of goods, services, investment, skilled labour and freer flow of capital (http://www.asean.org/communities/asean-economic-community). When this materialises, there will be greater transboundary movement amongst these neighbouring countries. Hence, it is crucial to assess and have an enhanced understanding of the current status of the epidemiology and clinical impact of parasitic infections in these 11 SEA countries.

Thus far, there has been no collective systematic appraisal of parasitic infections and their vectors in SEA. For these reasons, this book attempts to present a comprehensive review of all the accessible information/data and publications for individual SEA countries. Coverage of parasites in this book includes *Plasmodium*, *Entamoeba*, *Giardia*, *Cryptosporidium*, *Toxoplasma*, *Blastocystis*, free-living amoeba, filarial worm, soil-transmitted helminths, cestodes, trematodes, *Sarcocystis*, pentastomes and vectors for malaria and filariasis. For those who have always been intrigued by the diversity of the SEA communities, may this book inject some interest into the health aspects, in particular, the epidemiology of parasitic infections in this region. On a more serious note, it is hoped that the collation of these data will provide an extensive baseline information with crucial highlights on the significant gaps of knowledge. It is hoped that this understanding could then assist in formulating a solid scientific framework/platform for future integrated research in the field of infectious diseases, in particular, parasitic

infections amongst member countries. In short, may it spearhead a consolidated regional effort in public health and prepare the region as it launches into a borderless trade.

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Chapter 2
Plasmodium knowlesi: Emergent Human Malaria in Southeast Asia

Kim-Sung Lee and Indra Vythilingam

Abstract *Plasmodium knowlesi* is an emerging malaria parasite in humans and is unique to Southeast Asia. Since most countries in Southeast Asia are working towards elimination of malaria, it is important to have knowledge on this emerging simian malaria parasite affecting humans. The first case of simian malaria was reported in Malaysia in 1965. At that time extensive work conducted did not reveal other simian malaria cases in humans. However, in 2004, a large focus of *P. knowlesi* was reported from Sarawak, Malaysian Borneo and that led to many studies and cases being reported from most countries in Southeast Asia. In this chapter, the history, epidemiology, diagnosis, vectors and role of simian host are discussed. Malaria is now a zoonosis and the challenges facing the countries of Southeast in tackling the knowlesi malaria situation and the way forward have been documented.

2.1 Introduction

Malaria is a mosquito-borne disease caused by the protozoan parasite of the genus *Plasmodium*. To date, there are nearly 200 species of *Plasmodium* known to infect a wide range of hosts [1]. These include malaria parasite species that infect mammals, rodents, birds and reptiles. There are five species of *Plasmodium* known to infect and cause malaria in humans, namely *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* [2, 3]. Of these, *P. falciparum* is well known to be the deadliest form of human malaria, whereas *P. vivax* is the most prevalent

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and widely distributed species of human malaria [4, 5]. In general, malaria caused by *P. vivax*, *P. malariae* and *P. ovale* is milder and rarely fatal.

The fifth species of human malaria, *P. knowlesi*, which received much attention only in the last decade, is a malaria species of non-human primate origin [3, 6, 7]. *Plasmodium knowlesi* is prevalent in Southeast Asia and is the cause of human malaria with symptoms ranging from mild to severe disease [8]. Previously, naturally acquired human infections with malaria species of zoonotic origin were considered rare, and it was believed that humans are likely the accidental hosts. This perception changed after it was discovered that a large number of human cases of *P. knowlesi* malaria were routinely misdiagnosed as *P. malariae* in the Kapit division of Sarawak, Malaysian Borneo [2, 9]. Following this first report, it was later discovered that human knowlesi malaria is widespread as human cases were identified throughout Southeast Asia with the exception of Lao PDR.

In this chapter, a special focus is given to the epidemiology and emergence of *P. knowlesi* in Southeast Asia. Several aspects of this simian parasite including its discovery, incidence in countries of Southeast Asia, studies on its natural hosts, vectors, emergence as well as recent development in diagnosis are discussed.

2.2 Transmission and Parasite Life Cycle

Transmission of malaria parasites between vertebrate hosts occurs through the bite of infected female *Anopheles* mosquito. The sexual stages or gametocytes (macrogametocyte in female and microgametocyte in male) of the parasite ingested during a blood meal play an important role in this transmission cycle. Fertilization takes place inside the gut of the mosquito to form a zygote. The zygote develops into a motile ookinete, which penetrates the midgut wall of the mosquito before it grows into an oocyst. A matured oocyst contains thousands of infective sporozoites. When the oocyst ruptures, these sporozoites are released into the body cavity of the mosquito and migrate to the salivary gland. The sporogonic phase usually takes between 1 and 2 weeks depending on the species. In the following blood meal from another vertebrate host, the sporozoites are injected into the bloodstream together with the mosquito saliva, thus passing the parasite to the next host. Between ten and a few hundreds of infective sporozoites are usually introduced during the blood meal. Once inside the bloodstream, the sporozoites will reach the liver fairly quickly. Each sporozoite infects the hepatocyte or liver cell individually. The parasite inside each hepatocyte further develops to form merozoites, which forms the liver schizont. When the mature liver schizont bursts, the merozoites are released into the bloodstream and enter into the erythrocytic phase. The duration taken for the parasite to mature inside the liver cells before the merozoites are released into the bloodstream varies depending on the species of the parasite. On average, the pre-erythrocytic phase takes between 5 and 6 days for *P. falciparum*; 8 and 9 days for *P. vivax*, *P. ovale*, and *P. knowlesi*; and 13 days for *P. malariae*. In *P. vivax* and *P. ovale* infections, some of the sporozoite that invades the liver cell

may not immediately develop into merozoites but instead remain dormant and may remain so for a year or more in the liver before activating. This stage is known as a hypnozoite, and it is the cause of relapsed malaria infections. Relapsed infection may occur many months after an apparent cure of the first symptomatic infection.

The blood stage starts with the newly released merozoites infecting the red blood cells. Inside the red blood cell, the merozoite grows through several stages, namely early trophozoite or ring form, trophozoite and schizont as it divides to produce new merozoites. The schizont contains newly divided merozoites that will be released into the bloodstream when the red blood cell containing the schizont ruptures. This forms one cycle of the schizogonic phase. The newly released merozoites will infect more red blood cells and the cycles continue. It is the blood-stage parasites that cause the symptoms of malaria infection. During the schizogonic phase, only a small fraction of the merozoites develop into gametocytes (sexual stage) after infecting the red blood cells. The duration of each erythrocytic cycle is dependent on the species of malaria parasite: 48 h in *P. falciparum* and *P. vivax* and 72 h in *P. malariae* infections. *Plasmodium knowlesi* is by far the only parasite species with the shortest erythrocytic stage, 24 h, which is also associated with daily paroxyms or fever peaks.

2.3 Zoonotic Simian Malaria in Southeast Asia

At least 11 species of malaria known to infect non-human primates have been described in Southeast Asia (Table 2.1). Five of these simian malaria species are found naturally in macaques, whereas the others are malaria parasites of apes. In the natural hosts, these malaria species seldom cause serious illness, and the disease is usually mild or asymptomatic, very often with very low level of parasitaemia.

Since 1960, it was already known that at least seven species of simian malaria can be naturally transmitted to human through the bite of Anopheline mosquitoes [6, 7]. Three species of simian malaria in Southeast Asia are known to pose potential risk of zoonotic infection. Apart from *P. knowlesi*, which is now recognized as the cause of the fifth human malaria [3], *P. cynomolgi* and *P. inui* are the two other malaria species that are capable of infecting human [10, 11]. Both these species also share the same natural hosts with *P. knowlesi*, particularly the long-tailed and pig-tailed macaques [6, 7].

Incidences of human infections with *P. cynomolgi* through the bites of infected mosquito in the laboratories were reported in the early 1960s in the United States [10]. Together with a report of the first human case of *P. knowlesi*, it was believed that simian malaria is a potential zoonosis in nature, which could hamper the success of the malaria eradication programme launched during that time. This led to the initiation of a study in Malaysia, where more than 1,100 blood samples collected from local residents were tested in rhesus macaques by inoculation of blood [7, 12]. However, none of these rhesus macaques showed any signs of

Table 2.1 *Plasmodium* species of simian origin in Southeast Asia and their associated hosts and geographical distributions

Plasmodium species	Natural host	Geographical distribution
P. knowlesi	Macaques (*M. fascicularis*, *M. nemestrina*) Leaf monkeys (*Presbytis melalophos*)	Malaysia, Borneo, Philippines, Indo-China
P. cynomolgi	Macaques (*M. fascicularis*, *M. nemestrina*, *M. radiata*, *M. cyclopis*, *M. sinica*, *M. mulatta*) Langur (*Presbytis cristatus*, *Semnopithecus entellus*)	Malaysia, Borneo, Taiwan, Cambodia, Sri Lanka
P. inui	Macaques (*M. fascicularis*, *M. nemestrina*, *M. mulatta*, *M. cyclopis*, *M. radiate*) Langur (*Presbytis cristatus*, *P. obscurus*)	Indonesia, Malaysia, Borneo, Taiwan
P. coatneyi	Macaques (*M. fascicularis*)	Malaysia, Philippines
P. fragile	Macaques (*M. radiata*, *M. sinica*)	India, Sri Lanka
P. fieldi	Macaques (*M. nemestrina*, *M. fascicularis*)	Malaysia, Borneo
P. simiovale	Macaques (*M. sinica*)	Sri Lanka
P. hylobati	Gibbon (*Hylobates moloch*)	Indonesia, Borneo
P. youngi	Gibbon (*Hylobates lar*)	Malaysia
P. eylesi	Gibbon (*Hylobates lar*)	Malaysia
P. jefferyi	Gibbon (*Hylobates lar*)	Malaysia
P. silvaticum	Orang utan (*Pongo pygmaeus*)	Borneo
P. pitheci	Orang utan (*Pongo pygmaeus*)	Borneo

infections, and it was concluded that transmission of simian malaria to humans is extremely rare.

The infectiveness of *P. inui* in human was first demonstrated experimentally through blood passage in 1938 [13]. Subsequent *P. inui* infection studies in human volunteers demonstrated through the bites of infected *Anopheles* mosquitoes as well as blood passage resulted in all volunteers being infected and six of seven patients presented with fever [10]. While *P. cynomolgi* and *P. inui* are malaria species with zoonotic potential, cross transmission of these simian malaria species from macaques to humans in nature has not been reported.

2.4 *Plasmodium knowlesi*

2.4.1 History and Discovery

Plasmodium knowlesi was first isolated and studied in detail in the 1930s. In 1931, *P. knowlesi* was observed in the blood of a long-tailed macaque, *Macaca fascicularis*, which originated from Singapore by Napier and Campbell whose initial interest was on leishmaniasis [14]. They inoculated the infected blood into

three macaques: two long-tailed macaques and a rhesus macaque (*Macaca mulatta*). The infected rhesus monkey developed severe infection [14]. In the same study, they also investigated the tendency for the parasite to cause haemoglobinuria in *M. fascicularis* [14].

In the following year in 1932, the blood form of *P. knowlesi* was first described by Dr. Robert Knowles and his assistant Dr. Das Gupta from the Calcutta School of Tropical Medicine in India. Their study was based on the original infected monkey first studied by Napier and Campbell. The parasite was maintained in monkeys through sub-passaging of infected blood [15]. Knowles and Gupta also demonstrated the ability of the parasite to infect humans through inoculation of blood.

The parasite was further studied in the same year by Colonel John Alexander Sinton, who was the Director of the Malaria Survey of India at that time, and his co-worker Dr. Mulligan [16]. Using the parasite isolate from Knowles and Das Gupta and their own parasite isolate from a long-tailed macaque, also originated from Singapore, they noted some distinctive morphological features of the blood parasite stages and discovered its unique 24-h schizogonic cycle. These observations convinced them that they were dealing with a new species of *Plasmodium*. In honour of Dr. Robert Knowles, Sinton and Mulligan named the parasite, *Plasmodium knowlesi* [16, 17].

In 1934, the ability of *P. knowlesi* to infect humans was again demonstrated by Ionesco-Mihaiesti and co-worker, who mistakenly claimed to have found *P. inui* in baboon after inoculating it with emulsified spleen from *M. fascicularis* [18]. In 1935, Van Rooyen and Pile utilized *P. knowlesi* in the treatment of patients with neurosyphilis. They found that patients who had previous infections with *P. vivax* were less susceptible compared to those who had no past experience with malaria infection [19].

In the following year (1936), Chopra and Das Gupta carried out successful treatment of two patients with neurosyphilis through inoculation of *P. knowlesi* directly from *M. fascicularis* and thus demonstrating the potential of using *P. knowlesi* in malaria therapy for neurosyphilis [20]. The malaria therapy for patients with neurosyphilis was particularly successful in Romania until the 1950s. Ciuca and co-workers reported about 80 % of patients without prior experience with malaria infections developed infections with *P. knowlesi* [21]. However, the use of *P. knowlesi* in malaria therapy was abandoned in 1955 after it was found that the infection was becoming more virulent after 170 blood transfers and required drug treatment to terminate the infection.

The first evidence of *P. knowlesi* being transmitted to human in nature was only reported in 1965 [6, 22]. The infection was acquired by an American army personnel after spending 5 days on a working assignment in the primary forest at Bukit Kertau, Pahang. He developed symptoms on his way back to the USA. He was first diagnosed by microscopy as having *P. falciparum* infection. Instead of immediate treatment, he was referred to the Army's Walter Reed Hospital in Washington D.C. and later to the Clinical Centre of the National Institute of Health (NIH) in Bethesda. At this point, he was diagnosed by microscopy as having *P. malariae* infection. Fortuitously, the physician who saw him was interested in

malaria. His blood sample was given to a group of malariologist at NIH, who were interested in obtaining samples of *P. malariae*. Subsequently, his blood was inoculated into volunteers at the US Penitentiary in Atlanta, Georgia, and all were infected with the malaria parasite. His blood was also inoculated into rhesus macaques and all subjects died of severe infections. This observation provided a final confirmation that the infection in the original patient was due to *P. knowlesi*.

Six years later after the first report of natural human *P. knowlesi* infection, another human case of *P. knowlesi* was suspected based on presumptive diagnosis [23]. The diagnosis of *P. knowlesi* infection in this second case was based on microscopy and serological tests. Since then, no other human cases of naturally acquired *P. knowlesi* infections have been reported until 2004; perhaps no investigation was carried out due to the tedious method of using rhesus macaque for confirmation of *P. knowlesi* infections.

2.5 Epidemiology of Human Knowlesi Malaria in Southeast Asia

2.5.1 Malaysia

About 40 years after the first report of a natural *P. knowlesi* infection in human, a large number of humans naturally infected with *P. knowlesi* were discovered in the interior of Sarawak, Malaysian Borneo [2]. Microscopically diagnosed *P. malariae* appeared to be concentrated in the Kapit division of Sarawak, a region largely covered by primary and secondary forest. Singh and colleagues from the University Malaysia Sarawak observed that there were certain features of the infections that were not compatible with the classical description of *P. malariae*. The infections appeared to be atypical for *P. malariae* infection that is usually chronic and asymptomatic with low parasitaemia. Almost all patients who were diagnosed by microscopy as having *P. malariae* in the Kapit division presented clinical signs and symptoms and required treatment and hospitalization. Another peculiar feature was the elevated parasite counts [2].

Singh and colleagues utilized nested PCR assay to examine isolates from the Kapit division that were diagnosed by microscopy as *P. malariae*. Although the isolates were positive with *Plasmodium* genus-specific primers, subsequent nested PCR with species-specific primers showed negative results. This finding led to the sequencing of the small subunit ribosomal RNA gene in order to determine whether the *P. malariae* in the Kapit division is a variant form of *P. malariae* or an entirely new malaria species. A variant form of *P. malariae* has been reported elsewhere in Asia [24]. A preliminary sequencing analysis of the small subunit rRNA from a few isolates revealed that the *P. malariae* was actually *P. knowlesi*. Singh and colleagues undertook a detailed study on eight samples that were microscopically diagnosed as having *P. malariae*. Data generated from cloning and sequencing of

the small subunit rRNA(SSU rRNA) and circumsporozoite (*csp*) genes confirmed that all eight samples were phylogenetically indistinguishable from *P. knowlesi* and distinctly different from the other four known human malaria species, including the variant form of *P. malariae* [24].

Between March 2000 and November 2002, blood samples from 208 malaria patients admitted to Kapit hospital were collected and examined. A set of primers specific for *P. knowlesi* was designed and included into malaria-nested PCR assay. Using the newly designed *P. knowlesi*-specific primers, more than half (58 %) of 208 patients were tested positive for *P. knowlesi*. Blood films from *P. knowlesi* positive patients were also examined. Morphologically, the parasite resembles *P. falciparum* in the early trophozoite stage and *P. malariae* in the later stages including the gametocytes. One of the epidemiological characteristics among the cases confirmed by PCR as having *P. knowlesi* was that almost all (91.5 %) the patients were adults [2].

Following this discovery, it was suspected that human knowlesi malaria may not be limited to Kapit division since microscopy-diagnosed "*P. malariae*" have also been reported elsewhere in Malaysian Borneo. Extensive samples of unselected malaria patients collected throughout the state of Sarawak, including archival specimens in the form of blood films originated from Sabah (northern state of Malaysian Borneo) and the state of Pahang in the peninsular Malaysia, were studied [9]. In this study, four death cases due to malaria infections were also examined. All archival blood films examined were those originally diagnosed by microscopy as *P. malariae*. By employing nested PCR assay with *P. knowlesi*-specific primers, it was confirmed that *P. knowlesi* is widespread across the state of Sarawak. Cases of human knowlesi malaria were detected in almost all hospitals included in the study (11 of 12 hospitals). The study also revealed a high proportion of *P. knowlesi* among archival specimens from Sabah (83.7 %) including all five specimens from the state of Pahang. Of more importance from the perspective of clinical management of malaria was the detection of *P. knowlesi* in specimens from patients who died of complicated or severe malaria infections. Notably, all death cases were misdiagnosed by microscopy as having *P. malariae* with high parasite counts and suffered from remarkable liver and kidney failure. The study concluded the widespread distribution of human knowlesi malaria and that the infection with *P. knowlesi* can be potentially life threatening [9]. In view of the malaria death cases due to *P. knowlesi*, it is crucial that intensive clinical management be given to malaria patients who are microscopically diagnosed as "*P. malariae*" with high parasite count and who have history of travel to Southeast Asia.

Considering the widespread and the high prevalence of human knowlesi malaria particularly in the state of Sarawak, it seems unlikely that the malaria disease due to *P. knowlesi* is a newly emergent zoonotic disease. By using molecular methods, Lee and colleagues showed that the blood films collected in 1996 and previously diagnosed by microscopy as "*P. malariae*" were in fact *P. knowlesi* [25]. This clearly indicates that incidence of human infections with *P. knowlesi* is not new and that the infections have been misdiagnosed as *P. malariae* for many years.

The five archival specimens from Pahang found to be positive for *P. knowlesi* [9] only represent the tip of the iceberg. The research group from the Institute for Medical Research (IMR) in Kuala Lumpur led by the senior author of this chapter (the research group that incriminated the Anopheline vector of *P. knowlesi* in the Kapit division of Sarawak) initiated a study to determine the malaria situation in peninsular Malaysia [26]. Blood samples or Giemsa-stained blood films sent to IMR from hospitals and health centres across peninsular Malaysia for confirmation of malaria provided an opportunity to determine the distribution of human knowlesi malaria. Again, by using nested PCR assay, Vythilingam and colleagues found that human *P. knowlesi* infections occurred in most states of peninsular Malaysia and further concluded that human knowlesi malaria is widely distributed across the peninsular Malaysia [26].

In the northern state of Malaysian Borneo, extensive studies have also been carried out to further determine the incidence of human knowlesi malaria. A molecular epidemiological study led by a research group from the University of Malaysia Sabah (UMS) examined over 200 samples from patient suspected with malaria infections in the interior division of Sabah [27, 28]. The region in the interior of Sabah is hilly and mainly covered by primary and secondary rainforest, where the natural hosts of *P. knowlesi* are commonly found. By using nested PCR assay, *P. knowlesi* was detected in almost 60 % of the total number of samples that were positive for malaria. Importantly, it was confirmed that all samples that were originally diagnosed by microscopy as *P. malariae* were actually *P. knowlesi*, thus illustrating the misdiagnosis of *P. knowlesi* and its close resemblance to *P. malariae*.

A retrospective study led by a research group from Australia investigated the incidence of *P. knowlesi* in the northeastern region of Sabah [29]. Based on the examination of archival blood slides by molecular method, a high proportion of *P. knowlesi* positive cases (76 %) were confirmed, of which the majority were previously diagnosed by microscopy as *P. malariae*. *Plasmodium knowlesi* was the predominant malaria species in the Kudat region of Sabah. The same research group also described the clinical outcome of severe *P. knowlesi* infections through a retrospective review of malaria cases in a tertiary care hospital in Sabah [30]. By using the WHO criteria, it was demonstrated that the proportion of severe knowlesi malaria is higher than previously reported. Surprisingly, review of cases also observed the large proportion of severe knowlesi cases among female patients, although the reason behind such proportion is still unclear. Most notably, malaria infection in children is not uncommon in this region, and most cases among children are caused by *P. knowlesi* [29, 31]. However, their observation suggests that *P. knowlesi* infections in children are usually uncomplicated, and they responded adequately to conventional antimalarial drugs.

It appears that the population at risk of acquiring *P. knowlesi* infections are those living or travelled into the Malaysian forest, where the reservoir hosts and mosquito vectors are abundant. It also seems that a single infection may not confer immunity against the parasite, possibly due to the high antigenic diversity. In 2011, Lau and colleagues from the University of Malaya reported a case of *P. knowlesi* reinfection

in human, who acquired knowlesi malaria twice within a 1-year period [32]. Both occasions were associated with history of travel into the forest. The patient presented clinical symptoms about 2 weeks after travelling to the forest in the state of Pahang and Perak. Interestingly, genotyping of *P. knowlesi* parasites in this case based on the *csp* gene revealed that both infections were due to distinct parasite strains, suggesting that infection with a specific strain of *P. knowlesi* may not necessarily provide protective immunity towards another strain.

2.5.2 Thailand

The first case of human infection with *P. knowlesi* reported in Thailand provided further evidence on its widespread distribution and that reliance on examination of blood film by microscopy can lead to misdiagnosis of *P. knowlesi* infection. During an evaluation of PCR detection assay for human malaria parasites, Jongwutiwes and colleagues from Chulalongkorn University detected one of the positive control isolates that were negative for all four human malarias [33]. This particular case was originally diagnosed as *P. malariae* by microscopy given that every developmental stage seen under the microscope was similar to a typical *P. malariae* parasite. Some atypical morphological features for *P. malariae* such as fimbriated edges, irregularly shaped cytoplasm and tenue form of the parasite were also noticed. However, analysis of the SSU rRNA and cytochrome b genes revealed that the parasite was actually *P. knowlesi* [33].

The same research group subsequently carried out a large-scale study between 2006 and 2007 to investigate the prevalence of *P. knowlesi* in Thailand [34]. By using nested PCR assay, they examined blood samples from 1,874 febrile patients at four distinct regions near the Myanmar–Thailand border. The prevalence of *P. knowlesi* was surprisingly low at 0.57 %, although the parasite was found in all four regions studied. In the same study, mixed species infections especially between *P. falciparum* and *P. vivax* were quite common in regions bordering Myanmar. Nine out of ten patients infected with *P. knowlesi* were also co-infected with either *P. falciparum* or *P. vivax*. This study concluded that *P. knowlesi* mostly occurred as cryptic infections in Thailand despite its widespread distribution across Thailand [34].

2.5.3 Philippines

Early evidence of the presence of *P. knowlesi* malaria parasite in the Philippines was first reported in 1961 based on the isolation of the parasite from the blood of a long-tailed macaque [35]. In the early 1970s, a group of Japanese researchers who conducted a survey on simian malaria parasites and their vectors in Palawan Island found that long-tailed macaques with relatively higher parasite count had

P. knowlesi malaria parasite co-infected with other species of simian malarias such as *P. inui*, *P. cynomolgi* and *P. coatneyi* [36]. Although this observation was based on the examinations of blood smears, it is evident that the transmission of *P. knowlesi* continued to be maintained in the island at least among the wild macaques. In 2008, five human cases confirmed by molecular detection as *P. knowlesi* were reported from five distinct locations in the Palawan Island [37]. These cases were also misdiagnosed by microscopy as having *P. malariae* either single infection or mixed with *P. falciparum* or *P. vivax*. Evidence of human knowlesi malaria based on this report provides further evidence of the widespread of *P. knowlesi* in Southeast Asia.

2.5.4 Singapore

The isolation of *P. knowlesi* parasites from a monkey imported from Singapore in 1932 [15] was perhaps the earliest indication that the parasites have maintained its transmission for a considerable period of time at least among the macaques population within the island. It is also possible that *P. knowlesi* have been transmitted to humans, but the infections may have been misdiagnosed as human malaria especially *P. falciparum* or *P. malariae*.

Singapore is a highly urbanized city state and has been declared malaria-free by WHO since 1982 [38]. However, it appears that there is potential risk of acquiring *P. knowlesi* infections at the forested areas where army trainings are usually conducted. A total of six cases of human knowlesi malaria were reported between 2007 and 2008 [39–41]. All cases involved military personnel who had visited the same forested area, which is also the natural habitat of long-tailed macaques. Molecular analysis of the *csp* gene suggests these human cases were epidemiologically linked to the infected long-tailed macaques caught at the same area. On the other hand, peri-domestic macaques from nature reserve park were free of malaria infections, suggesting the presence of competent Anopheline mosquitoes may be limited to the forested area [39].

2.5.5 Vietnam

Examination of blood samples derived from cross-sectional surveys conducted in 2004 and 2005 in Ninh Thuan Province, a forested region of central Vietnam, to screen for the presence of *P. knowlesi* in humans yielded interesting findings in terms of clinical presentations and case demographic [42–44]. Only 5 out of 95 samples selected for screening with *P. knowlesi* primers were positive by PCR assay, and only three samples were further confirmed by sequencing. Two of these confirmed cases were young children aged 2 and 3 years old, whereas the other case was a 27-year-old man. Interestingly, all three cases had low parasite counts and

were asymptomatic and co-infected with *P. malariae*. Nevertheless, the findings in Vietnam indeed proved the presence of *P. knowlesi* and provided additional perspective on *P. knowlesi* infections in terms of its asymptomacity and occurrence among young children. The finding of persistent *P. knowlesi* infections in one of the children who was identified as having *P. knowlesi* infection even 1 year later strongly suggests that *P. knowlesi* may be more common in Vietnam than previously known.

Plasmodium knowlesi also appeared to be widespread across Vietnam. Epidemiological surveys conducted in southern Vietnam through a 12-month active case detection and a cross-sectional survey showed that *P. knowlesi* cases were detected across 8 of 12-month period, suggesting the continuous transmission of *P. knowlesi* parasite [45]. Most notably, the majority of patients with *P. knowlesi* infections were asymptomatic (81.3 %), and only 6 of 32 (18.7 %) patients were symptomatic. Interestingly, all these patients were also co-infected with other malaria species, particularly *P. vivax*.

2.5.6 Myanmar

The prevalence of malaria at the border region of Myanmar has been documented [46]. An isolated case report in 2006 indicates that distribution of *P. knowlesi* extends to the region bordering Myanmar and People's Republic of China [47]. Further investigation was conducted by Jiang and colleagues to determine the prevalence of *P. knowlesi* at the border region between southern Myanmar and Yunnan Province, China [48]. Examination of 146 microscopy confirmed malaria samples by nested PCR assay and sequencing of the small subunit rRNA gene revealed a prevalence of 21.9 % for *P. knowlesi*, with majority of these infections occurring as mixed infections with *P. falciparum* or *P. vivax* or both.

2.5.7 Indonesia

The distribution and prevalence of human knowlesi malaria in Indonesia are not well studied, considering the geographical scale that covers a large part of the Southeast Asia region. So far, a few human malaria cases confirmed as *P. knowlesi* have only been reported from Kalimantan, Indonesian Borneo [49, 50]. In one study focusing on the molecular epidemiology of malaria in Indonesia, *P. knowlesi* were detected in 4 out of 22 samples tested [50]. Of these, at least one case of *P. knowlesi* was confirmed by nested PCR assay and sequencing of the PCR amplicon. The remainder three samples were possibly mixed infections of *P. falciparum*, *P. vivax* and *P. knowlesi*. Further analysis of the PCR amplicons for these three samples revealed the sequences of *P. vivax*, although the size of the amplicons appeared to be identical to that of *P. knowlesi*. Similar to the observation made in Thailand

(described in Sect. 2.9.2), it seems that the original *P. knowlesi*-specific primers developed by Singh and colleagues [2] have cross-reacted with the DNA of *P. vivax* from south Kalimantan. In another reported case, *P. knowlesi* was detected in an Australian who frequently travelled to the forested area in South Kalimantan Province [49]. Recently, a molecular epidemiology study carried out by researchers from University of Airlangga revealed that a group of workers at oil palm plantations in Central Kalimantan (mostly migrants from the Java Island) were infected with *P. knowlesi* (Kasmijati, Sukmawati and YoesPrijatna, unpublished data). These limited reports of human knowlesi malaria indicate that the distribution of *P. knowlesi* extends to the Indonesian side of the Borneo Island. The prevalence of *P. knowlesi* in human as well as in macaque population in the other parts of Indonesian archipelago remains largely unknown. Certainly, it would not be surprising to find more human cases of *P. knowlesi* if extensive malaria surveillance covering a larger geographical area is carried out.

2.5.8 Cambodia

Epidemiologic study in Cambodia provides further evidence on the widespread of *P. knowlesi* in the Southeast Asia region. In a cross-sectional prospective study conducted between 2007 and 2010, a total of 1,475 patients were examined, of which 754 patients were positive for malaria infections [51]. Two cases of *P. knowlesi* originating from two distinct locations in the Pailin Province were detected. Both patients frequently travel to the forested area, where long-tailed macaques are usually found. Clearly, the macaques in Cambodia are likely the reservoir host of *P. knowlesi*. However, further epidemiologic study at a wider scale is necessary to stratify the potential risk of *P. knowlesi* infections in Cambodia.

2.6 Natural Hosts of *Plasmodium knowlesi*

The long-tailed and pig-tailed macaques are the two main natural hosts of *P. knowlesi* [6, 7]. Study conducted in the 1960s reported that banded leaf monkeys (*Presbytis melalophos*) in the peninsular Malaysia are also naturally infected with *P. knowlesi* [52]. The distribution of long-tailed and pig-tailed macaques in Southeast Asia is extensive. They are found mainly in the forest covering most of the mainland of Southeast Asia, Borneo, Sumatra, Java, Philippines and Singapore [53, 54].

Studies conducted in the 1970s have shown that the macaques in Cebu and Palawan islands in the Philippines were harbouring *P. knowlesi* parasites [36]. *Plasmodium knowlesi* parasite is highly prevalent among the wild macaques in Sarawak, Malaysian Borneo [55]. Molecular study conducted on wild macaques caught in the Kapit division showed that 94 % of the macaques caught were infected with *P. knowlesi*.

Interestingly, the transmission of simian malaria among the wild macaques in the forest of Sarawak appeared to be very intense as almost all wild caught macaques were also co-infected with other species of simian malaria. In fact, *P. inui* was the most common malaria species (82 %) found followed by *P. knowlesi* (78 %), *P. coatneyi* (66 %), *P. cynomolgi* (56 %) and *P. fieldi* (4 %) [55]. The notion of high intensity of transmission is further supported by the analysis of mtDNA and *csp* gene of *P. knowlesi*, where the number of mtDNA haplotypes and *csp* alleles was significantly higher in the wild macaques as compared to those found among human patients [55].

A study utilizing molecular tools conducted in the peninsular Malaysia showed that the long-tailed macaques are also the natural hosts of *P. knowlesi* [26]. Ten out of 75 (13.3 %) long-tailed macaques trapped in Kuala Lipis were positive for *P. knowlesi*. In contrast, none of the 29 long-tailed macaques trapped from the urban areas in Kuala Lumpur was infected [26]. This observation suggests the strong linkage between the presence of competent mosquito vectors, humans and monkeys and thus highlighting the potential risk of human infections with knowlesi malaria in areas where the reservoir hosts and mosquito vectors are present.

The presence of a competent Anopheline species in areas where there are natural hosts of *P. knowlesi* are found is essential for maintaining the transmission cycle of the parasite. Similar results were also reported in a malaria survey conducted on the macaques in Singapore [39]. Wild long-tailed macaques caught from the forested area were positive for *P. knowlesi*, whereas none of the peri-domestic monkeys, caught from the nature reserve park were infected with any malaria parasites. Although the vectors of *P. knowlesi* have not been identified in Singapore, it is known that Anopheline mosquitoes are limited or virtually absent in areas where peri-domestic monkeys are commonly seen [39].

Long-tailed and pig-tailed macaques in Thailand are also the natural hosts for *P. knowlesi*, although its prevalence appeared to be much lower compared to those reported in Malaysian Borneo and peninsular Malaysia. A prospective malaria survey conducted on macaques caught near the Thai–Malaysian border (Yala Province and Narathiwat Province) showed that *P. knowlesi* was only detected in 5.3 % of long-tailed macaques and 2.3 % of the pig-tailed macaques [56]. Interestingly, one langur (*Semnopithecus obscurus*) caught in the same study was also found to be positive for *P. knowlesi*.

2.7 Mosquito Vectors of *Plasmodium knowlesi*

Mosquitoes belonging to the Leucosphyrus group have been incriminated as vectors of simian malaria. The Leucosphyrus group is divided into three subgroups: Leucosphyrus, Hackeri and Riparis. The Leucosphyrus subgroup consists of Leucosphyrus complex which is made up of five species, namely *An. leucosphyrus* Donitz, *An. latens* Sallum and Peyton, *An. introlatus* Colless, *An. balabacensis* Baisas and *An. baisasi* Colless, and the Dirus complex is made up of eight species, namely *An. dirus* Peyton and Harrison, *An. cracens* Sallum and Peyton, *An.*

scanloni Sallum and Peyton, *An. baimaii* Sallum and Peyton, *An. elegans* (James), *An. takasagoensis* Morishita, *An. nemophilous* Peyton and Ramalingam and *An. mirans* Sallum and Peyton. The subgroup Hackeri consists of four species, namely *An. hackeri* Edwards, *An. pujutensis* Colless, *An. recens* Sallum and Peyton and *An. sulawesi* Koesoemawinangoen, and the last subgroup Riparis consists of three species *An. riparis* King and Baisas, *An. cristatus* King and Baisas and *An. macarthuri* Colless [57].

Studies carried out in the 1960s incriminated *An. hackeri* as the vector of *P. knowlesi* and was found in the mangrove area of Selangor [58]. *Anopheles hackeri* was also incriminated as vector of four other simian malarias, namely *P. cynomolgi*, *P. inui*, *P. coatneyi* and *P. fieldi* [59]. This mosquito was zoophagic and was never found biting humans. Other studies conducted in the Northern region of peninsular Malaysia in the state of Perlis incriminated *An. balabacensis* (now known as *An. cracens*) as vector of *P. inui* and *P. cynomolgi* [60]. In Hulu Lui and Gombak in Selangor, *An. latens* was incriminated as vector for *P. inui* and *An. introlatus* as vector of *P. cynomolgi* and *P. fieldi,* respectively. At that time it was postulated that knowlesi malaria would not infect humans since *An. hackeri* was found only biting monkeys.

Studies carried out in Kapit, Sarawak, Malaysian Borneo, incriminated *An. latens* to the vector of *P. knowlesi* [61, 62]. Using molecular tools, *Anopheles latens* was also incriminated as vector of *P. cynomolgi*, *P. inui*, *P. coatneyi* and *P. fieldi* [63]. *Anopheles latens* will feed on either humans or monkeys; monkey to human biting ratio was 1:1.3 [62]. *Anopheles latens* is also the vector of human malaria in Sarawak [64] but during forest clearing vectors had been replaced by *An. donaldi*.

In Kuala Lipis, Pahang, *An. cracens* was incriminated as the vector of *P. knowlesi* [26, 65]. *Anopheles cracens* was the predominant species in the study area comprising 66.2 % of the collection. The study showed that *An. cracens* was more attracted to humans than monkeys, with human to monkey biting ratio of 2 to 1. Generally in peninsular Malaysia, it is now known that Anopheline mosquitoes of the leucosphyrus group are more commonly collected compared to decades ago when human malaria was high.

Besides Malaysia, studies have been conducted only in Vietnam to determine the vector of simian malaria [45, 66]. In Vietnam, *An. dirus* has been incriminated as the vectors of *P. knowlesi*, and there was mixed infection of *P. knowlesi* and *P. falciparum* and *P. vivax* sporozoites in the same mosquito [45]. This is the first instance where the mosquito has both the human and simian malaria sporozoites.

2.8 Emergence and Evolutionary History of *Plasmodium knowlesi*

Previous molecular phylogenetic studies on malaria parasites have demonstrated the close relationship of *P. knowlesi* to *P. coatneyi* [67], another species of simian malaria that also naturally infects long-tailed macaques and behaves almost similarly like *P. knowlesi* when inoculated in rhesus macaque [6]. However, the evolution and the emergence of *P. knowlesi* are still not well understood. Much of the recent understanding of the emergence and evolutionary history of *P. knowlesi* derived from studies conducted in the Kapit division, where a large number of human cases of knowlesi malaria have been reported. The most recent estimation of the age of *P. knowlesi* suggests that the extant parasite population could be as old or older than *P. falciparum* and *P. vivax* [55]. By using the Bayesian coalescent approach to analyse of the complete mtDNA genomes of a population of *P. knowlesi* parasites that derived from human cases as well as infected macaques in Sarawak, it was estimated that *P. knowlesi* emerged approximately 257,000 years ago (95 % range 98,000–478,000) [55]. Previous studies estimated that *P. falciparum* emerged sometime between 50,000 and 330,000 years ago [68, 69] whereas for *P. vivax*, sometime between 53,000 and 265,000 years ago [70, 71]. Most interestingly, the mtDNA dataset also revealed that *P. knowlesi* underwent a rapid population expansion between 30,000 and 40,000 years ago. This period directly overlapped with the previously estimated time of human population expansion in Southeast Asia. On the other hand, similar analysis conducted on macaque populations in Southeast Asia based on the cytochrome b sequences in public database did not reveal a parallel signature of population expansion with that of *P. knowlesi* or human populations. There may be limitations to the result interpretation when cyt b sequences alone are used for such analysis as the lack of resolution may not reveal an accurate estimate of the time of population expansion for the macaque population. While it seems that the population growth of *P. knowlesi* parasite and human population is correlated, it is also possible that this observation is purely coincidental. Other factors especially the expansion of mosquito vectors and their adaptation may potentially play a role in changing the demographic history of *P. knowlesi* [55]. However, these factors are still poorly understood and necessitate in-depth population studies on the mosquito vectors in Malaysian Borneo.

Given the widespread distribution of *P. knowlesi* in this region, there is still limited understanding of the evolutionary and phylogeographic relationship among the *P. knowlesi* parasite populations in different localities. Sequence analysis of the mitochondrial *coxI* gene of *P. knowlesi* from peninsular Malaysia, Sarawak and Thailand revealed a distinct phylogeographic structure among the *P. knowlesi* parasites in these three locations [72]. Although preliminary, this finding probably suggests that *P. knowlesi* evolved independently in these three locations. Perhaps, the population and evolutionary histories of *P. knowlesi* in the mainland of Southeast Asia may be different from that observed in Sarawak. In-depth molecular

studies that employ sampling of *P. knowlesi* at a wider geographical scale across Southeast Asia will shed further light on the understanding of the emergence and evolution of parasite. Molecular evidence based on data from Sarawak indicates *P. knowlesi* probably represent an ancient zoonosis, and human population and macaques may have been infected since its emergence.

Data generated from whole genome sequencing of malaria parasites is anticipated to provide new avenues for advancing the understanding of the parasite's biology and evolution. The 23.5 megabase genome sequence of *P. knowlesi*, which is made up of 14 chromosomes, has been described [73]. The genome consists of a total of 5,188 protein-coding genes, of which approximately 80 % of the predicted genes in *P. knowlesi* can be identified with both *P. falciparum* and *P. vivax*. The *P. knowlesi* SICAvar and *kir* genes formed the two major variant antigen gene families that are randomly distributed across all 14 chromosomes. Most notably, the *kir* genes revealed a high degree of molecular mimicry to the host cell receptor CD99 in macaques and thus supporting the notion that *P. knowlesi* is adapted to macaque hosts [73].

2.9 Laboratory Diagnosis of *Plasmodium knowlesi*

2.9.1 Microscopy

Microscopy examination of stained blood films is regarded as the "gold standard" for diagnosis of human malaria [74]. It is still the preferred and reliable method for the detection of malaria parasites in malaria-endemic countries. The method is relatively simple, rapid and cheap as it only requires preparation of stained thin and thick blood smears followed by examination using a standard microscopy technique under a 100× objective. Microscopy is also a sensitive method and allows parasite density to be quantified. This is particularly useful for monitoring the effectiveness of malaria treatment. However, accurate detection by microscopy requires experienced microscopist, a proper microscope and staining reagents, which are often lacking in developing countries [74]. Although each species of human malaria possesses certain morphological characteristics that allow one species to be distinguished from the other under the microscope, diagnosis can be difficult when parasitaemia is low and only certain stages such as early trophozoites are present. For instance, the early trophozoites of most malaria species appear to be almost identical, and it is not possible to definitively confirm the species of malaria on the basis of early trophozoites.

Accurate identification of malaria species by microscopy becomes more difficult when there are identical morphological features at various stages of the erythrocytic cycle that are shared by more than 1 species. Such is the case for *P. knowlesi* as the parasite shares several morphological characteristics with *P. falciparum* and *P. malariae*, which makes accurate diagnosis by microscopy

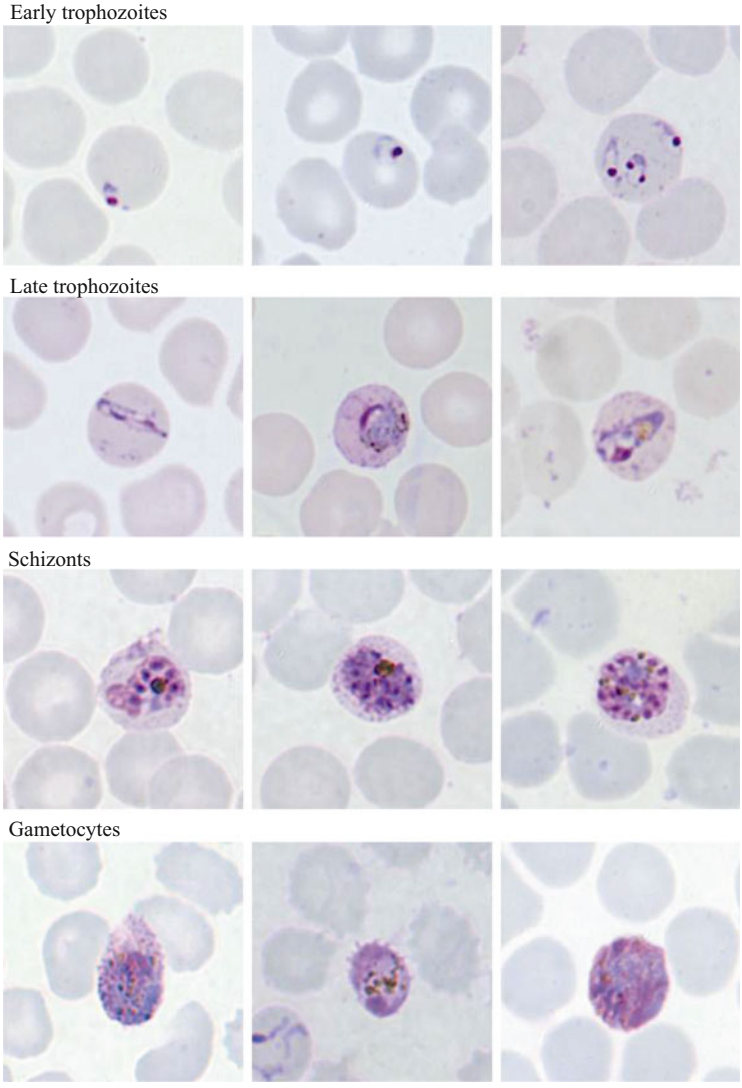

Fig. 2.1 Morphological features of the blood stages of *Plasmodium knowlesi* in Giemsa-stained thin blood films. The figure is reproduced from reference [75] with permission from Biomed Central

virtually impossible [2, 75]. The early trophozoite of *P. knowlesi* and *P. falciparum* are totally identical. Morphological features commonly seen in falciparum malaria such as double chromatin dots, multiple parasites in single erythrocyte, appliqué forms and no changes to the size of infected erythrocytes are also seen in knowlesi malaria infections (Fig. 2.1). In the later developmental stages including late trophozoites, schizonts and gametocytes, the morphology of *P. knowlesi* becomes generally indistinguishable from *P. malariae*, with very minor differences [75]. In a

study to carefully examine Giemsa-stained blood films from patients having low to high parasitaemias, it was observed that the cytoplasm of some late trophozoites of *P. knowlesi* appeared to be amoeboid [75]. Other minor differences between *P. knowlesi* and *P. malariae* include the maximum number of 16 merozoites per schizont and the absence of "rosette pattern" at the mature schizont stage of *P. knowlesi*. Certainly, these minor differences cannot be used to distinguish *P. knowlesi* from *P. malariae* as these features can be easily missed or absent in most knowlesi malaria infections, especially among those with low parasitaemia [75].

While microscopy diagnosis of *P. knowlesi* remains a challenge at routine laboratories, it is of utmost importance that the parasite is accurately identified and quantified at the clinical setting. Human infections with *P. knowlesi* can be potentially life threatening, as a result of the parasite's ability to replicate rapidly leading to hyperparasitaemia. Accurate diagnosis is therefore important so that prompt and suitable treatment with proper clinical management can be carried out.

2.9.2 Nested PCR

The development of molecular tools such as PCR has revolutionized the field of pathogen diagnostic. Molecular methods have been developed and widely used to detect infectious microorganisms such as bacteria, viruses and parasites. Detection of the four human malaria parasites using molecular tools has been developed and established since the 1990s [76–78]. Nested PCR assay for the detection of malaria parasites has been shown to be far superior compared to conventional microscopy in terms of sensitivity and specificity. This method targets the small subunit ribosomal RNA gene of malaria parasites by utilizing the conserved regions for first-round amplification with genus-specific primers, followed by amplification with species-specific primers that targets the variable regions in separate PCR reactions. It was the application of nested PCR malaria detection assay in the initial molecular epidemiological study in Sarawak that led to the discovery of human infections with *P. knowlesi* in the Kapit division of Sarawak, Malaysian Borneo [2]. A set of *P. knowlesi*-specific primers, Pmk8 and Pmkr9, was designed and incorporated into the existing nested PCR malaria detection assay. In a molecular epidemiologic study in Sarawak, the application of these *P. knowlesi*-specific primers revealed that 27.7 % of 960 malaria patients across Sarawak were infected with *P. knowlesi*. The majority of these patients were misdiagnosed by microscopy as having *P. malariae* [2]. The same set of primers were also used to examine 108 wild macaques caught from 17 locations in the Kapit division of Sarawak. The findings revealed that 78 % of these macaques were harbouring *P. knowlesi* parasites [55].

The Pmk8 and Pmkr9 primers were subsequently used to detect for the presence of *P. knowlesi* infections in malaria patients in other parts of Southeast Asia [9, 26, 37, 40, 47, 48, 51]. However, these primers appeared to be unspecific, particularly

when tested against some isolates of *P. vivax* in other regions [50, 79]. In a study conducted in Thailand, Imwong and colleagues found that these primers also amplified the target gene of some *P. vivax* isolates. Further investigation through analysis of the primer sequences demonstrates that the false positive was likely due to stochastic cross-reactivity of the primers with *P. vivax* DNA [79]. A new set of three primers was designed for specific amplification of *P. knowlesi* small subunit rRNA gene, either as semi-nested PCR reaction or combined with previously designed genus-specific primers in a nested PCR assay. Similarly, the cross-reactivity of Pmk8 and Pmkr9 primers with *P. vivax* DNA was also observed as weak amplification in other study in Myanmar [48].

To address the specificity and sensitivity of using small subunit rRNA gene in nested PCR assay, Lucchi and colleagues recently reported a new single-step PCR that targets novel genomic sequences [80]. By using data mining approach on the parasite genome database, a primer set based on a multicopy genomic sequence of unknown function was identified and shown to be highly specific and sensitive for *P. knowlesi*. Although this novel primer set was shown to be 100 % specific, the finding was based on one clinical isolate of *P. knowlesi*. Considering the genetic diversity of *P. knowlesi* parasites, a proper validation of these novel primers with a larger set of clinical *P. knowlesi* samples is necessary [80].

Using the small subunit rRNA gene target, a research group from the University of Malaya recently developed a rapid, single-step multiplex system for the detection of all five human malaria species, including *P. knowlesi* [81]. Due to multiplexing, this assay is less labour intensive and requires significantly less time for preparation compared to semi-nested or nested PCR assay. However, the limitation of this multiplex system is that it can only detect mix infections up to two species level. It has also not been fully validated with significant numbers of naturally acquired mixed infections. Currently, this multiplex assay is commercialized under the trade name PlasmoNex^{TM}.

2.9.3 Real-Time PCR

The advancement of PCR technology from end-point detection to real-time detection has enhanced the diagnosis of pathogens by providing a more rapid detection and quantitative data. Previously, there were several reports describing the use of real-time PCR for the detection of *P. falciparum, P. vivax, P. malariae and P. ovale* [82–85]. More recently, real-time PCR assays for the detection of *P. knowlesi* have also been described [86–88]. To date, all real-time PCR assays developed for *P. knowlesi* are based on detection of the small subunit ribosomal RNA gene. Several protocols of real-time PCR assays that were described utilized either SYBR Green dye [88], FRET probes [86] or TaqMan probes [87] and with sensitivity of detection between 5 and 100 copies of template per micro litre. However, most of these real-time PCR protocols for *P. knowlesi* were only tested with a small number of reference samples. So far, the real-time PCR assay that

utilizes TaqMan probe for the detection of *P. knowlesi* is the only assay that has been validated with a wide range of clinical samples of *P. knowlesi* [87].

2.9.4 Lamp PCR

In the recent development of PCR technique, a novel nucleic acid amplification approach termed loop-mediated isothermal amplification (LAMP) has started to gain considerable interest among molecular microbiologist for its potential applications in pathogen detection. Unlike the conventional PCR method, LAMP employs DNA polymerase with strand displacement activity (e.g. *Bst* DNA polymerase) and a set of four different primers that target six specific regions of the targeted genomic region [89]. These primers consist of forward and backward inner primers as well as outer primers that work through the amplification reaction process at constant temperature to form specific double-stranded structure with loops at both ends. The loops serve as binding sites for the inner primers to initiate amplification through a new cycling step, and the process continues until targeted DNA structures with multiple loops are produced [89]. LAMP method has been described to be highly specific, and its sensitivity is comparable to that of conventional PCR. It is also being described as simple and easy to perform without requiring expensive thermocycler [90]. The detection time for LAMP is also significantly shorter as the result can be visually interpreted based on turbidity of the reaction caused by the precipitation of magnesium pyrophosphate, a by-product from the amplification process [90].

The use of LAMP method for the detection of *P. falciparum* has been described previously [91, 92]. Iseki and colleagues extended this approach to the detection of *P. knowlesi* and evaluated its sensitivity, specificity and potential use at the clinical setting [93]. The LAMP assay for the detection of *P. knowlesi* was based on the β-tubulin genes of malaria parasites. They demonstrated that the primer set for *P. knowlesi* was highly specific after evaluating nine species of simian malaria parasites including *P. knowlesi* and four human malaria species. The detection with LAMP assay was 100-fold more sensitive when compared to single-round conventional PCR with detection limit up to 100 copies of DNA template per sample. It appears that the *Bst* DNA polymerase used in LAMP assay is highly robust as inhibitors in the blood do not seem to affect its performance. An evaluation using different DNA preparation from whole blood and genomic extracts showed identical results, and thus highlighting its usefulness as a new tool for malaria diagnostic and surveillance. Another LAMP assay developed based on the apical membrane antigen 1 (AMA-1) of *P. knowlesi* was shown to have higher sensitivity [94]. The AMA-1-based LAMP assay was able to detect up to ten copies of DNA template per sample.

2.9.5 Rapid Antigen Kit

The development of rapid diagnostic tests (RDTs) based on the principle of immunochromatography has opened up possibility of a more rapid and yet less labour-intensive approach for the detection of malaria parasites. Immunochromatographic test as applied in many commercially available RDTs for malaria used either monoclonal or polyclonal antibodies to capture the parasite's antigen in infected blood before it is conjugated to a bioactive particle in a mobile phase [95]. Another component applied to the RDT strip is another monoclonal antibody that acts as the immobile phase. As the antigen–antibody complex migrates in the mobile phase along the strip, the antibody on immobile phase will capture the labelled antigen to produce a visible coloured line [95]. The current RDTs for the detection of malaria parasite target histidine-rich protein 2 (HRP-2), lactate dehydrogenase (pLDH) and aldolase [95]. While the test is rapid and simple, there are several limitations to the use of RDTs for malaria detection, which include low sensitivity especially when blood with low parasitaemia is tested, false positivity due to cross-reactions with autoantibodies such as rheumatoid factor or with persisting targeted antigens even after parasites are cleared from circulation and also false negativity [95].

In the first evaluation of RDT on *P. knowlesi*, McCutchan and colleagues demonstrate that, to some extent, a pLDH-based RDT commonly used for detecting *P. falciparum* and *P. vivax* can also be used to detect *P. knowlesi* [96]. Although the pLDH-based RDT is able to differentiate *P. knowlesi* from *P. malariae* and *P. ovale*, it cross-reacts with both *P. falciparum*- and *P. vivax*-specific pLDH antibodies and therefore cannot be used to differentiate between *P. knowlesi* and mixed infections of *P. vivax* and *P. falciparum* [96].

Thus far, the use of RDTs for the detection of *P. knowlesi* has been mostly demonstrated on travellers with knowlesi malaria [49, 97–102]. Based on the limited number of reports, RDT based on detection of both *P. falciparum*-specific HRP-2 and aldolase antigen appeared to have lower sensitivity. Out of eight case reports that described the use of this RDT [41, 49, 97–102], only three cases indicated positive results for pan-malaria antigen including one case that was also positive for *P. falciparum*. In one of these case reports, an evaluation between *P. falciparum* HRP-2/pan-malaria-based RDT and pLDH-based RDT for the detection of *P. knowlesi* showed that LDH-based detection is more sensitive as it was able to detect the parasite's antigen in samples with lower level of parasitaemia [98].

2.10 Key Gaps and Way Forward

The increasing number of studies conducted to investigate the human cases of *P. knowlesi* in recent years has contributed to our understanding on its epidemiology and widen our perspective on how we viewed malaria in this region. However,

the key gaps remain in terms of the understanding of its actual burden, its transmission, its pathogenesis and the mosquito vectors involved. While the clinical aspects and treatment are not covered in this chapter, it is known that most cases responded well to treatment based on the existing guidelines for human malaria. Few studies have been conducted particularly in the Malaysian Borneo to investigate the pathogenesis of *P. knowlesi* infections [103, 104]; however, the current understanding on the disease development and its potential risk in causing severe infection are still limited.

Human cases of *P. knowlesi* malaria will continue to be uncovered in new areas in Southeast Asia and further extending its distribution. However it remains a challenge to estimate the true burden of human knowlesi malaria in this region. The potential risk of *P. knowlesi* infections among humans in many areas where human *P. knowlesi* infections have been reported remains largely unknown. Most reported cases were symptomatic cases derived from cross-sectional studies with the utilization of molecular tools. There is also limited understanding of asymptomatic *P. knowlesi* infections in humans, and it is currently unknown how much asymptomatic cases contribute to the overall epidemiology of *P. knowlesi*. To estimate the actual prevalence of *P. knowlesi*, it is therefore essential to conduct large-scale longitudinal surveillance in *P. knowlesi* endemic areas. In this surveillance, the application of molecular tools and serological assays for *P. knowlesi* will provide useful information not only on symptomatic cases but will also shed further light on the past exposure to *P. knowlesi* infections.

While new molecular assays continue to be developed and simplified, it can never replace the conventional microscopy diagnosis due to its cost and feasibility in the rural settings where most malaria cases are usually reported. Considering the potential life-threatening infection due to *P. knowlesi*, it is important to diagnose the human knowlesi malaria quickly and accurately. Therefore, there is a need for development of highly sensitive and specific antigen-based rapid diagnostic test for *P. knowlesi* to complement microscopy diagnosis.

At this juncture, it is still unclear whether the increasing prevalence of *P. knowlesi* was largely due to transmission between human to human and monkey to human. The current studies that involved surveillance of human cases or malaria parasites in macaques have yet to yield any evidence of host switching by *P. knowlesi*.

Although it is widely known that *Anopheles* mosquitoes of the leucosphyrus group are potential vectors of *P. knowlesi*, the vector species responsible for transmission to human host is still not known in many areas where *P. knowlesi* have been reported. To date, the vectors that have been incriminated are mostly exophilic, which makes vector control very challenging. A more extensive entomological surveillance is essential to understand the mosquito vectors and their bionomics. Data on mosquito vectors will also provide invaluable information for assessment of potential risk of acquiring *P. knowlesi* infections at different places throughout Southeast Asia.

2.11 Conclusion

The fifth human malaria species *P. knowlesi* is now a predominant species affecting humans especially in Malaysia. While countries in Southeast Asia are now working towards eliminating malaria, the daunting task ahead is how to control the spread of *P. knowlesi*. Early detection and treatment are important because *P. knowlesi* is deadly and mortality can occur if early diagnosis and treatment are not carried out. The current vector control tools that is indoor residual spraying and insecticide-treated bed nets would not be effective because most vectors have shown exophilic and exophagic tendencies. Thus, now that malaria is a zoonosis, it will be difficult to eliminate the disease, and more work is needed on every aspect of the parasite, vectors and hosts.

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Chapter 3
Filarial Worms in Southeast Asia

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Abstract The lymphatic filariae, namely *Brugia malayi*, *B. timori* and *Wuchereria bancrofti*, are of medical importance in Southeast Asia. Brugian filariasis is predominant in Indonesia, Malaysia, Brunei Darussalam and Vietnam; bancroftian filariasis is common in Lao PDR, Philippines and Myanmar, while both types of filariasis are found in Cambodia, Southern Philippines, Thailand and Timor Leste. The Global Programme for the Elimination of Lymphatic Filariasis (GPELF) began in year 2000 and targeted to be achieved by 2020. These countries are at different phases of the programme, and most are showing successes in terms of health and economic benefits. The traditional thick blood smear examination using night blood is still being used for diagnosis; however, more sensitive, rapid and field-applicable tests that allow blood sampling at anytime of the day, such as Brugia Rapid and ICT card tests, are important tools for GPELF. An integral part of the programme is the mass drug administration (MDA) for a minimum of 5 years to stop transmission of the infection. It comprises an annual dose of diethylcarbamazine and albendazole, and in children this has also been shown to reverse the subclinical lymphatic

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pathology. The commonly recognised clinical manifestations of brugian filariasis are chronic lymphoedema of the limbs, which may lead to elephantiasis and repeated attacks of acute dermatolymphangioadenitis (ADLA). Limb hygiene is a simple and effective method for morbidity management to prevent ADLA and has become the mainstay for disability management in GPELF. The current trend is adoption of an integrated approach to the control of Neglected Tropical Diseases (NTD), such as combining elimination programmes for lymphatic filariasis and soil-transmitted helminths. In addition, a surveillance programme after elimination of lymphatic filariasis is crucial to prevent reemergence of this disease in the future.

3.1 Epidemiology of Lymphatic Filariasis in Southeast Asia

Filarial worms are classified under the superfamily Filarioidea of nematodes. There are eight important filariae species that infect humans; however, only the lymphatic filariae, namely *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, are of medical importance in Southeast Asia (SEA) [1].

Lymphatic filariasis (LF) affects about 120 million people in 73 countries, and about 1.4 billion live in endemic areas where chemotherapy prevention or mass drug administration (MDA) is required to eliminate the disease [2]. About 90 % of LF worldwide is caused by *W. bancrofti*, while most of the rest are confined in Asia and caused by *B. malayi* with minor endemic areas of *B. timori* [3]. The adult thread-like worms dwell in lymphatic system of humans. Copulation between mature male and female worms produces a gravid viviparous female, which releases microfilariae (mf) or first-stage larvae into the host circulation, which are then taken up by mosquitoes [4]. More than 70 different species and subspecies of mosquitoes are known to transmit LF [5]. One mf moults twice within 10–14 days to develop into a single infective L3. During feeding, L3 are released from the mosquito proboscis onto the host skin, it then gains entry into the host lymphatic system via the punctured skin. Ironically, despite the ineffective mode of transmission, LF still affects millions of humans worldwide.

The periodic and subperiodic forms of *B. malayi* can be differentiated based on the mf tendency to shed sheaths. About 50 % of the periodic form cast their sheaths, compared to <8 % in the sub-periodic form [6]. In future, it would be interesting to develop molecular tools to distinguish the two forms and utilise them for molecular epidemiological studies. This is relevant as different mosquito control strategies are required to curb the two types of filaria transmission [7]. The subperiodic *B. malayi* is a zoonotic parasite. Besides humans, other mammals such as domestic cat, *Presbytis* monkeys and wild mammals are reservoirs for the parasite [7]. Hence MDA may not be as effective in controlling the zoonotic form of lymphatic filariasis.

3.2 Southeast Asian Countries

SEA has 11 countries, in which 10 are members of the Association of Southeast Asian Nations (ASEAN) except Timor Leste, which is currently applying to be a member. Similar to other parts of this book, the geographical definition of SEA is being used in the present chapter. However, it needs to be highlighted that for logistic and political reasons, the World Health Organization (WHO) region or regional programme review group for implementation of preventive chemotherapy or MDA classify Indonesia, Myanmar, Thailand and Timor-Leste under *WHO Southeast Asia Region*, which includes Bangladesh, India, Maldives, Nepal and Sri Lanka. Other SEA countries such as Brunei Darussalam, Cambodia, Lao People's Democratic Republic (Lao PDR), Malaysia, Philippines and Vietnam are considered to be in Western Pacific and grouped under *WHO Mekong-Plus Region*. Singapore is the only country in the region which is not endemic for LF [2].

About 15 million people in SEA suffered from LF and >64 million were at risk of acquiring the disease [8]. Almost half of those at risk live in the *WHO Mekong-Plus Region*; and the rest are in Indonesia, Myanmar, Thailand and Timor-Leste in the *WHO Southeast Asia Region* [2, 9]. Lymphoedema of the limbs and/or sexual organs are reported in 15 million people worldwide, while urogenital swelling, primarily scrotal hydrocele, caused problems to 25 million men [2].

3.3 Southeast Asian Countries at Risk

Brugia malayi is the predominant species in the SEA countries like Brunei Darussalam, Indonesia, Malaysia and Vietnam, and *W. bancrofti* is commonly found in Lao PDR, Philippines and Myanmar, whereas both the filarial species are predominant in Cambodia, the islands of Southern Philippines and Thailand. Timor Leste is endemic for all the three filarial worms, but *B. timori* is the predominant species.

Brunei Darussalam is a small country situated at Northwestern region of Borneo, with Sarawak, an East Malaysian state, as its neighbour. Like in Malaysia, the predominant *B. malayi* is transmitted by *Mansonia* spp. LF is not a public health problem and the World Health Organization (WHO) foresees that this country is unlikely to require MDA [1]. Nevertheless, the Brunei Ministry of Health launched the National Programme for Eradication of Lymphatic Filariasis where MDA is implemented at some of the risky mukims (subdistricts) in the districts of Belait, Tutong and Temburong.

The northeastern provinces of Cambodia were reported to be endemic for bancroftian and brugian filariasis, with about 430,000 people at risk of LF [10, 11]. Twenty districts in four provinces (i.e. Ratanakiri, Stung Treng, Siem Reap and Preah Vihear) were covered by MDA [9]. In Vietnam, brugian filariasis was predominantly found in the northern part of the country, where 675,000 individuals in six districts of the provinces of Song Hong (Red River Delta) and Quang Binh

were at risk of the infection, whereas bancroftian filariasis was confined to a few locations in the southern region [12]. Cambodia and Vietnam had completed and subsequently stopped their MDA in all endemic areas in 2010 and 2009, respectively [2].

Indonesia is a widespread country where LF is endemic in all provinces. A total of 316 districts with about 119 million people were reported to be at risk of infections caused by *B. malayi*, *W. bancrofti* and/or *B. timori*. Environmental changes and human migration have been implicated for the fluctuations of incidences in some localities. Although the overall prevalence appears to be decreasing, endemic foci still exist in urban, rural and remote areas [13]. Indonesia has the largest population as compared to the other SEA countries; hence, the total number of LF cases is the highest in the region. Besides MDA, health education and advocacy programmes have been implemented to curb LF transmission.

In Lao PDR, the population at risk was estimated to be around 11,000 [9]. During the third national survey in 2007, four positive cases were detected in three provinces. The infected persons were treated, but no MDA was instituted [12]. Subsequently, another national survey was carried out in the same affected area, and none of the 10,000 individuals screened were positive [9].

Malaysia has a rich historical record in LF epidemiological research. LF is no longer a notifiable communicable disease in Malaysia [14]. *B. malayi* is still the endemic species in Malaysia but in recent years the detection rate of *W. bancrofti* mf has exceeded that of *B. malayi*. In 2011, *W. bancrofti* contributed to 45.5 % of the infections, followed by 36.2 % from *B. malayi* (periodic form) and the remainder by *B. malayi* (subperiodic form) [15]. However, all of the bancroftian filariasis cases were imported cases by migrant workers from Nepal, Myanmar, Bangladesh, Indonesia, India and Philippines. The total number of mf cases in Malaysia has decreased from about 1,000 cases in 1987 to less than 300 in 2003, 172 in 2006 and 156 in 2010; the cases were mostly in Sabah and Sarawak. The incidence rate reduced to 0.4 per 100,000 population in 2008 from 2.1 per 100,000 population in 2000. However, in 2011, an incidence rate of 1.4 per 100,000 population was reported where 387 new cases were detected. MDA is being instituted in endemic areas in Malaysia and 2018 is the latest target for filariasis elimination. By the fifth round of MDA, the coverage achieved has been more than 90 % [14].

In 2007, 45 of the 65 regions/provinces in Myanmar were endemic for LF, in which 46,994,323 persons were at risk of acquiring the infection [16]. The mf rates in Magway Division, Mandalay Division, Sagaing Division and Rakhine State were >10 %; while the rates in Bago and Tanintharyi Divisions were between 5 and 10 %. In Irrawaddy Division, Yangon Division and Kayin State, the microfilaraemia rates were between 1 and 5 % (http://www.whomyanmar.org/LinkFiles/Publications_MLymphaticFilariasis.pdf).

In the Philippines, *W. bancrofti* is the predominant species and about 21 million people in 40 endemic provinces were at risk of acquiring LF [9]. Both *W. bancrofti* and *B. malayi* were found in most of the southern half of the Philippine archipelago [17]. The disease is mostly confined to the rural areas and not much data is available on the epidemiology, social and economic impact of LF on women, children,

marginalised groups, farmers, abaca workers and indigenous and ethnic minorities [17]. Thailand is also endemic for both *W. bancrofti* and *B. malayi* infections. Areas along the western region of the country bordering Myanmar are endemic for bancroftian filariasis, while the southern province of Narathiwat is endemic for nocturnal subperiodic form of *B. malayi*. In 2010, 87 villages with a total population of 80,930 were mapped [18, 19].

Timor-Leste has a population of about 1 million people who live in all 14 endemic regions. All the three lymphatic filariae are present, but the predominant species is *B. timori*, which accounted for about 95 % of the cases. *An. barbirostris*, the vector for timorian filariasis, breeds in clean water and also the irrigated rice-paddy fields where there are many human activities. The regions of Dili, Liquica, Oecusse and Manatutu with a total population of about 320,000 people are at higher risk of acquiring the disease [13].

3.4 Other Filarial Worms

Other less significant filarial worms in SEA are the zoonotic *Brugia pahangi*, *Dirofilaria repens* and *Dirofilaria immitis*. In the natural environment, adult *B. pahangi* resides in the lymphatic vessels of cats, dogs and wild carnivores [20]. The innenkorper lengths of *B. pahangi* and *B. malayi* are about 53 % and 30 % of its total length, respectively [21]. In Malaysia, the first zoonotic transmission of *B. pahangi* in nature was reported in five patients from suburbia of Kuala Lumpur [22]. Infrequently, adult filarial worms that reside in the subcutaneous tissues of dog and cat such as *D. repens* were reported in humans in SEA countries like Thailand and Malaysia [23, 24]. Four cases have been reported in Malaysia; in one incidence an adult female worm was recovered from a swollen eyelid [25]. Shekhar et al. [26] reported two cases of *D. repens* infections based on histology, one in a cervical lymph node and another in the left inguinal nodule. In the fourth case, an immature female worm was recovered beneath the conjunctiva of a patient [24]. A prevalence study on *D. immitis*, a dog heartworm, was carried out in Chiang Mai, Thailand; about 18 % (107/589) of the dogs screened were found to be infected; however, not a single case of human infection was reported in the province. It was then suggested that there was a high probability of misdiagnosis of suspected human lung tumour in the province [27].

3.5 Diagnosis of Lymphatic Filariasis

The understanding of the epidemiology, parasite life cycle, pathology and immune response to the infection is important in the choice and interpretation of the diagnostic test for LF [28]. The diagnosis of LF can be categorised as follows: (1) mf detection, (2) antibody detection, (3) antigen detection, (4) DNA detection

and (5) radiological diagnosis. The selection of the diagnostic test is influenced by the purpose of performing the test, whether it is for patient diagnosis, follow-up post-treatment or for filariasis elimination programme. With regard to the first two, before presumptive diagnosis of lymphatic filariasis patient is undertaken, it is important to establish that the patient has a probable history of sufficient exposure to the infection. The variety of clinical manifestations of LF has implication on the selection of the diagnostic method. A symptomatic microfilaraemic individual is most easily diagnosed by microscopy, while someone with occult infection is not easily identified and may need more than one diagnostic test.

For use in the Global Programme for the Elimination of Lymphatic Filariasis (GPELF), the choice of diagnostic tools affects the decisions where MDA should be performed, how to measure its effects, how to determine endpoints for stopping MDA and how to perform surveillance post-MDA [29]. Various diagnostic tests have been recommended for the different phases of the GPELF, which includes parasitological diagnosis, rapid antigen and antibody tests and molecular diagnostics [30]. Nevertheless, a field-applicable diagnostic tool is preferred since the rapid results obtained allow for timely programmatic decisions to be made.

3.5.1 Microfilaria Detection

The thick blood smear examination provides the definitive diagnosis since it allows the visualisation of mf under the microscope. It is a time-tested and relatively easy method to perform. Thus, despite the introduction of several other highly sensitive tests, thick blood smear is still carried out routinely in many countries in SEA. Due to its unique features and relatively large size, mf is rarely misdiagnosed. Usually about 50–60 µl blood is taken from a fingerprick and spread as a circular thick smear on a clean microscope slide. It is dried overnight, dehaemoglobinised in water, fixed in methanol and then stained with Giemsa. A recent modification of the thick smear is the use of three parallel lines on a slide, approximately 20 µl blood per line. This has been successfully field-tested in a large multicentre study evaluation of several diagnostic tools [31]. Nevertheless, mf detection from thick smears suffers from low sensitivity due to the use of a small volume of blood and loss of mf during dehaemoglobinisation. Moreover individuals with either very low mf density or at the amicrofilaraemic stage of the infection will probably be missed by this method. The phenomenon of mf periodicity is a major concern when using this method since blood collection should coincide with the time the mf circulates in the peripheral blood. *B. malayi* in Malaysia and Brunei Darussalam is nocturnal subperiodic, thus it can be detected in the peripheral blood both day and night but the density is much higher at night between 10 pm and 2 am. In Indonesia and most other SEA countries with nocturnal periodic filaria, mf is only detected in the peripheral blood at night.

To increase the sensitivity of parasitological diagnosis, a larger volume of blood obtained by venous sampling is used in concentration techniques i.e. Knott

technique, membrane filtration or counting chamber. In the Knott concentration, a minimum of 1 ml of blood is needed which is collected in a tube with anticoagulant, then mixed with 9 ml of 2 % formalin. After 15 min, the mixture is centrifuged and the sediment examined under the microscope [32]. Due to its low cost, it is a popular choice in diagnostic laboratories in SEA. Since formalin helps preserve the mf, sample processing can be delayed, thus is useful for field research. A modification of the technique in which the formalin is replaced by 2 % Triton X-100® has been reported to reduce the amount of sediment formation, thus making it easier and faster for microscopic examination [33]. Membrane filtration [34] is a widely used technique which involves a minimum of 1 ml of anti-coagulated blood being passed through a polycarbonate membrane (3–5 μm pore size) placed on a syringe filter. After flushing with 20 ml distilled water to lyse the blood cells, the filter is removed and examined directly under the microscope or stained with Giemsa before microscopic examination. To enable longer preservation of mf before performing the technique, Dickerson et al. [35] proposed adding a mixture of 10 ml Teepol/2 % formalin to the blood. Counting chamber is a method of choice in many countries in Eastern and Southern Africa but infrequently used in SEA [36, 37]. A 100 μl finger-pricked blood is mixed with 0.3 % acetic acid, the latter preserves as well as lyses the red blood cells. The sample is then transferred to a Sedgewick Rafter counting chamber and examined microscopically [37]. A related procedure performed in conjunction with blood microscopy is the diethylcarbamazine (DEC) provocative test [38]. This is performed in cases where blood needs to be taken in the day or there is strong suspicion of LF but the thick blood smear or concentration method gives negative results. A single dose of 50–100 mg DEC is administered to provoke the mf to enter the peripheral blood, then blood sample is taken for examination 30–45 min later.

In performing microscopic examination, familiarity with the morphological features of the different species of lymphatic filariae is necessary. The details on the differential characteristics are available in standard medical parasitology textbooks. Figure 3.1 shows a typical morphology of *B. malayi* with overlapping body nuclei and two terminal nuclei separated by a space from the tapered portion of the tail.

3.5.2 Antibody Detection

There are many studies on detection of antibody against LF with most focusing on IgG and IgG4 responses against the infection. The concern has always been whether antibody detection assays detect past infections. However, in recent years numerous studies have shown that anti-filarial IgG4 antibodies is a marker of active infection, particularly in children [39–41]. A major advantage of an IgG4 test over parasitological diagnosis is that blood can be taken at any time of the day. This is a major relief for field workers in remote areas. Not only is the logistics a major challenge

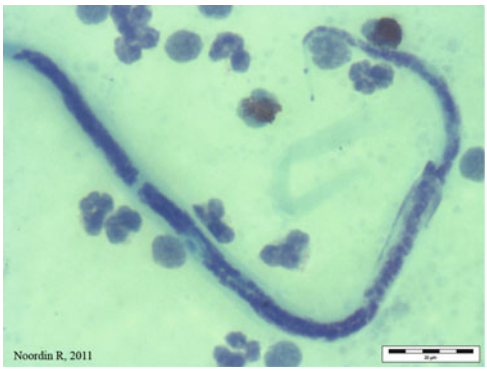

Fig. 3.1 Microfilaria of *B. malayi*

for night blood sampling, the cooperation from villagers is much affected at night since people in the remote areas often go to bed early, particularly children.

Although various native antigen preparations have been reported to be useful for antibody detection of LF, cross-reactivity is a problem [42]. Thus, recombinant antigens are preferred since they allow for standardised assays to be produced, as well as reproducibility of results by other investigators. Several recombinant antigens have been reported to be good diagnostic reagents especially using IgG4 as the probe. For brugian filariasis, the notable ones are *Bm*R1 [41, 43] and *Bm*14 [44]. Meanwhile for bancroftian filariasis, the well-reported recombinant antigens are *Bm*SXP [45] and WbSXP [46].

Wb123 [47] and Bm33 [48] have also been used for the detection of bancroftian filariasis [49, 50]. Synthetic peptides of Wb-SXP1 showed good reactivity in recognising sera of mf positive patients, but further studies are needed before it can replace its recombinant form [51]. Antibody testing is more sensitive than antigen testing; thus for bancroftian filariasis, despite the availability of an antigen detection test, detection of anti-filarial antibody is preferred for surveillance at the post-certification period of the GPELF. Anti-filarial antibody assays have been reported in urine for the detection of bancroftian filariasis but the results are varied [31, 52–54]. Thus, there is still a need for a validated and highly sensitive field-friendly rapid lateral flow antibody test to detect bancroftian filariasis.

*Bm*R1 has been developed into an ELISA and Brugia Rapid tests, but only the latter is commercially available (Reszon Diagnostics International Sdn. Bhd., Malaysia). Laboratory evaluations of ELISA using *Bm*R1 have shown it to be a highly sensitive and specific antigen [41]. Brugia Rapid, a lateral flow cassette test based on *Bm*R1, has also been shown to be a good diagnostic tool [18, 19, 43, 55]. It can be used with either serum, plasma or blood sample, and the anti-filarial antibodies are captured by the recombinant *Bm*R1 antigen lined on the strip in a cassette. A second line on top of the test line is a control line (goat anti-mouse IgG) which is a control for the test integrity. The antigen–antibody complex is then detected by monoclonal anti-human IgG4 conjugated to colloidal gold. Two pink lines denote a positive result, while one pink line denotes a negative result. For a disease such as LF in which the diagnosis needs to be

performed in the field or in places with very limited resources in terms of equipment, skilled personnel and reliable electrical supply, availability of a rapid field-applicable test such as Brugia Rapid is a necessity, especially in the context of the elimination programme. Such a test will allow transportation at room temperature, ease of performance and interpretation and does not require any equipment other than a pipettor (which may also not be needed if a calibrated capillary tube is used).

Another rapid test called PanLF Rapid (Reszon Diagnostics International Sd. Bhd., Malaysia) has been used to detect both brugian and bancroftian filariasis [45]. It is lined with two recombinant proteins i.e. *Bm*R1 to detect brugian filariasis and *Bm*SXP to detect bancroftian filariasis. In a multicentre trial in an exclusively *W. bancrofti* endemic area, it was found to be not as sensitive as the ICT card test [31], thus it has limited use in such areas but would still be useful in brugian areas with some mixed bancroftian filariasis. A rapid flow-through immunofiltration test using Wb-SXP-1 has been reported for diagnosis of brugian and bancroftian filariasis, however varied evaluation results have been reported [43, 56].

3.5.3 Antigen Detection

There has been an attempt to develop a good antigen test for brugian filariasis [57], but it has not been translated into use in patient diagnosis or the elimination programme. The commercially available antigen detection tests developed for bancroftian filariasis do not cross-react with serum samples of patients with brugian filariasis. For bancroftian filariasis, two antigen detection tests are well established, namely Og4C3-ELISA (TropBio Pty Ltd, Queensland, Australia) and the antigen card test [presently called the Binax NOW® Filariasis Immunochromatographic Test (ICT) (Alere, USA)]. In the former, the microtitre plate is coated with Og4C3 monoclonal anti-filarial antibody, and concentration of the CFA is determined using a standard curve. It has been reported to be very sensitive compared to mf detection, both in laboratory- and field-based studies [31, 43]. It is very useful as a research tool, but the need for laboratory facilities, equipment and skilled personnel limits its use in elimination/control programmes. The ICT test is a rapid lateral flow test that has been established for use in diagnosis and elimination programmes in bancroftian filariasis endemic areas [18, 19]. It uses colloidal gold-conjugated to polyclonal anti-filarial antibody dried on a pad that binds to filarial antigen in the blood of an infected person. This complex is then detected by anti-filarial monoclonal antibody AD12.1 lined on the strip in the card. Recently, a new filarial antigen test, the Alere Filariasis test strip, has been produced as an improvement over the ICT test. It showed greater stability and higher sensitivity when tested in the field than its predecessor [58].

3.5.4 DNA Detection

Molecular diagnosis which detects specific DNA of lymphatic filarial species has been reported. For *B. malayi* detection, the conserved and highly repetitive 322 base pairs *Hha*1 DNA sequence is usually targeted in PCR assays and known to be highly sensitive and specific [59–62]. Real-time quantitative PCR based on the same target has also been well-reported [63–65]. In addition, a non-PCR method using loop-mediated isothermal amplification (LAMP) has been developed to detect *B. malayi* or *B. timori* and showed to detect 1 pg of genomic DNA of *B. malayi* [66]. This could lead to the field applicability of molecular diagnosis for brugian filariasis. Differentiation among *B. malayi*, *B. pahangi* and *D. immitis* can be made by amplifying 114-bp region of mitochondrial 12S rRNA genes of these worms, followed by a High Resolution Melting point (HRM) assay which shows a specific melting temperature for each species [67]. Other than for patient diagnosis, this assay will be useful in epidemiological studies of reservoirs and vectors. For molecular diagnosis of *W. bancrofti*, several DNA targets have been used in conventional, multiplex and real-time PCR, with notable ones being *Ssp*1 repeat [68, 69], LDR repeat [63, 64] and ITSI [70]. In addition, LAMP has also been developed that can detect one thousandth of *W. bancrofti* DNA from one mf [71].

3.5.5 Radiological Diagnosis

Research on bancroftian filariasis in Brazil using ultrasonography showed the presence of live, adult worms in 'nests' (dilated lymphatics) exhibiting rapid movements called 'filarial dance sign' [72]. It has been used for diagnosis and to assess the adulticidal efficacy of anti-filarial drugs in bancroftian filariasis patients [73, 74]. In brugian filariasis, whereby the adult worms tend to be in deeper lymphatics, technological refinement using colour and pulse wave Doppler succeeded in the detection of infection even in children [75–77] Lymphoscintigraphy is another very useful imaging technique for diagnosis of LF by observing abnormal and dysfunctional lymphatics in infected individuals. It has been used in both brugian [78–80] and bancroftian filariasis [81].

3.6 Clinical Manifestations of Brugian Lymphatic Filariasis

The commonly recognised clinical manifestations of brugian lymphatic filariasis (BLF) are chronic lymphoedema of the limbs, which may lead to elephantiasis and the repeated attacks of acute dermatolymphangioadenitis (ADLA). Tropical pulmonary eosinophilia syndrome (TPE) is an uncommon presentation. However, in

an endemic area for BLF, a spectrum of other asymptomatic forms is known to exist. First among them are the 'true endemic normals' that form the largest group, who neither harbours filarial-specific IgG4 antibody (FSIA) nor mf in their blood. The next two asymptomatic groups are (1) those having no mf in their blood but positive for FSIA indicating either active or sometimes past filarial infection and (2) those who have mf in their blood and are mostly positive for FSIA. Field studies conducted in *B. malayi* endemic areas have previously reported that IgG4-ELISA using *Bm*R1 antigen detected 4.9–9 times more positive individuals in comparison to mf detection methods [60, 61, 82]. In a recent study among children, this proportion was 27 times more [79, 80].

3.6.1 Pathogenesis

The common clinical manifestations of BLF are chronic and generally evolve slowly over the years. Acute attacks of ADLA are very common and occur mostly in the limbs in association with lymphoedema or even earlier when there is underlying subclinical lymphatic damage [76, 77]. In BLF the lymphoedema involves only the legs below the knee and upper limbs below the elbow, without any genital or breast involvement [83]. The earliest pathology is the dilation of the lymph vessels where the adult worms live. This has been demonstrated even in children who are clinically asymptomatic except for the presence of mf or FSIA, by ultrasound examination of the lymphatics and by lymphoscintigraphy of the limbs [76, 78]. In course of time this damage caused by the adult parasite results in lymphatic dysfunction, leading to lymphoedema usually in the lower limbs and sometimes in the upper limbs. The damage to lymph vessels causes stagnation of lymph, which is aggravated by acute bacterial infections of the limb, prolonged standing or strenuous exertion. Stagnation of lymph encourages growth of bacteria invading the region. Injuries, even trivial ones, resulting in wounds or abrasions; fungal or bacterial infections; fissuring of the skin; paronychia and eczema are the lesions of the skin that favour entry of such bacteria into the tissues [84, 85]. These bacteria cause the ADLA attacks, commonly seen in the limbs. Several studies have implicated pathogenic bacteria, mainly streptococci, as the causative agents [86]. ADLA attacks worsen the lymphoedema, which in turn favours more of such attacks and a vicious cycle is thus established [87, 88].

3.6.2 Acute Manifestations

3.6.2.1 Acute Dermatolymphangioadenitis

This is the most common acute clinical manifestation in BLF. This is usually associated with fever, chills, headache and pain in the involved region. In severe

cases, there may be toxaemia, altered sensorium and urinary incontinence. Though seen during the early stages of the disease, these attacks are more frequent in higher grades of lymphoedema. The affected limb is extremely painful, warm, red, swollen and tender. Red streaks may be visible along the inflamed lymph vessels. The draining lymph nodes in the groin or axilla may become swollen and tender. The presentation may be with lymphangitis, lymphadenitis, cellulitis or abscess formation.

It is now recognised beyond doubt that the acute episodes are caused by bacterial infections [84, 85, 87, 88]. It is also evident that the filarial worms do not directly cause them [89]. In the affected limbs, lesions favouring entry of bacteria can be demonstrated, either in the form of fungal infection in the interdigital spaces (Fig. 3.2a), minor injuries, wounds, abrasions, infections, eczema or cracks in the feet [84, 85]. In higher grades of lymphoedema, fungal infections occur in the webs of the toes and get aggravated during rainy season or due to household work where the feet are soaked in mud. In such situations the ADLA attacks are more frequent, abetting the progression of lymphoedema to elephantiasis [87, 88].

3.6.2.2 Acute Filarial Lymphangitis

Rarely, when the adult worms die in the lymphatics, either spontaneously or by DEC administration, acute lymphangitis can occur. This is directly caused by the death of adult worms and is a comparatively uncommon and transient acute manifestation in BLF. At the site where adult worms die, small tender nodules are formed, usually in the lymphatics of the groin or axilla. These nodules are very difficult to locate, unless carefully palpated. Affected lymph nodes may become tender [89]. Inflamed large lymphatics may stand out as long tender cords underneath the skin in the axilla, along the sides of chest, medial aspect of thigh or arm, restricting the movement of the affected limb [87]. Transient oedema may occur at the affected region. Unlike in ADLA, these episodes are not associated with fever, toxaemia, entry lesions or bacterial infection. Rarely, sterile abscesses occur at the site of dead adult worms, usually in the inguinal region leaving typical scars on healing that takes time [90].

3.6.3 Chronic Manifestations

3.6.3.1 Lymphoedema and Elephantiasis

Lymphoedema of the extremities is the most common chronic manifestation of BLF, which on progression results in elephantiasis. Usually the lower limbs are involved, either unilaterally or sometimes bilaterally in which case the swelling tends to be asymmetrical. The upper limbs may also be affected. The lymphoedema of the limbs is commonly graded as follows [91]:

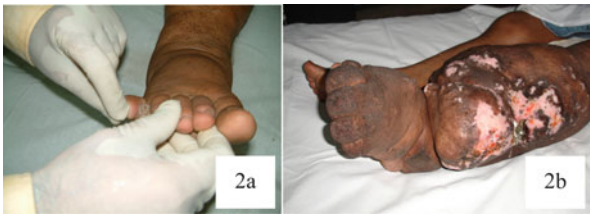

Fig. 3.2 (**a**) (*left*): Skin lesion caused by candidiasis of the interdigital region in a filarial limb, which acts as entry site for bacteria causing acute attack of dermatolymphangio adenitis; (**b**) (*right*): Grade IV lymphoedema in brugian filariasis showing large swelling of the leg with deep folds, warty changes, ulceration and depigmentation

Grade I—Pitting oedema, reversible on elevation of the affected limb
Grade II—Pitting or non-pitting oedema, which does not reverse on elevation of the affected limb and there are no skin changes
Grade III—Non-pitting oedema that is not reversible, with thickening of the skin
Grade IV—Non-pitting oedema that is not reversible, with thickening of skin along with nodular or warty excrescences—the stage of elephantiasis (Fig. 3.2b)

In advanced stages of lymphoedema, the skin is thickened and thrown into folds, often with hypertrichosis, black pigmentation, nodules, warty growth, intertrigo in the webs of toes or chronic non-healing ulcers [92]. The swelling may be so huge and grotesque that the patient is incapacitated requiring help even for personal needs.

3.7 Treatment of Brugian Lymphatic Filariasis

3.7.1 Drugs Acting Against B. malayi

3.7.1.1 Diethylcarbamazine

Brugia malayi infections are said to be more sensitive to this drug, which is very effective against mf, but only partially effective against the adult worms. Its action on the parasite is mediated through the host immune system. Previously, the standard dose of DEC recommended in BLF infection was 6 mg/kg daily for 12 days [83].

Recent studies have shown that a single dose of 6 mg/kg is as effective as the 12 days course. Single annual administration of 6 mg/kg DEC results in sustained lowering of blood mf levels even at the end of 1 year [93, 94]. Ultrasonography has shown that a single dose of DEC kills ~50 % of adult worms when they are sensitive to the drug. If they are insensitive, even repeated administrations of the drug do not kill the parasite [95, 96]. In adults, treatment with DEC did not reverse the

lymphatic damage once it is established [97]. But in a recent study in BMF infection, it was shown that in children, the subclinical lymphatic pathology was reversed by treatment with anti-filarial drugs DEC and albendazole used in single doses as recommended in MDA in the GPELF [79, 80].

The indication to use DEC is when a person has active BMF infection, as evidenced by presence of mf in his blood, presence of adult parasites in the lymphatics on ultrasonography or when there is filariasis specific IgG4 antibody (FSIA). The effective dose of the drug is 6 mg/kg in single dose, which may be repeated once in 6 or 12 months, if the above conditions persist [97]. DEC has no role either in the treatment or prevention of the acute dermatolymphangioadenitis (ADLA) attacks which are caused by bacterial infections or in the treatment of chronic filarial lymphoedema occurring in BLF where there is no active filarial infection [85, 87, 88]. DEC is the drug of choice in the treatment of tropical eosinophilia syndrome (TPE) where the drug has to be given for longer periods.

Due to its sustained microfilaricidal action even in single annual doses, DEC is a good tool to prevent the transmission of BLF [94, 98]. The adverse effects noticed on treatment with DEC are mostly in subjects who have mf, due to their rapid destruction. These symptoms, like fever, headache, myalgia, sore throat or cough last from 24 to 48 h, are usually mild, self-limiting and require only symptomatic treatment [93]. Direct adverse effects related to the drug are very rare. Even though DEC is conventionally not recommended for administration during pregnancy, in several earlier community studies where this drug was used for mass distribution, no adverse effect has been reported in pregnant women. As such, there is no evidence that single dose of 6 mg/kg of DEC is detrimental during pregnancy [98].

3.7.1.2 Ivermectin

This drug is an effective microfilaricidal agent, which acts directly on the parasite. In single annual doses of 200–400 mg/kg, ivermectin keeps the blood mf counts at very low levels, even at the end of 1 year. The adverse effects are noticed only in microfilaraemic patients and are similar to those produced by DEC, though milder due to the slower clearance of the parasitaemia [93, 94]. Ivermectin has no proven action against the adult parasite and it is not effective in ADLA attacks or TPE syndrome [87, 88, 99]. Ivermectin is also effective against human ectoparasites like head and body lice, scabies and many intestinal helminths [98].

3.7.1.3 Albendazole

This well-known anthelmintic drug was shown to destroy the adult filarial worms when given in doses of 400 mg twice daily for 2 weeks [100]. This drug has no direct action on mf and does not immediately lower the mf counts. But when given in annual single dose of 400 mg in combination with DEC or ivermectin, there is sustained lowering of blood mf levels [101]. Consequent on this effect and its action

against many intestinal parasites, albendazole combined either with DEC or ivermectin is recommended for the filariasis elimination programme [98].

3.7.2 Treatment of Acute Dermatolymphangioadenitis

In BLF, the most distressing disability is caused by the acute attacks of ADLA. In severe cases, the subject is prevented from attending his daily activities for several days. These attacks can be easily treated and it is also possible to prevent such episodes. Bed rest and symptomatic treatment with simple analgesics and antipyretics till the symptoms subside is enough in mild cases. Local precipitating factors in the affected limb like injury and bacterial or fungal infection, especially in the webs of the toes, should be looked for and treated with appropriate local antibiotic or antifungal ointments. Additional prompt administration of oral or parenteral antibiotics is required in moderate or severe ADLA. Commonly used antibiotics like penicillin, tetracycline, ampicillin, amoxycillin or cotrimoxazole may be given in adequate doses till the infection subsides. In severe cases, bacteriological examination of swabs from the entry lesions may help in choosing proper antibiotic [85, 87, 88].

3.7.3 Prevention of ADLA

This is the sheet anchor of management of disability in BLF. A regularly carried out 'foot care programme' or 'limb hygiene' is a simple, effective, cheap and sustainable method available for prevention of ADLA [84, 85, 87, 88]. Foot care aimed at prevention of fungal and bacterial infections has become the mainstay for disability alleviation in GPELF [98, 102]. This procedure needs only the common facilities available for washing in any household and hence can be carried out in their homes itself by the patients or by trained community health workers or 'home care' providers, when the patients are unable to carry out the procedure themselves due to the massive swelling. The 'local hygiene' procedures that can be performed are as follows: washing the affected area, especially the webs of the toes and deep skin folds with soap and water twice a day or at least once before going to bed and wiping dry with a clean cloth; clipping the nails at intervals and keeping them clean; prevention or prompt attention to any local injuries or infection using antibiotic ointments; prevention or treatment of fungal infection in the webs of the toes, skin folds and sides of the feet by applying antifungal ointment and regular use of proper foot wear to relieve the swelling of limb and keeping the affected limb raised at night, using bricks to elevate the foot end of the cot [85, 87, 88].

In patients with huge swelling of the legs, proper local care of the limb is not always possible due to deep skin folds, nodules or warty changes. To prevent

recurrent ADLA in such situations long-term antibiotic therapy using oral penicillin or long acting parenteral benzathine penicillin is indicated [85].

3.7.4 Treatment and Prevention of Lymphoedema

In early stages of the disease when there is active filarial infection, if the adult worms are sensitive to DEC, treatment with this drug might destroy them and thus logically prevent the later development of lymphoedema. Equally important is the prevention of ADLA attacks in these patients since the occurrence of lymphoedema and its progression are related to these repeated infections [85, 87, 88]. Once lymphoedema is established, there is no permanent cure and as mentioned above, treatment with DEC does not seem to reverse the existing lymphatic damage [97]. To alleviate the lymphoedema and to prevent further progression of the swelling, the following treatment modalities are indicated depending on the stage of the disease: applying elastocrepe bandage or tailor made stockings while ambulant; keeping the limb elevated at night, after removing the bandage; regular exercising of the affected limb; regular light massage of the limb to stimulate the lymphatics and to promote flow of lymph towards proximal larger patent vessels; intermittent pneumatic compression of the affected limb using single or multicell jackets and heat therapy using either wet heat or hot ovens and surgical procedures. There are various surgical options available to offer relief of lymphoedema, like lymph nodo-venous shunts, omentoplasty and excision with skin grafting [103]. Even after surgery the local care of the limb should be continued for life, so that ADL attacks and recurrence of the swelling are prevented.

3.8 Global Programme for the Elimination of Lymphatic Filariasis

About 20 years ago, LF was little known to the global public health community. There was little appreciation of the burden and loss on affected individuals and communities, inadequate means of diagnosis, inadequate tools for treatment, insufficient understanding of how to alleviate the suffering and disfigurement caused by the disease, inadequate strategies to control the infection, insufficient knowledge of the parasite and its pathogenesis and little hope or anticipation that things would change soon. The GPELF targets the global elimination of LF as a public health problem by 2020. The programme recommends a comprehensive strategy for achieving the elimination goal through a two-pillar approach, namely interruption of transmission of filarial infection in all endemic countries through a minimum of five annual MDA and prevention and alleviation of disability and suffering in individuals already affected by LF [1].

3.8.1 Population at Risk of LF and World Poverty Statistics

Around the globe, 1.3 billion people are estimated to be at risk of LF. It is well appreciated today that LF is not only a disease of adults but also a disease of children. Worldwide 500 million children are at risk of LF. Early detection and treatment are particularly important as they can prevent disabling consequences of LF. The highest disease burden among children is found in South Asia where nearly 300 million children are at risk. There are an estimated 50 million people with overt disease (elephantiasis, genital damages, etc.) and an estimated 70 million are living with hidden lymphatic damage [104].

LF is considered a disease of the poor and there is a strong correlation between disease burden and poverty. The distributions of population at risk and population living below US$1 a day are associated. For example, countries in South Asia (e.g. India, Bangladesh, Myanmar and Indonesia), where a large number of people live in poverty, remain highly endemic. Many years were spent on Research and Development in LF including multicentre drug trials in many parts of the world with DEC, ivermectin, and albendazole, combination therapy trials, development of microfilaricide and macrofilaricide, development of immunodiagnostics, research on pathology and epidemiology including studies to better understand social aspects of the disease. These efforts, addressing a wide range of issues, were critical to the development of the global programme. Today GPELF is 10 years old, active in 53 of the 80 odd endemic countries.

3.8.2 Breakthroughs and New Developments

Effective interventions that made the global programme possible are developments of drugs effective in decreasing microfilaraemia (i.e. DEC and ivermectin) and combination therapy with albendazole-improved clinical disease management and new diagnostics such as antigen detection test for *Wuchereria bancrofti* (BinaxNOW® Filariasis ICT card test) and Brugia Rapid antibody cassette test for *Brugia* infections.

3.8.3 Milestones Towards Elimination and Progresses Made

In 1994, a consultative meeting was held at Universiti Sains Malaysia, Penang to discuss LF–Global Control strategies, and in 1997 the World Health Assembly passed a resolution on elimination of LF as a public health problem. In 2000, the birth of GPELF made it possible to implement elimination strategies on a global scale in order to achieve the two goals of LF elimination i.e. MDA to interrupt transmission and clinical management and health promotion to reduce and prevent

disabilities. Epidemiological mapping of LF was completed in most endemic countries by 2007 except for some countries experiencing political and conflict problems. By 2009, the majority of endemic countries have initiated MDA. More than 2.8 billion cumulative number of treatments had been delivered by GPELF, most of them (2.4 billion) were delivered to WHO's Southeast Asia Region [1]. By sixth round of MDA, mf prevalence was reduced by nearly 95 % compared to pre-MDA and nearly two-thirds of sentinel sites where five rounds of MDA were completed experienced a reduction in mf prevalence to zero [1]. The progresses made could not have been possible without effective funding. More than 50 % of MDA operational cost has been borne by the Ministries of Health. For example, Brazil, India, Malaysia, the Philippines and Thailand have totally funded their national MDA programmes. In addition, various organisations have provided financial assistance to GPELF; they include but are not limited to Australian Agency for International Development (AusAID), Bill & Melinda Gates Foundation, GlaxoSmithKline (GSK)—Donation of Albendazole, Japanese International Cooperation Agency (JICA), Merck & Co. Inc—Donation of Ivermectin, non-governmental development organisations, UK Department for International Development (DFID) and WHO.

3.8.4 Impact

In its first 9 years, more than 50 % of endemic countries are actively involved in annual MDA. Nearly 2 billion treatments, which include to 176 million children, have been delivered to more than 560 million people in 48 countries. The benefits of GPELF (Table 3.1) have not been limited to LF alone, additional health benefits were provided from more than 310 million treatments of albendazole delivered to women of child-bearing age and school-age children. It provided sustained relief from the negative consequences of soil-transmitted helminths (STH) infections that include maternal anaemia, low-birth weight newborns, excess infant mortality, inhibited growth and development and diminished intellectual performance. In African communities, almost 150 million treatments of ivermectin have been delivered, which provided sustained relief from onchocercal skin disease, scabies, lice and important STH infections [104].

3.9 Challenges and Way Forward

Since 2005, there has been a growing interest in adopting an integrated approach to Neglected Tropical Disease (NTD) control, and in particular for diseases targeted by preventative chemotherapy such as LF and STH ('deworming') and LF and malaria. This 'paradigm shift' has resulted in several favourable changes, among others are commitment of endemic countries to create budget lines for NTD

Table 3.1 Health and economic impact of the GPELF (first 9 years)

Health impact	Individuals protected	Disease prevented	DALYs averted	Economic cost prevented
Prevention of infection in newborns	6.6 million babies	1.4 million cases of hydrocoele	3.2 million DALYs	US$924 million
		800,000 cases of lymphoedema	2.8 million DALYs	US$80 million
Prevention of progression from subclinical to clinical disease	9.5 million people	6.0 million cases of hydrocoele	14 million DALYs	US$7.7 million
		3.5 million cases of lymphoedema	12 million DALYs	US$6.8 million
Prevention of worsening of morbidity or reversal	2.3 million people	1.2 million cases of hydrocoele	3.2 million DALYs	US$2.0 million
		1.1 million cases of lymphoedema	4.2 million DALYs	US$2.9 million
				Total US$21 billion

[104]; Ottesen, personal communication

including LF, US$30 million provided by Asian Development Bank for NTD in Mekong countries and President Obama's recent pledge of over US$80 billion for global NTD control [1].

A number of challenges still exist for LF elimination in SEA, which include scaling up interventions in areas with complex political situations or resources are scarce, ensuring the quality of interventions (e.g. high MDA coverage and drug quality), documenting the impact of LF programmes on LF and on other diseases including STH and defining 'endpoints' of MDA based on data and experiences from the field and implementing post-MDA surveillance capable of detecting disease resurgence early. In addition, monitoring and evaluation, which has been an integral part of GPELF, will become increasingly important as a number of countries prepare for elimination and verification exercises [1].

In conclusion, significant progress has been made in LF control in Southeast Asia and other parts of the world. Following China, which declared LF elimination in 2006, Republic of Korea was declared free of LF in 2008. In the South Pacific, Tonga, Cook Islands, Vanuatu, Palau and Niue are in their final stages of achieving elimination. Many others are expected to follow. GPELF is one of the most rapidly expanding public health programmes in the world. Of the 73 countries where LF is endemic, 53 countries have started implementation of MDA, of which 12 countries have implemented more than five rounds of MDA and transitioned to post-MDA surveillance [2]. When GPELF reached its half-way point in 2010, WHO reviewed the progress made during 2000–2009 and developed a strategic plan to address the challenges in the next 10 years [1]. Since then, GPELF has progressed towards the targets and milestones set in the Strategic Plan and supported endemic countries to start and scale up MDA, conduct Transmission Assessment Survey (TAS) in order

to decide whether MDA can be stopped, phase into post-MDA surveillance and achieve verification of elimination [2].

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Chapter 4
Vectors of Malaria and Filariasis in Southeast Asia: A Changing Scenario

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Abstract Malaria and filariasis are two mosquito-borne diseases that are in the pipeline for elimination in most countries in Southeast Asia. In this review, the bionomics of the important vectors are discussed in relation to the changing environment and landscape. Due to good control programmes, the cases of malaria and filariasis have diminished in numbers and so have the vectors. However, behaviour of vectors has changed. The challenges faced in maintaining the diseases at low levels are discussed.

4.1 Introduction

Malaria and filariasis are two important vector-borne parasitic diseases in Southeast Asia. Although both diseases are on the decline, they are still considered a public health problem in most countries of Southeast Asia perhaps with the exception of Singapore. There are about 4,000 species of mosquitoes in the world, but only about 10 % are vectors for diseases [1]. The Southeast Asia region has one of the most numbers of vector species and species complex compared to other regions, and they are found in various ecological sites [2]. Only the *Anopheles* mosquitoes are vectors of malaria while mosquitoes belonging to the genera *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Downsiomyia* and *Mansonia* can be vectors of filariasis [3]. It is possible for both of these diseases to occur simultaneously in a patient, and the same vector can also carry both the malaria and filarial parasites [4].

The epidemiology of both of these diseases is closely linked to the physical environment of the area. The different species of mosquitoes have adapted to different ecological conditions, and with development and deforestation, many of the forest species have now colonised plantations and farms, and two good

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examples are *Anopheles dirus* in Thailand [5] and *Anopheles latens* in Sarawak, Malaysian Borneo [6]. In some areas due to land changes, certain species of mosquitoes have been displaced, and other species have taken their place [7, 8].

Both Singapore and Brunei Darussalam were declared malaria free by the World Health Organisation (WHO) in November 1982 [9] and August 1987, respectively (http://www.wpro.who.int/countries/brn/3BRUpro2011_finaldraft.pdf). WHO has now embarked on an elimination programme for both malaria and filariasis on a global scale. During the last decade, valuable information on the bionomics of vectors have been obtained from the countries in the Mekong region which include Cambodia, Lao PDR, Thailand and Vietnam [10–15]. Of late more information is also available from Timor-Leste [16].

Due to the implementation of control operations on vectors, the vector mosquitoes in general have changed their behaviour, and those that used to be endophilic have now become exophillic and exophagic and also bite much earlier [17]. Thus, it is important to study the changes in the bionomics of vector mosquitoes as evidence has shown that changes are taking place all the time.

The aim of this review is to consolidate the published documents on the important vectors of malaria and filariasis in Southeast Asia and determine what is lacking in each of the countries in the region. This can lead to networking among scientists in the region to better understand the important vectors and fill in the gaps in knowledge now that both of these diseases are in the pipeline for elimination.

4.2 Bionomics of Malaria Vectors

4.2.1 Distribution of Anopheles *Vectors*

Table 4.1 shows the important *Anopheles* vectors and suspected vectors of malaria/filariasis in Southeast Asia.

Although there are many vectors in Southeast Asia, each country actually has only one or two important primary vectors [18], while the rest are secondary vectors and may not play a major role in the transmission of the disease. The vectors that play a major role are *An. aconitus* [19], *An. balabacensis* [20, 21], *An. dirus* [11, 13, 15, 22], *An. epiroticus* [23], *An. latens* [6, 24, 25], *An. maculatus* [26–28], *An. minimus* [15, 29], *An. subpictus* and *An. sundaicus* [30]. Thus, only the bionomics of these species will be described. However, it is possible that when a primary vector has been reduced to very low levels due to control activities, secondary vectors may play a major role.

Table 4.1 Important *Anopheles* vectors and suspected vectors of malaria/filariasis in Southeast Asia

Anopheles spp.	Brunei Darussalam	Cambodia	Lao PDR	Indonesia	Malaysia	Myanmar	Philippines	Singapore	Thailand	T-Leste	Vietnam
An. aconitus				+		+			+	+	+
An. annularis						+			(+)	+	
An. baimaii									+		
An. balabacensis	+			(+)	+ f		+				
An. barbirostris		(+)		+ f						+	
An. campestris					+ f				(+)		
An. culicifacies						+					
An. dirus		+	+			+			+		+
An. donaldi					(+) f						
An. epiroticus		+			+			+			+
An. farauti				(+)							
An. flavirostris					+ f		+				
An. hodgkini									(+)		
An. jamesii		(+)									
An. jeyporensis			(+)			+				+	+
An. kochi									(+)		
An. latens					+ f						
An. letifer					+ f						
An. litoralis							+				
An. maculatus		(+)	(+)	(+)	+ f	+		+		+	
An. minimus		+	+			+			+		+
An. nimpe											(+)
An. nivipes									(+)		
An. philippinensis			(+)			+					
An. pseudowillmori									+		
An. sawadwongporni									(+)		
An. sinensis						+					

(continued)

Table 4.1 (continued)

Anopheles spp.	Brunei Darussalam	Cambodia	Lao PDR	Indonesia	Malaysia	Myanmar	Philippines	Singapore	Thailand	T-Leste	Vietnam
An. subpictus				+ f						+	
An. sundaicus				+		+				+	+
An. vagus				f					(+)	+	
An. whartoni					f						

+, vector; (+), suspected vector; f, vector for filariasis
Modified from [3]

Table 4.2 Larval habitats of the important *Anopheles* vectors in Southeast Asia

Species	Light intensity	Water type	Water movement	Natural water collection	Man-made water collection
An. aconitus	Heliophilic	Clear, can be turbid or slightly cloudy	Stagnant or slow-flowing water	Slow-moving streams, ricefields	Wells, burrow pits, hoof prints (these are rare)
An. balabacensis	Heliophobic	Clear fresh water	Stagnant	Stagnant pools	Wheel tracks, animal hoof prints
An. dirus	Heliophobic	Clear fresh water	Stagnant	Temporary water collection, pools that remain as long as there is rain	Natural containers, wheel tracks, animal hoof prints
An. epiroticus	Heliophilic	Highly brackish 0–11 %	Stagnant	Ponds with vegetation esp. algae	Shrimp/fish ponds
An. latens	Heliophobic	Fresh water	Still or stagnant	Small shallow running streams	Muddy pools, cart track, elephant foot prints
An. maculatus	Heliophilic	Clear fresh water	Slow flowing	Slow-moving streams, clear rock pools, clean water pockets, muddy water pockets [31]	–
				Shallow pools formed in gravel beds of receding rivers [16]	Shallow water in rice fields post harvest, Timor-Leste [16]
An. minimus	Heliophobic	Fresh water	Still or stagnant	Stream margins	Rice fields
An. subpictus	Heliophilic	Brackish	Still or stagnant		Rice fields
An. sundaicus	Heliophilic	Brackish	Still or stagnant		

Adopted from [18]

4.2.2 *Larval Habitats*

Each *Anopheles* vector species has its own ecological niche. Some are fresh water breeders while others are brackish water breeders. Table 4.2 shows the different larval habitats for the various important malaria vectors in the Southeast Asia region.

As shown in Table 4.2, some species breed in water open to sunlight while others need shade. It is also possible for *Anopheles* to breed in different types of habitat in different countries. Some species like *An. epiroticus* can breed in water of varying degree of salinity from almost that of sea water to almost fresh water. Most intense breeding occurs between 10 and 20 % salinity [32–34]. In Sarawak, it was found breeding inland in fresh water [23]. Molecular studies carried out proved that it was *An. sundaicus* and is found in Borneo island [23]. In Cambodia, peninsular Malaysia, Thailand and Vietnam, this species has been given the new name of *An. epiroticus* [23].

Anopheles maculatus breeds in slow-flowing streams exposed to sunlight [26]. Recent studies showed that *An. maculatus* was found breeding in clear ground pools, rock pools and water pockets [31]. It was also found breeding nearer houses. About 200 m away from houses, very few breeding habitats had *An. maculatus* [31]. In Timor-Leste, it was also found in shallow water in ricefields postharvest [16].

Anopheles dirus is mainly a vector in Cambodia, Laos, Myanmar, Thailand and Vietnam and is found in forested regions with larvae typically breeding in shady places in small often temporary pools of water created by humans or animals [22]. *Anopheles dirus* has also been found breeding in wells in Myanmar [35].

Anopheles minimus is essentially a mosquito of the hilly region, either low or rolling foothills in mountain ranges. It is also associated with extensive irrigation systems [35]. In Myanmar, it is one of the most important vectors responsible for hyperendemic and stable malaria in the foothill and submountainous regions [35, 36]. It is an important vector in Thailand and Vietnam [13, 37–39], but its status as a vector in the Southern part of Laos is still under consideration [10, 13, 40, 41].

4.2.3 *Biological Characteristics of* Anopheles *Vectors*

Table 4.3 shows the important characteristics of the main *Anopheles* vectors. In parts of Indonesia (Java and West Timor), *An. aconitus* is considered a vector for malaria [19, 42]. The peak biting activity for *An. aconitus* was in the early part of the night before midnight and at dusk rather than dawn [43]. Although more were biting outdoors, there was no significant difference between in- and outdoors. In some areas of West Java in the upland districts in hilly ricefields and highland areas, it seems to be an important vector of malaria [42, 44, 45]. However, none of the recent studies have shown the presence of sporozoites in *An. aconitus*. It has been

Table 4.3 Important biological characteristics of *Anopheles* vectors

Species	Endophagic	Exophagic	Peak biting time	Host preference	Reference
An. aconitus	+	More +	18.30–19.30; 22.30–23.30	Zoophilic	[43, 46]
An. balabacensis	+	More +	19.00–20.00	Anthropophagic	[8] [21]
An. dirus	+	More +	19.00–20.00; 24.00–01.00	More anthropophagic	[47]
An. epiroticus	+	+	20.00–03.00	Anthropophagic/ zoophagic	[33]
An. latens	+	More +	19.00–20.00; 01.00–02.00	More anthropophagic	[25]
An. maculatus	+	More +	21.00–22.00	Anthropophagic	[28]
An. minimus	+	+	00.00–02.00	Anthropophagic	[37]

incriminated as a vector due to large numbers being caught. Actual vectors are *An. subpictus*, *An. sundaicus* (in coastal plains) and *An. balabacensis* in mountainous areas of Lombok. In Sumba island, *An. subpictus*, *An. sundaicus* and *An. vagus* were the predominant species; however, none were positive for malaria parasites by ELISA [30]. In their study *An. aconitus* was the predominant species only in the upland interior of one study site [30].

In Timor-Leste, it was found that there was a definite peak in the early hours between 19.00 and 21.00 for all anopheline species [16].

In Thailand, at least 21 species are reported as primary, secondary and suspected vectors of malaria. The primary vectors are *An. dirus*, *An. baimaii*, *An. minimus* and *An. maculatus*, while *An. aconitus*, *An. pseudowillmori* and *An. epiroticus* are considered secondary vectors based on detection of sporozoites in salivary glands [22, 48, 49]. The *An. barbirostris* and *An. campestris* groups are considered as potential vectors based on their anthropophilic behaviour and high oocyst and sporozoite rate in laboratory experiments [50, 51]. *Anopheles baimaii* bites outdoors, and its peak biting activity is around the latter half of the night around 01.00–03.00 h. *Anopheles baimaii* is incriminated as vector of vivax and falciparum malaria [3, 22]. *Anopheles minimus* and *An. epiroticus* showed that the peak biting activity was from 18.00 to 20.00 and increased at midnight 21.00–24.00 h. It has been found positive for *P. falciparum* and *P. vivax*, and EIR and parous rates were found to be 76.6 and 74, respectively [49]. Laboratory studies on *An. barbirostris* complex showed them to be susceptible to *P. vivax* [49]. Thus, the importance of these species as vectors cannot be dismissed.

Recent studies in Western Thailand showed that *An. minimus* had greater activity occurring in the second half of the night after midnight between 02.00 and 04.00 h. However, outdoor biting occurred earlier in the evening with peaks at 21.00–00.00 h [37]. The current study has also shown that *An. minimus* has more anthropophilic tendency, as only lesser numbers were caught off cattle bait [37].

Anopheles dirus still remains an important vector in the Mekong countries of Cambodia, Laos, Thailand and Vietnam and also in Myanmar [10, 13]. In Myanmar,

Table 4.4 Sporozoite rate, EIR, vectorial capacity of major *Anopheles* vectors

Species	Country	Parous rate	Sporozoite	EIR	VC	Reference
An. dirus	LAO PDR	62.4–76.0	0.55–2.5	0.12–0.25	2.37–6.5	[10]
	LAO PDR	60.2–73.2	0.17–2.69	0.01–0.22	1.28–7.13	[11]
	LAO PDR	16.7–100	1.45–2.56	0.31–0.32	0.58–13.8	[12]
	LAO PDR	33.3–66.6	–	0.05–0.14	0.01–0.43	[41]
	Myanmar	–	1.33–2.66	–	–	[35]
	Vietnam	44.0–96.0	1.1–10.7	1.10–5.21 (AEIR)	–	[13]
	Thailand	42.0–83.0	0.4–1.0	0.01–0.91	0.03–4.55	[53]
	Thailand	21.0–70.0	1.66	0.03–0.29	0.001–2.60	[22]
	Thailand	–	0.88	–	–	[54]
	Thailand	0–65	4.8	0–0.05	0–3.21	[55]
An. flavirostris	Philippines	–	0.75–1.55	–	–	[56]
	Malaysia	–	0.75–2.92	0.24–0.94	0.19–15.80	[21]
An. balabacensis	Indonesia	–	1.97	–	–	[57]
	Malaysia	–	1.15–6.38	0.59–8.78	1.44–19.7	[21]
An. maculatus	Malaysia	44.1–62.0	0.02–1.24	0.002–0.25	–	[28]
	Thailand	–	0.22	–	–	[54]
An. epiroticus	Vietnam	–	0.18–4.4	–	–	[33]
An. minimus	Thailand	32.0–84.0	0.5–6.3	0.01–0.06	0.01–0.81	[53]
	Thailand		0.15			[54]
	Myanmar		1.33–2.66			[35]
An. barbirostris	Timor-Leste		0.26			[16]
An. vagus genotype B	Timor-Leste		0.28			[16]

it is a major vector and is mostly associated with forested foothills, deep forest and domestic wells [52]. It peaks in the post-monsoon season in Myanmar in October [35]. *An. dirus* is a more endophagic, in/out ratio being 1.6 [12].

Anopheles maculatus is the main vector of malaria in peninsular Malaysia, but it may not be the predominant vector mosquito in some areas. For instance, in a recent study, Sg. Ular, which used to be a predominant *An. maculatus* area, was now colonised by *An. cracens* [17]. Perhaps due to changes in land use, other vectors may be playing a major role.

Table 4.4 shows the sporozoite rates and the vectorial capacity of the main vector mosquitoes. The sporozoite rate is an indicator of how extensive transmission is taking place in an area. The parous rate is another indicator to show if control measures instituted against vectors are having an effect on them. Most studies carried out in the early years provided such data as shown in Table 4.4. However, recent studies do not seem to have these data. Perhaps with control activities, number would have reduced, but it is essential to detect sporozoites in mosquitoes so that current status will always be known.

4.3 Species Complex of *Anopheles* Vectors

In Southeast Asia, the *Anopheles* vectors belong to species complexes, and this region has the most number of complexes compared to other regions in the world [58]. Species belonging to a complex are difficult to identify morphologically, and it is important to correctly identify them because some may be vectors while others are not. Earlier studies were based on cross-mating experiments and cytogenetics [59, 60]. However, new molecular techniques have been established for most of the species complexes in the region. The species complexes are as follows: *An. dirus* complex, *An. minimus* complex, *An. maculatus* complex, *An. sundaicus* and *An. leucosphyrus* complex.

4.3.1 An. dirus Complex

The *Anopheles dirus* complex consists of seven species, namely *An. dirus*, *An. cracens*, *An. scanloni*, *An. baimaii*, *An. elegans*, *An. nemophilous* and *An. takasagoensis* [61]. Currently two PCR assays have been designed to identify the five species except *An. elegans* and *An. takasagoensis*. [62] developed the multiplex PCR based on the ITS2 region and [63] developed the SCAR-PCR to identify the same five species.

4.3.2 An. minimus Complex

The *Anopheles minimus* complex consists of *An. minimus* and *An. harrisoni* and closely related species of *An. aconitus*, *An. pampani* and *An. varuna*. To identify these species, molecular tools such as restriction length polymorphism (RFLP-PCR) have been developed [64, 65]. This is a two-step PCR where the PCR product is digested with a restriction enzyme before running the agarose gel. There is also the AS-PCR assay which has been developed to identify these species [66, 67].

4.3.3 An. maculatus (Group) Complex

The *Anopheles maculatus* group comprises two subgroups—the Maculatus subgroup and the *An. sawadwongporni* subgroup. The species belonging to the Maculatus subgroup includes *An. pseudowillmori*, *An. willmori*, *An. dispar*, *An. greeni*, *An. dravidicus* and *An. maculatus* [68] while the Sawadwongporni subgroup includes *An. notanandai* and *An. sawadwongporni* and chromosomal form K [69]. Of these two species, *An. dispar* and *An. greeni* are found only in the

Philippines, and RFLP-PCR has been established to detect the two [70]. A PCR-based identification method was also developed to distinguish five species of the group: *An. maculatus*, *An. dravidicus*, *An. pseudowillmori*, *An. sawadwongporni* and chromosomal form K [71].

4.3.4 An. sundaicus Complex

Anopheles sundaicus breeds in both fresh and salt water, and thus, it was believed to be a complex [26]. In 2005, Linton and co-workers proved that *An. epiroticus* was actually *An. sundaicus* A based on morphology and molecular characterisation of the ITS2 and C01 mtDNA loci [23]. This species occurs from Southern Vietnam to peninsular Malaysia [23], while *An. sundaicus* s.s was found in Lundu district in Sarawak, Malaysian Borneo, based on ITS2 and CO1 [23]. *Anopheles sundaicus* forms B and C are actually only a single form based on molecular characteristics [72], and thus, it is now called *An. sundaicus* E. An allele-specific PCR has been developed to identify these three species—*An. sundaicus* s.s, *An. epiroticus* and *An. sundaicus* form E [73].

4.4 Vectors of Filariasis

Filariasis in Southeast Asia is caused by three main species of filarial worms, namely *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. Of these *B. timori* is mainly confined to the Timor-Leste. In most other countries of Southeast Asia, *B. malayi* plays a major role followed by *W. bancrofti*. The mosquitoes involved in the transmission of filariasis belong to the following genera: *Anopheles*, *Aedes*, *Culex*, *Downsiomyia* and *Mansonia* [3]. The genus *Mansonia* is divided into two subgenera: *Mansonia* and *Mansonioides*. The subgenus *Mansonioides* includes the important vectors of lymphatic filariasis caused by *Brugia malayi* in Southeast Asia. Six species of this genus occur in the Southeast Asia region, and they can be vectors for the two types of Brugian filariasis: periodic and subperiodic. The six species are *Mansonia bonneae*, *M. dives*, *Ma. uniformis*, *Ma. annulifera*, *Ma. annulata* and *Ma. indiana*.

4.4.1 Larval Habitats

Mansonia mosquitoes are associated with a large number of aquatic plants in different habitats. They breed profusely in open swamps, ponds, rivers and canals associated with various types of plants such as *Eichornia*, *Salvinia* and *Pistia* spp. and also in swamp forest with various plants that include rattan, *Dillenia*, *Eugenia*

and grasses, herbs and arums. With these types of vegetation, it is difficult to get rid of plants as they cover wide areas. In Sarawak it was found that *Ma. bonneae/dives* was associated with plants belonging to family Araceae, *Homalomena cordata*, *Homalomena rostrata* and *Hydrostemma motley* [74]. However, in a larval survey in Malaysia a tall grass plant (*Setaria geniculata*) which looks like a lalang was positive for *Ma. dives*, *Ma. bonneae* and *Ma. uniformis* [75]. An important finding in Thailand showed that *Ma. uniformis* was recovered from most of the host plants while *Ma. bonneae* preferred submerged plants and was not obtained from floating aquatic plants [76]. In Quezon, Palawan, Philippines, breeding areas for *Ma. bonneae* were wide, deep swamp covered with pandanus plants. In other areas the breeding sites for both *Ma. uniformis* and *Ma. bonneae* were shallow swampy areas planted with rice [77].

Studies carried out in Sarawak showed that there were no significant monthly fluctuations in larval density *of Ma. bonneae* and *Ma. dives* in swamp forest with *H. cordata* as the host plant [74]. However, the larvae per plant were approximately halved during the dry season (June to August) compared to the wet season (December to February).

It is well known that the larvae of the *Mansonia* mosquitoes attach themselves to the roots of plants and get their oxygen through them. In swamp forest, it is difficult to carry out control measures to reduce the breeding sites of *Mansonia* mosquitoes. However, many of the breeding sites were destroyed due to development, thus, reducing the breeding sites of the mosquitoes.

4.4.2 Biological Characteristics

Mansonia are attracted to a wide range of host, and man is not always the preferred host. The average human blood index (HBI) of *Ma. bonneae*, *Ma. dives* and *Ma. uniformis* in subperiodic *B. malayi* area in Sarawak were 0.25, 0.26 and 0.69, respectively [78]. However, in an endemic area in peninsular Malaysia the HBI for *Ma. bonneae* and *Ma. uniformis* were 0.50 and 0.40, respectively [79].

In swamp forest fringed areas of Thailand, the most abundant species was *Ma. bonneae* (47.5 %) followed by *Ma. annulata* (32.8 %), *Ma. uniformis* (9.8 %), *Ma. indiana* (6.0 %) and *Ma. dives* (3.9 %) [76]. The biting rates of *Ma. bonneae* in Thailand ranged from 4.3 per man hour to 24.3 per man hour depending on the season [76]. However, some species are very dependent on climatic conditions, for example, *Ma. annulata* was not obtained in the month of April, but the biting rate was highest in October (31.7 bites/man/hour) after heavy rainfall in September [76]. In Indonesia it was found that 70.9 % were attracted to human bait while only 29.1 % were attracted to cat bait [80]. This was from specific studies carried out during choice experiments. However, blood meal analysis of *Ma. uniformis* showed that only 11.9 % fed on humans while 66.7 % fed on bovids and small percentage fed on cats and birds, while *Ma. indiana* showed that 50.9 % fed on humans, 26.3 on bovids [81]. *Mansonia* mosquitoes are known to rest outdoors during the

day [82, 83]. Currently there is not much information about the resting habits of the *Mansonia* mosquitoes.

In Thailand it was found that both *Ma. bonneae* and *Ma. uniformis* showed bimodal patterns in biting activities with peaks in October and January following low and high rainfall, respectively [76]. Similar findings were obtained in Malaysia in swamp forest where the density of *Mansonia* was high over a long period from July to December which coincided with a steady rainfall during that period [84]. However, in Sarawak, Malaysian Borneo, and in Indonesia, there was no clear correlation between the biting density of some *Mansonia* species and total rainfall [78, 81, 85].

In general it is known that *Mansonia* mosquitoes are nocturnal. However, studies in Thailand have shown that in the forest in shaded areas the biting activities of *Ma. annulata* and *Ma. bonneae* showed two peaks during the daytime 08.00 to 11.00 hours, followed by a smaller peak at 13.00–16.00 hours and a prolonged peak at night from 17.00 to 23.00 hours [76]. In other studies, it has been shown that the peak biting times of *Mansonia* occurred at late evenings and early morning hours [78, 81–83, 85]. These mosquitoes are mostly exophagic, but in Sarawak, it was shown to be less exophagic perhaps due to the structure of the houses which had gaps on the bamboo flooring, and the mosquitoes gained access through the gaps [78].

The gonotrophic cycle of *Ma. uniformis* was determined in the field by mark release recapture cycle to be 3–4 days [86]. A cycle of 4 days was also estimated for *Ma. bonneae*, *Ma. dives* and *Ma. uniformis* [78]. In Indonesia [81] showed that the gonotrophic cycle of *Ma. uniformis* was 3.3–4.1 days and for *Ma. indiana* was 3.4–3.8 days.

Studies carried out in peninsular Malaysia showed that generally, *Ma. uniformis* survivorship was lower compared to the other five vector species of *Mansonia*; it ranged from 0.65 in *Ma. uniformis* to 0.72 in *Ma. annulifera* [87]. However, in Sarawak, Malaysian Borneo, the daily survival rates of *Ma. bonneae*, *Ma. dives* and *Ma. uniformis* were 0.90, 0.89 and 0.86, respectively [78]. Similar observations were also made in Sabah, Malaysian Borneo, where both *Ma. bonneae* and *Ma. dives* had survival rate of above 0.90 [83].

Mansonia generally have strong flying abilities. In Thailand the flight ranges of *Ma. annulata*, *Ma. indiana* and *Ma. uniformis* were observed to be between 1.0 and 1.7 km [88]. In Malaysia, studies have shown that *Mansonia* can fly between 1.45 and 2.4 km [86, 89].

4.4.3 Vector Parasite Infection Rates

In Thailand the infection rates of *Ma. bonneae* and *Ma. annulata* were 1.1 % and 0.6 %, respectively, for subperiodic *B. malayi* [76]. These infection rates are higher than those reported in the earlier years which were 0.18 % for *Ma. bonneae* and 0.20 % for *Ma. dives* [90]. In the Philippines, *Ma. uniformis* and *Ma. bonneae* were

considered vectors with an infective rate of 2.9 % and 12.9 %, respectively [77]. In 2009 it was found that the infection rates of *Ma. annulata* and *Ma. bonneae* were 0.47 and 0.25 %, respectively, in Thailand [91]. In the same study, they also found *An. letifer* as a vector for the first time, and the infection rate was 2.2 % [91]. In Indonesia, in areas endemic with periodic *B. malayi*, *Ma. bonneae* was the most efficient vector followed by *Ma. dives*, *Ma. uniformis* and *Ma. annulata* [85, 92, 93]. In peninsular Malaysia, *Ma. uniformis*, *Ma. bonneae* and *Ma. dives* were efficient vectors. However, in Sabah and Sarawak, Malaysian Borneo, *Ma. bonneae*, *Ma. dives* and *Ma. uniformis* were incriminated as vectors of subperiodic *B. malayi* [78, 83, 94].

As mentioned previously, some of the *Anopheles* mosquitoes are vectors of filariasis. In a study in Grik, Perak, Malaysia, *Anopheles donaldi* was incriminated as the vector of periodic form of *B. malayi* with the infection rate ranging from 3.65 to 13.3 % [95]. The infective bites per man night ranged from 2.4 to 6.3 in the above-mentioned area. In Sabah infection rate of *An. balabacensis* for *W. bancrofti* was 1.14 % [96]. These rates are higher than those in *Mansonia*-infected areas.

In Thailand studies were carried out to determine the susceptibility status of the *Anopheles hyrcanus* group, and it was found that *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus*, *An. argyropus*, *An. pursati*, *An. sinensis*, *An. paraliae and An. nitidus* to *B. malayi* were 70–95 %, 70–100 %, 80–85 %, 50–65 %, 60 %, 60 %, 10 %, 5 %, and 0 %, respectively [97].

4.5 Challenges

The role played by various vectors in the transmission of malaria and filariasis has been well studied in the past in most of the countries in Southeast Asia. However, with changes in the ecology of landscape and also deforestation, the distribution of the vectors may not be the same. Thus, it is not possible to depend on past data and assume the presence of vectors in those areas. Since the elimination of both malaria and filariasis is in the pipeline for most countries in Southeast Asia, it is of importance to map out the vector distribution in each country.

Indoor residual spraying (IRS) and insecticide-treated bednets (ITN) are the two main tools used for vector control. These tools were selected based on the behaviour of the mosquito vectors. It had an impact in some countries like Singapore and Brunei Darussalam which have eradicated malaria and filariasis. However, currently it was observed that vectors are biting outdoors and in the early part of the night where people are still active, and thus, these tools may not be effective. Thus, there are gaps in our control measures, and these need to be addressed before elimination can be considered. Newer tools for vector control such as repellents and insecticide-treated clothing have to be considered for people working outdoors early in the morning or late in the evenings.

Both IRS and ITN are dependent on insecticides. Currently the only insecticides being used are pyrethroids, and resistance to insecticides needs to be monitored.

Development of newer insecticides is urgently required so as to have reserves in the eventuality of development of resistance to those currently being used [98]. It is also stressed that resistance monitoring has always been a problem especially with the *Anopheles* mosquitoes, and thus, there needs to be formal training or development of new tools for quantitative monitoring of different forms of resistance in different vectors [98].

Air travel has become affordable, and thus, it is easy for people from endemic countries to bring in the parasites. Foreign workers are also crisscrossing various countries carrying parasites in them. Thus, it is important to study the susceptibility status of various vectors to the different species of parasites. For example, in Malaysia, filariasis caused by *W. bancrofti* is on the increase, and the local strain of *Cx. quinquefasciatus* is susceptible to the Myanmar strain of *W. bancrofti* [99].

While working towards elimination of a disease, it is also important to study the status of the vectors and the potential vectors. The landscape and ecology are always changing, and with this, it is possible for changes in vector distribution to occur. Thus, before elimination it is important to map the distribution of the vectors and know the ecology of the vectors [100]. Thailand is a good example, where various species of mosquitoes are being colonised and their susceptibility to various parasites is being tested [97]. This is important because it is possible for a secondary vector to become the main vector especially when changes in the environment have taken place.

Currently it has been shown that malaria is a zoonosis and also there is potential for *Brugia pahangi* to infect humans [101]. Thus, in order to determine the parasites in vectors, dissection still remains the gold standard. Molecular tools should be used to identify the parasites seen during dissection [17, 101, 102]. This will provide many new findings before we actually have an epidemic.

Species complexes are predominant in the Southeast Asia region, and thus, it is important to identify the mosquitoes correctly using molecular techniques so that vectors can be separated from the nonvectors.

4.6 Conclusion

It is obvious that there are changes in the epidemiology of both malaria and filariasis in Southeast Asia. With the elimination of both of these diseases in the pipeline, it is important to consider not only the known vectors but also the potential vectors. Many factors affect the distribution and ecology of vectors, and thus, this has to be updated all the time. With vectors being more exophilic, one has also to determine newer control strategies to control the vectors. Although the IRS and ITN along with the treatment of cases had helped to reduce the burden of malaria in many countries, there are now emerging numbers of vectors which are exophilic and exophagic and thus would not be affected by these tools. Thus, new tools to target outdoor-biting mosquitoes are needed for the elimination of malaria/filariasis to be possible.

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Chapter 5
Unravelling *Cryptosporidium* and *Giardia* in Southeast Asia

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Abstract In Southeast Asia, *Cryptosporidium* and *Giardia* have been reported in countries such as Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, the Philippines, Singapore, Thailand and Vietnam. Some of the factors encouraging the transmission of *Cryptosporidium* and *Giardia* infections include rapid modernization, exponential population growth, greater human movement and the escalating numbers of HIV/AIDS individuals. The aim of this chapter is to consolidate available published reports on *Cryptosporidium* and *Giardia* in Southeast Asia and determine available information, fill up lacunae of knowledge and propose potential efforts that can lead to fruitful collaborations among scientists in the region in pursuit of a better understanding of these infections.

5.1 *Cryptosporidium*

5.1.1 Historical Background and Taxonomy

Cryptosporidium is an intracellular but extracytoplasmic protozoan parasite, which infects human and a wide range of animals. The genus *Cryptosporidium* was first named by Ernest Edward Tyzzer in 1907 [1], and 5 years later, *Cryptosporidium parvum* was described [2]. For the next 60 years, the medical significance of this protozoan parasite was not recognised until two cases of human cryptosporidiosis were reported in 1976 in an immunosuppressed patient [3] and a 3-year-old child [4]. With the emergence of AIDS, *Cryptosporidium* infection was reported as a causative agent of diarrhoea among immunocompromised patients [5]. *Cryptosporidium* is a member of the phylum Apicomplexa, class Sporozoasida, subclass

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Coccidia, order Eucoccidiida, suborder Eimeriina and family Cryptosporiidae. To date, isolates of *Cryptosporidium* have been assigned to 27 species and more than 60 genotypes which cannot be distinguished based on morphology [6–11]. The *Cryptosporidium* species include *Cryptosporidium parvum* (human, mouse, cattle, pig, sheep, horse, goat), *Cryptosporidium hominis* (human), *Cryptosporidium meleagridis* (human, turkey), *Cryptosporidium felis* (human, cat), *Cryptosporidium muris* (human, mouse), *Cryptosporidium canis* (human, dog), *Cryptosporidium suis* (pig, human), *Cryptosporidium andersoni* (cattle, human), *Cryptosporidium baileyi* (chicken, human), *Cryptosporidium bovis* (cattle), *Cryptosporidium xiaoi* (sheep), *Cryptosporidium ducismarci* (tortoise), *Cryptosporidium fayeri* (kangaroo and human), *Cryptosporidium fragile* (black-spined toad), *Cryptosporidium galli* (chicken), *Cryptosporidium macropodum* (kangaroo), *Cryptosporidium molnari* (fish), *Cryptosporidium ryanae* (cattle), *Cryptosporidium saurophilum* (lizard), *Cryptosporidium scopthalmi* (fish), *Cryptosporidium serpentis* (corn snake, lizard), *Cryptosporidium varanii* (emerald monitor lizard), *Cryptosporidium wrairi* (guinea pig), *Cryptosporidium ubiquitum* (ruminants, humans), *Cryptosporidium cuniculus* (rabbit, human), *Cryptosporidium tyzzeri* (domestic mice) and *Cryptosporidium viatorum* (human) [12]. Subtyping based on 60 kDa glycoprotein (*gp60*) gene identified at least 11 subtype families (IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh, IIi, IIk and III) for *C. parvum* and 6 subtype families (Ia, Ib, Id, Ie, If and Ig) for *C. hominis* which have different host preference and public health significance [13].

5.1.2 Biology and Life Cycle

Cryptosporidium completes its developmental stages in a single host. Infection begins with the ingestion of viable oocysts containing four naked sporozoites. These ingested oocysts undergo excystation process: attachment to the intestinal epithelial cells, merogony, sexual reproduction and oocysts formation. The excystation process may be induced by host factors such as temperature, pH, pancreatic enzymes and bile salts and parasite-derived molecules [14–16]. The attachment of sporozoites to host cells may be enhanced by several adhesive molecules secreted from the apical complex [17]. Sexual reproduction produces thin and thick wall oocysts. While thick wall oocysts exit in the stool, thin wall oocysts excyst in the lumen, causing autoinfection.

5.1.3 Pathology and Clinical Manifestation

Cryptosporidiosis in immunocompetent patients is either asymptomatic or causes self-limiting diarrhoea which may be accompanied with abdominal cramps and mild fever. In the acute phase, the diarrhoea is watery and may continue for 4–7 weeks. Children under 2 years may show severe dehydration and increased

diarrhoea. The symptomatology of cryptosporidiosis depends on a combination of host factors such as age, previous exposure and dose of infection and parasite factors such as *Cryptosporidium* species and age of oocysts. In immunocompromised patients, the severity of cryptosporidiosis depends on the level of the CD4 T-cell count; for instance, life-threatening diarrhoea has been reported in patients with CD4 $< 150/\mu L$ [18]. Protracted infection lasting for months or years may spread to the hepatobiliary, causing cholangiohepatitis, cholecystitis or choledochitis, or to pancreatic duct, leading to pancreatitis [19, 20]. Although the mechanism of diarrhoea caused by *Cryptosporidium* is poorly understood, several mechanisms have been suggested including dysfunction of microvilli, secretion of enterotoxin by the parasite and the adhesive factors that enhance the attachment of the parasite to the enterocyte [21–23].

5.1.4 Source of Infection and Mode of Transmission

The sources of *Cryptosporidium* infection are either infected humans or animals. The mode of transmission includes direct (faecal–oral) or indirect through contaminated water (waterborne) or food (foodborne). The infection circulates among humans (anthroponotic), animals or across from animals to humans (zoonotic). *Cryptosporidium parvum* is the most common species circulating among humans and a wide range of animals and has been considered as a zoonotic species. *Cryptosporidium hominis* is the most common species responsible for human infection. *Cryptosporidium meleagridis*, *C. felis*, *C. muris*, *C. canis* and *C. suis* are generally species that infect animals, but they have been reported to infect humans as well, highlighting a possibility of zoonotic transmission [24]. The *Cryptosporidium parvum* subtype families IIa, IId and III infect humans and animals and may potentially be responsible for some zoonotic infections. The *Cryptosporidium parvum* subtype family IIc is responsible for human infection and has been considered as anthroponotic subtype. The subtype families, IIb and IIe, are reported in humans and have never been seen in animals [13].

5.1.5 Diagnosis and Treatment

The diagnosis of *Cryptosporidium* infection depends on the detection of oocysts in faecal specimens. Modified acid-fast stain (AFS) is the most common staining technique used [25]. The fluorescein isothiocyanate (FITC)-labelled anti-*Cryptosporidium* oocysts that use monoclonal antibodies to detect the intact oocysts have been developed and are commercially available. Antigen detection is another approach using ELISA technology and immunochromatographic formats [26, 27]. More recently, advanced PCR-based technologies provide specific diagnosis up to species and genotype levels with high sensitivity. However, it is

expensive and not practical to be used for routine diagnosis especially in the Southeast Asian region at this point of time. Management of human cryptosporidiosis should initially focus on managing the dehydrated patients due to diarrhoea. This goal could be achieved by oral supplementation of nutrients and fluids. Antiviral treatments of AIDS patients with cryptosporidiosis result in dramatic improvement from diarrhoea [28]. Antiparasitic drugs including nitazoxanide, paromomycin, macrolide, spiramycin, azithromycin and rifaximin have been approved for treating cryptosporidiosis. However, their efficacy is still limited [29–31]. In the severely affected immunocompromised patient, drug treatment is uncertain with probably limited efficacy, and the infection responds best to an improved host's immune status, for example, by means of HAART.

5.1.6 Epidemiology of Cryptosporidiosis in Southeast Asia

5.1.6.1 In HIV/AIDS Individuals

Presently, a number of Southeast Asian countries face a severe and likely underestimated problem with HIV/AIDS [32]. Individuals with HIV/AIDS are more susceptible to other opportunistic pathogens, including *Cryptosporidium* spp. (e.g. [33]). There is an increased migration of people within and among these neighbouring countries due to economic and political reasons [34], suggesting that the dissemination of cryptosporidiosis and other pathogens associated with people with HIV/AIDS could escalate. Currently, SEA has published studies on cryptosporidiosis in HIV-infected individuals, with data available from 5 (of 11) countries (i.e. Thailand, Malaysia, Indonesia, Cambodia and Vietnam). Iqbal et al. [12] noted in her review that these studies have shown a wide range of *Cryptosporidium* infection rates (i.e. 3.0–52.5 %) in HIV-infected individuals with or without diarrhoea.

Cryptosporidiosis in **Thailand** has been quite substantial, with reports on HIV-infected individuals (with mean CD4 T-cell counts of <100 cells/mm^3) documenting prevalence ranging from 8.8 to 34.4 %. Clinical signs commonly reported include chronic, watery diarrhoea and weight loss [35–37]. Subsequently, genetic analysis on 29 faecal samples from these immunocompromised patients found infections associated with *C. hominis* (82.8 %), *C. meleagridis* (10.3 %), *C. felis* (3.4 %) or *C. muris* (3.4 %). It was interesting to note that only *C. meleagridis* and *C. muris* were present in HIV-infected children, whereas *C. hominis* predominated in HIV-infected adults [38]. In another investigation, RFLP analysis and DNA sequencing of the 18S rRNA of 34 *Cryptosporidium* isolates from symptomatic patients indicated that 17 were *C. hominis*, with the rest being *C. meleagridis* (seven isolates), *C. parvum* (five isolates), *C. felis* (three isolates) and *C. canis* (two isolates). This was the first report of *C. canis* and *C. parvum* in HIV-infected Thai patients [39]. Predominance of *C. hominis* (27 of 41) followed by *C. meleagridis* (4) was also noted in adult HIV/AIDS patients in a combined AIDS care centre and hospice in Thailand [40].

In **Malaysia**, cryptosporidiosis case was initially highlighted in 1984 [41]. Following that, recorded prevalence in HIV individuals ranged from 3 to 23 % [42–46]. The highest was in a study among 168 asymptomatic HIV intravenous drug users [42]. Prevalence rates were however lower among hospitalised HIV patients (i.e., 3–16 %) [43–46]. With regards to CD4 counts, there were two studies which indicated that HIV patients with CD4 T-cell counts of <200 cells/mm were more likely to have cryptosporidiosis with diarrhoeal symptoms [44, 46]. In 2011, the sequencing of amplicons derived from 18S rRNA revealed that *C. parvum* was the most commonly detected species at 64 % of 25 cases (16 isolates), followed by *C. hominis* (six isolates), *C. meleagridis* (two isolates) and *C. felis* (one isolate) [44]. Further subgenotyping analysis targeting gp60 gene demonstrated *C. parvum* subgenotype IIdA15G2R1 and *C. hominis* subgenotypes IaA14R1, IbA10G2R2, IdA15R2, IeA11G2T3R1 and IfA11G1R2 [44]. Another study on subgenotype analysis targeting the same gene (i.e. gp60) identified 18 (5.2 % of 346 cases) isolates as *Cryptosporidium*-positive, with 72.2 % of the 18 identified as *C. parvum* and 27.7 % as *C. hominis*. Gp60 analysis revealed high diversity as represented by *C. parvum* subgenotypes IIaA13G1R1 (two isolates), IIaA13G2R1 (two isolates), IIaA14G2R1 (three isolates), IIaA15G2R1 (five isolates) and IIdA15G1R1 (one isolate) and *C. hominis* subgenotypes IaA14R1 (two isolates), IaA18R1 (one isolate) and IbA10G2R2 (two isolates) [47].

In **Indonesia**, cryptosporidiosis (4.9 % of 318 cases) in HIV-infected individuals has been associated with chronic diarrhoea and CD4 T-cell counts <50 cells/mm^3 [48]. In contrast to the low prevalence, a more recent study in Indonesia however indicated a high percentage (i.e., 52.5 %) of cryptosporidiosis among 100 HIV-infected individuals presenting with diarrhoea [49].

Although **Cambodia** has high rates of HIV infections (i.e. 0.8 % of the global numbers of HIV infection) [50], there was only one case-control study involving 80 HIV-infected individuals (40 individuals with HIV/AIDS whose median CD4$^+$ cell count was 11.5 cells/mm^3 and 40 HIV-negative individuals = control group). Using PCR-RFLP, this study showed the presence of *C. hominis* in chronic diarrheic patients and both *C. hominis* and *C. parvum* in asymptomatic patients [51]. Interestingly, the percentage of *Cryptosporidium*-infected people was similar between the HIV/AIDS cohort and the control group (40 % and 53 %, respectively).

Over in **Vietnam**, molecular data was first reported for *Cryptosporidium* from humans in 2003 with the discovery of *C. hominis* in three isolates of HIV-infected adults [52].

5.1.6.2 In Non-HIV/AIDS Individuals

Currently, data on cryptosporidiosis in non-HIV/AIDS individuals have been generated from Thailand, Malaysia, Indonesia, the Philippines, Lao PDR and Myanmar [53]. In **Thailand**, children were the main focus of the early studies [54–61] with recorded prevalence of 0.5–7.1 % [56, 57]. It was noted that in paediatric cases, the main clinical presentation included acute or prolonged

diarrhoea with a mean duration of 6.6 days of fever [56, 57, 61]. For children living in orphanages, the prevalence of *Cryptosporidium* was higher, estimated at 7–12 % [55, 58–60]. The likely contributor towards the relatively high prevalence of cryptosporidiosis in institutional setting was the acute depressed nutritional status in orphans [55].

In **Malaysia**, cryptosporidiosis was first discovered in a young man presenting with diarrhoea [41]. Symptomatic cryptosporidiosis was recorded in 1–11 % of hospitalised children [62–66], 2 % of immunosuppressed children suffering from cancer and receiving chemotherapy [67] and 11 % of diarrhoeic, young children in rural communities [62]. However, higher range of prevalence (i.e., 2.7–20.1 %) was observed in asymptomatic Orang Asli (indigenous) communities [68–71]. Recently, cryptosporidiosis was detected in 7.2 % of 276 aboriginal children (i.e. 2–15 years), and this infection was significantly associated with low birthweight ($\leq$2.5 kg), being part of a large household (with more than seven members) and prolonged breast-feeding (>2 years). The output of a binary logistic regression confirmed that large household size was a significant predictor of *Cryptosporidium* infection (giving an odds ratio of 2.15, with a 95 % confidence interval of 1.25–5.02) [72].

Cryptosporidiosis was first described in a premature baby in **Indonesia** [73]. A subsequent study in Surabaya revealed that in a hospital cohort, approximately one-third of all cryptosporidiosis cases (2.1 % of 1,960 patients) did not present with diarrhoea. Those who were most susceptible to cryptosporidiosis and likely to be symptomatic were children of less than 2 years of age [74]. Meanwhile in the communities, the prevalence of *Cryptosporidium* was lower (1.1 %; 49 of 4,368), with oocysts being detected frequently in diarrhoeic samples (8.2 % of 257 people) during the rainy season (June to October). *Cryptosporidium* oocysts were also detected in 2.4 % of 13 faecal samples from cats in these communities [74].

The earliest investigation on cryptosporidiosis in the **Philippines** was in 1985. In that study which was conducted at San Lazaro Hospital in Manila, 2.6 % of 735 babies (6–20 months of age) were found to be *Cryptosporidium* positive [75]. Besides humans, *Cryptosporidium* oocysts have also been detected in cattle and carabao (i.e. water buffalo) in a rural area in the Philippines [76]. Another study in hospitalised children detected 2.5 % of 236 were positive for cryptosporidiosis [77]. More recently, an extensive study throughout the Philippines involving 3,456 diarrhoeic human patients indicated lower prevalence (1.9 %) of cryptosporidiosis and the disease was more preponderant among paediatric patients on Luzon Island during the rainy season [78].

Hitherto, there is only one study of cryptosporidiosis reported from Lao PDR [79]. The study involved Hmong and other Laotian hill-tribe refugees at the Ban Vinai camp located at the Thailand–Laos border. It was discovered that *Cryptosporidium* oocysts was detected in 5 % of 324 faecal samples and the parasite was noted to be one of the most common enteric pathogens besides *Escherichia coli*, *Campylobacter* and rotavirus) in young children (of <2 years of age) with acute diarrhoeal disease [79].

In **Myanmar**, cryptosporidiosis was first detected in 1994 among infants below 1 year old presenting with acute diarrhoea [80]. There were 3.4 % of 203 infants that were found to be positive with cryptosporidiosis [80]. Similarly, another investigation among 472 Karen, Thai, Mon and Burmese preschool children (3 months to 5 years of age) conducted in Sangkhlaburi, a rural district in the west of Thailand along the Thai–Myanmar detected an overall prevalence of 3.4 % [81].

5.1.6.3 In Animals

In contrast to information in humans, there are lesser (i.e. from Thailand, Malaysia and Vietnam) accessible information among animals. A survey of *Cryptosporidium* in 363 Holstein-Friesian (dairy) cows (varying from 6 months to 5 years of age) from 108 farms in Nong Pho, **Thailand**, detected 9.4 % (of 363) of cattle and 31.5 % of the farms being *Cryptosporidium*-infected [82]. By age group, the prevalence was 7.8 % and 5.2 % in cattle of <1 year and 1–5 years of age, respectively. It was noted that proper farm management was a significant contributing factor towards fewer *Cryptosporidium* infections [82]. A subsequent investigation utilising a nested PCR approach (targeting SSU gene) determined *C. parvum* in all eight positive samples [83]. In northern Thailand, the overall seroprevalence of *C. parvum* was 4.4 % (of 642 dairy cows). According to provinces, there were 3.3 % in Chiang Mai, 5.1 % in Chiang Rai and 3 % in Lumpang. These results suggest that cattle could play a role in zoonotic cryptosporidiosis in Thailand [84]. Besides northern Thailand, the prevalence of *Cryptosporidium* infection in dairy cows in central Thailand was 7 % (of 200 samples; 95 % CI 3.5–10.5) by acid-fast staining and 15.5 % (95 % CI 10.5–20.5) by PCR-RFLP. This is the first report of genetic identification of the *C. parvum* bovine genotype in dairy cows in Thailand. It was also noted that calves less than 2 months old were more frequently infected by *Cryptosporidium* than others (OR 13.82, 95 % CI 3.67–51.97, $p = 0.001$) [85]. These studies have reinforced the notion that cattle may be a potential source of human cryptosporidiosis. Besides cattle, *Cryptosporidium* oocysts were detected in green mussel population of Samut Prakan (15.6 % of 32) and Bangkok market (8.3 % of 24) indicating that mussels may act as a reservoir of *Cryptosporidium* foodborne infections for humans [86].

Earlier epidemiological studies among **Malaysian** livestock [87–90] indicated a prevalence of 14.5–36 % in goat kids, neonatal lambs and cattle. Subsequently, an investigation utilizing PCR-coupled restriction fragment length polymorphism (RFLP) of SSU on cattle samples identified both *C. parvum* and *Cryptosporidium* 'deer-like'genotypes in positive cattle [91]. Another study found the evidence of infected cattle contributing towards environmental contamination. This was established via the detection of *Cryptosporidium* oocysts in wastewater ponds (20–3,100 oocysts per litre) on the farms and in rivers (3–240 oocysts per litre) receiving effluent from these wastewater ponds [87]. Recently, a cross-sectional study among pre-weaned and post-weaned dairy calves reported an overall *Cryptosporidium* prevalence of 27.1 %. Among pre-weaned calves, 32.4 % were

infected with *C. parvum*, 26.5 % with *C. bovis*, followed by 20.6 % with *C. andersoni*, 11.8 % with *C. ryanae* and 8.8 % with mixed sp. As for the post-weaned calves, 35 % were infected with *C. bovis*, followed by 30 % with *C. andersoni* and *C. ryanae* and 5 % mixed sp. Subtyping analysis of 8 of the 11 *C. parvum* isolates at the gp60 locus identified IIdA15G1 (five isolates), IIa18A3R1 (one isolate) and IIa17G2R1 (two isolates). Management factors that increased the risk of *Cryptosporidium* infection included having other cattle farms close by, feeding calves with saleable milk, keeping pre-weaned calves in pens with slatted floors and keeping post-weaned calves in pens with a sand floor [92]. Besides ruminants, six species of birds displayed in a zoo namely wrinkled hornbill, great argus pheasant, black swan, swan goose, marabou stork and moluccan cockatoo were found to excrete *Cryptosporidium* oocysts in the faeces [93]. In a subsequent study, cryptosporidiosis was detected both in birds (3.4 % of 116) and a bird handler at the same zoo [94] suggesting a possibility of zoonotic transmission. Another study based on molecular analysis conducted on 90 samples from 37 different species of birds determined that eight out of nine samples were *C. parvum* [95]. Given that *C. parvum* has not been reported to cause infection in birds, therefore the role of birds in this study was postulated mainly as mechanical transporters [95]. Another study on other animals in the same zoo revealed 14.1 % of 99 primates (seven species), 5.7 % of 70 ungulates (three species) and 14.3 % of 28 felids (four species) to be positive for *Cryptosporidium* oocysts [96]. Elsewhere, *Cryptosporidium* oocysts were detected in 4.1 % of 49 rats (*Rattus exulans*) captured in an Orang Asli community [97].

In **Vietnam**, the prevalence and epidemiology of *Cryptosporidium* infection in 266 cattle in the central region identified *C. parvum* (33.5 %), *C. andersoni* (5.6 %) and mixed infections (3.4 %) of both species [98]. Interestingly, it was shown that the infections was age-related with *C. parvum* being more common in diarrhoeic calves of <6 months of age whilst *C. andersoni* infection more preponderant in asymptomatic adult cattle [98]. More recently, a study on the prevalence and genotype distribution of *Cryptosporidium* isolates in native beef calves 2–6 months old in Dac Lac Province, central Vietnam, discovered an overall prevalence of 18.9 % (44/232) on the sample and 50 % (20/40) on the herd levels. Genotyping based on PCR and sequence analysis of the 18S rRNA gene revealed occurrence of the two non-zoonotic species *Cryptosporidium ryanae* and *Cryptosporidium bovis*, with the former as a dominant species in the animals [99]. Besides calves, an epidemiological investigation in pigs revealed that prevalences in the animal and at the farm levels were 18.1 % (134/740) and 71.9 % (64/89), respectively. Risk factor analysis showed that pre-weaned piglets were at the highest risk for infection, followed by post-weaners, sows and finishing pigs. Good sanitary conditions showed positive effects in decreasing oocysts shedding. Topographically, *Cryptosporidium* was more common in the mountainous zone than that in the coastal delta zone. There was an association between the occurrence of diarrhoea and the level of *Cryptosporidium* oocyst excretion within infected pigs [100]. Subsequent to that, 193 pig faecal samples were screened with 28 (overall prevalence 14.5 %) identified as positive by microscopy and genetic identification based on the 18S rRNA

and 70 kDa heat-shock protein genes revealed that pigs in Vietnam are infected with *Cryptosporidium suis* and *Cryptosporidium* pig genotype II. The presence of these host-adapted species/genotypes suggests that pigs may not pose a significant public health risk in this area [101]. Other than that, 23.7 % of 464 ostrich faecal samples were found with *Cryptosporidium* in a farm in Khanh Hoa Province, central Vietnam. Prevalences of *Cryptosporidium* in animals of <45 days, 45–60 days, 61–90 days, 91 days to 12 months and >12 months were 23.5 % (16/68), 33.3 % (22/66), 35.2 % (68/193), 0 and 5.8 % (4/69), respectively ($p < 0.05$). Majority of positive samples scored as the 3+ level of intensity of infection were from 61 to 90 days ostriches. Molecular analysis in the 18S rRNA, 70 kDa heat-shock protein and actin genes demonstrated the presence of only *Cryptosporidium avian* genotype II in ostriches in central Vietnam [102]. Besides domestic mammals, *Cryptosporidium* has also been detected in Asian seabass (Lates calcarifer) in nurseries in Vietnam [103].

5.1.6.4 Contamination in the Environment

In **Thailand**, concentration of *Cryptosporidium* oocysts was found in two canals (i.e. Klong Nueng and Klong Song) with levels of between 0 and 95 oocysts per litre, and confirmed specific identity of these oocysts was *C. parvum* by real-time PCR and sequencing of the *Cryptosporidium* oocyst wall protein (COWP) gene. Importantly, it was determined that there was a substantially point to point oocyst concentration variation along these canals, highlighting a requirement for multiple location testing [104]. More recently, the geographic information system (GIS) was used to evaluate and map point and, particularly, non-point pollution sources in urban canals in Thailand [105]. With regards to the contamination of *Cryptosporidium* oocyst in bulk water used in frozen food industry in Thailand, *Cryptosporidium* oocysts were isolated from 6 (35 %) untreated water samples of the 20 industrial sites at an average of 29 oocysts per 1,000 L [106]. In early 2005, 118 water samples of different origins were collected from six Tsunami-affected southern provinces of Thailand. Of these, 15 samples (12.7 %) were positive for *Cryptosporidium* spp. and nine (7.6 %) positive for *Giardia* spp. Three years later, in 2008, five out of 42 (11.9 %) samples from the two same areas were examined positive again for *Cryptosporidium* spp., and three out of 42 (7.1 %) were positive for *Giardia* spp. Both protozoans were found in reservoir, river/canal and pond waters [107]. Most recently, 144 water samples were collected with 72 samples from the Chao Phraya River, Thailand, collected in the summer, rainy and cool seasons and 72 samples from sea water at Bang Pu Nature Reserve pier, collected before, during and after the presence of migratory seagulls. Total prevalence of *Cryptosporidium* contamination in river and sea water was 11 % and 6 %, respectively. The highest prevalence in river water was 29 % and in sea water was 12 %. These were observed at the end of rainy season continuing into the cool season. During the rainy season, prevalence of *Cryptosporidium* was 4 % in river and sea water samples, but none in summer season. All samples from the river were

C. parvum-positive, while *C. meleagridis* (1) and *C. serpentis* (1) were obtained from sea water. To the best of our knowledge, this is the first genetic study in Thailand of *Cryptosporidium* spp. contamination in river and sea water locations and the first report of *C. serpentis*, suggesting that humans, household pets, farm animals, wildlife and migratory birds may be the potential sources of the parasites [108].

Cryptosporidium oocysts have been detected to be ubiquitous in the **Malaysian** environment [109]. For instance, in Kelantan where wells are common, 7.1 % of 28 well water samples were contaminated with *Cryptosporidium* oocysts [109, 110]. Pertaining to river water, 11.5 % of 174 river water samples analysed were positive with *Cryptosporidium* oocysts (ranging from 0.4 to 246 oocysts per litre). More importantly, when the quality of drinking water from eight treatment plants was evaluated; *Cryptosporidium* oocysts were only found in the raw water (0.05–3 oocysts per litre) and backwash water (1,200–1,600 oocysts per litre) [109, 111–113]. Besides that, sewage treatment works can also contribute *Cryptosporidium* oocysts into receiving waters, whereby 20–80 *Cryptosporidium* oocysts per litre have been detected in treated sewage effluent [114, 115]. In soil, *Cryptosporidium* oocysts have also been detected [109, 116] and the viability of the *Cryptosporidium* oocysts in water and soil in the Malaysian environment assessed using fluorogenic vital dyes, 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) indicated that it takes 1–2 months for *Cryptosporidium* oocysts to be rendered non-viable in a tropical climate [87, 116]. In a study on recreational rivers (i.e. Congkak River and Batu River), located in Selangor state, it was discovered that both *Giardia duodenalis* and *Cryptosporidium parvum* (oo)cysts were higher in Congkak River (50 % or 15/30 and 10 % or 3/30, respectively) than Batu River (16 % or 5/30 and 3.3 % or 1/30, respectively). The mean density of cysts/L was 0.72 in Sungai Congkak and 0.023 in Sungai Batu and that of oocysts/L was 0.023 in Sungai Congkak and 0.0033 in Sungai Batu, showing that the occurrence of *Giardia* was higher and more frequent than *Cryptosporidium* in both rivers. Sungai Congkak also showed higher faecal coliform count (ranging from 0.48×10^3 to 73×10^3 CFU/100 mL) than Sungai Batu (0.41×10^3 to 16×10^3 CFU/100 mL) [117]. More recently, biomonitoring of *Giardia* cysts and *Cryptosporidium* oocysts in river water corresponding to five villages situated in three states in peninsular Malaysia discovered that *Giardia* cysts were contaminating rivers from four villages (e.g. 33.3–100 %) while *Cryptosporidium* oocysts were only detected in two villages (66.6–100 %). All the physical and chemical parameters did not show significant correlation with both protozoa [118].

The use of wastewater in the production or processing of food in **Cambodia** was evaluated for the presence of *Cryptosporidium* oocysts. A recent study investigated the levels of faecal pathogens in Boeng Cheung Ek Lake, the main recipient of wastewater from the city of Phnom Penh. Water spinach (*Ipomoea aquatic*), a popular vegetable grown in that lake for both human consumption and animal feed was found to be contaminated with *Cryptosporidium* oocysts in 6 of 35 (17 %) water spinach samples, with an average concentration of 0.5 oocyst per gram [119]. Besides *Cryptosporidium*, *Giardia* (56 %) and *Cyclospora* (8 %) as well as helminths (11 %) were also isolated.

Based on these findings, there can be a potential occupational exposure of these parasites to farmers during harvest and subsequent handling and transport of vegetables from production sites to the markets [119].

To date, no cryptosporidiosis cases have yet been reported from **Brunei Darussalam and Timor-Leste**. In **Singapore**, the only available report of cryptosporidiosis was from an AIDS child with profuse diarrhoea (up to 15 episodes a day) over a one week period [120]. Nonetheless, due to scarcity of water sources in Singapore, a lot of focus has been placed on developing and enhancing methods for the detection of *Cryptosporidium* in water samples [121–123]. For instance, the use of flow-through immunomagnetic separation system [124] and the fabrication of the high-flux isopore micro-fabricated membrane for effective concentration and recovering of waterborne pathogens [125].

5.2 *Giardia*

5.2.1 Historical Background and Taxonomy

Giardia duodenalis (synonyms: *Giardia intestinalis* and *Giardia lamblia*) is a flagellated protozoan infecting humans and a wide range of animals. *Giardia* was used as a genus name for the first time in 1888 [126]. Based on morphological differences using light microscopy, *Giardia* has been classified into *Giardia agilis*, *Giardia intestinalis* and *Giardia muris* [127]. The application of electron microscopy has assigned additional species based on differences from *G. intestinalis*, which are *Giardia ardeae*, *Giardia psittaci* and *Giardia microti* [128–130]. Further genetically distinct assemblages, assemblage A–H, within *G. duodenalis* have also been reported which are likely to represent different species [131].

5.2.2 Biology and Life Cycle

Giardia is considered a primitive eukaryote. It possesses some basic characters of eukaryotes and lacks classical mitochondria, peroxisomes and morphologically evident Golgi apparatus that are organelles typical to higher eukaryotes [132]. Life cycle occurs in a single host and includes two major steps: excystation and encystation. The excystation starts when susceptible host ingests the cysts, which are exposed to the acidic environment of the stomach and pass to the proximal small intestine where they develop to trophozoites which cause the disease. Encystation is a mechanism of adaptation to survive outside the host by the formation of the resistant cyst wall.

5.2.3 Pathology and Clinical Manifestation

Giardiasis is most often asymptomatic and the symptomatology differs from person to person, depending on factors such as number of cysts ingested, duration of infection, host immune status and perhaps parasitic factors [133]. Acute giardiasis, which lasts 3 or 4 days, is characterised by diarrhoea, bloating, abdominal pain, nausea and vomiting. Chronic infection may develop which may last for 2 or more years, and patients may suffer intermittent diarrhoea and persistent or recurrent mild to moderate symptoms. Chronic giardiasis in early childhood is associated with poor cognitive function and failure to thrive [134]. Several mechanisms behind the pathogenesis have been demonstrated including enterocyte apoptosis, increases in intestinal permeability due to disruption of the apical junctional complex and $CD8^+$ lymphocyte-dependent microvillus shortening which decrease mucosal surface area and cause malabsorption and maldigestion [135].

5.2.4 Transmission

In developing countries where the infection rate of *G. duodenalis* is high with poor personal hygiene, faecal–oral is the most common route of transmission [136]. Person-to-person transmission has also been suggested in disadvantaged communities in developed countries [137, 138]. Waterborne transmission is common and is enhanced by some features such as the small size of cysts to penetrate the physical barriers of water treatment, insensitivity to some disinfectants used in the water industry, the ability to survive in water for 1–2 months and low host specificity. The risk of zoonotic transmission may be from zoonotic assemblage A (subassemblage AI) which can infect humans and animals. The host-adapted assemblages such as assemblages C and D (dog), assemblage G (rats), assemblage F (cat) and assemblage E (calves) may not represent risk to humans [139].

5.2.5 Diagnosis and Treatment

Microscopic identification of *G. intestinalis* trophozoites or cysts using permanent staining such as trichrome staining technique after concentration method has been considered the gold standard diagnostic method. The fluorescein isothiocyanate (FITC)-labelled anti-*Giardia* cyst, which targets the intact parasite, needs immunofluorescence microscope. The sensitivity of microscopy examination could be affected by sampling issues related to the intermittent excretion of intestinal parasites which can be overcome by examining triple faeces samples collected in three consecutive days [140]. Immunoassays that detect soluble antigens have also been introduced including ELISA test and nonenzymatic solid-phase qualitative

immunochromatographic assay. PCR assays are more specific and sensitive. However, they are expensive and time consuming, and the laboratory technologists need to be trained. Metronidazole has been the drug of choice of giardiasis at a single high dose or multiple low doses. Tinidazole is an alternative drug used as single dose or as multiple doses. Albendazole, the drug of choice for helminthiasis, has been found to be effective for the treatment of giardiasis [141].

5.2.6 Epidemiology of Giardiasis in Southeast Asia

Giardiasis is prevalent in Southeast Asian countries. The prevalence rate is as high as 30 % in some urban capitals, perhaps due to the high intensity of environmental contamination, poor sanitation and hygiene condition and the lack of clean water for domestic consumptions. Children in these highly endemic areas contracted giardiasis as early as before school-going age. Generally, there is no obvious association between the occurrences of giardiasis during wet or dry seasons of the year in these countries.

Giardia duodenalis is the only species found in man incriminated for giardiasis in Southeast Asia, although it is found in other mammals including domestic pets and livestock [109]. There has been an increased interest in the molecular epidemiological studies conducted on giardiasis in the last 10 years in Southeast Asia. This has significantly improved our understanding on giardiasis transmission in these countries. This includes a better knowledge of the parasite phenotypic and genotypic diversity, the roles of various transmission routes in giardiasis epidemiology and the significance of parasite genetics in clinical manifestations. To date, only *G. duodenalis* assemblages A and B are associated with human infections.

5.2.6.1 In Humans

In **Indonesia**, studies and surveys to determine the prevalence of giardiasis in this large archipelago of SEA are a very daunting task. With the current population exceeding 260 million, the different geographical conditions and cultures in the far outer islands, various life styles and habits, differences in basic living facilities and different social-economic status, the prevalence rate varies from 2.9 to 22.3 % [142, 143]. Initial studies on giardiasis in humans conducted in Jakarta from 1983 to 1990 recorded 2.9 %, and a separate survey in 1990 showed a prevalence rate of 4.4 % [144]. In a study of 903 women from all over Indonesia before emigrating as foreign workers, stool samples were examined by the Ritchie methods to isolate for parasitic infections. Of them, 319 workers were sampled for *Giardia* infection and 22.1 % showed presence of cysts [143]. It is notable that this group of population comes from different islands in Indonesia with varied geographical conditions and cultural differences. Among the 318 HIV patients recorded with diarrhoea between

2004 and 2007 in Jakarta, 95 % of the samples were males in the 21–40 age group having CD4(+) counts of 50 cell/mm and 1.9 % was positive for *Giardia* [48].

A survey on giardiasis in **Malaysia** was first conducted by Bisseru and Aziz [145] among residents in the rural areas. Further studies showed a prevalence rate ranging from 0.21 to 25 % with unsustainable reduction (reviewed in [109]). Rural areas had higher infection rates (4.8–10.8 %) [145–149] compared to urban areas (0.21–2.6 %) [150, 151] with plantation dwellers (11.3 %) [152] and Orang Asli communities (8.5–23.7 %) [68, 149, 153–157] being more endemic for giardiasis. Hospital patients have registered infection rates of 5.7–6.0 % [47, 67]. Several studies have aimed at identifying factors associated with giardiasis which included children $<$12 years old, drinking piped water, eating raw vegetables, low socio-economic standing [155] and the presence of an infected family member with the parasite [154].

In **Thailand**, a cross-sectional study was performed to determine the prevalence of giardiasis in hill-tribe children of two different remote districts (Mae-chaem and Hod), Chiang Mai, northern Thailand, from November 2006 to April 2007 [158]. The overall prevalence of giardiasis was 5.2 %. In 2005, a cross-sectional study was conducted to determine the prevalence and the risk factors of giardiasis in 531 primary schoolchildren of a rural community, the Chachoengsao Province. The prevalence of giardiasis was 6.2 %. The significant risk factors for giardiasis were children aged 5–9 years, households with $\geq$3 children under the age of 12 years, low parental educational level, drinking bottled water and living in close contact with dogs. Washing hands before meals had a protective effect. These significant risk factors tend to suggest that multiple modes of transmission of *G. duodenalis* take place in this population [159]. Another study was conducted to investigate the presence of intestinal parasites among preschool children (aged 3 months to 5 years) in Sangkhlaburi, a rural district in the west of Thailand along the Thai–Myanmar border. Stool specimens were collected from 472 preschool children in the period from October 2001 to October 2002. Of them, 107 individuals (22.7 %) were infected with intestinal parasites. The most frequent parasites identified in positive cases were *G. duodenalis* and *Cryptosporidium* spp., and eighteen specimens (3.8 %) showed mixed parasite infections. Highest proportion of intestinal parasites occurred during the rainy season (June–October) [81]. Another study examined 189 preschool children at Sanamchaiket District, Chachoengsao Province, central Thailand, in February 2007 [160]. The prevalence of *G. duodenalis* in preschool children was 5.8 %. In this study, children who kept cat(s) at home were at 5.1 times (95 % CI, 1.3–20.3) greater risk of acquiring giardiasis. The possibility of zoonotic transmission of *G. duodenalis* between cats and preschool children is likely to take place. However, *G. duodenalis* infection in cats was not determined. Recently, Prasertbun et al. [161] examined parasitic infections in the stool samples of children living on the Thai–Myanmar border and discovered that 10 (7.69 %) and 40 (30.77 %) of 130 stool samples were positive for *G. duodenalis* by microscopy and real-time PCR, respectively. Only three out of nine liquid stools revealed *G. duodenalis*-positive using microscopy, but all of them were *G. duodenalis*-positive using real-time PCR. Inpankaew

et al. [162] isolated faecal samples collected from 204 humans and 229 dogs from 20 different temples in Bangkok, as well as communities in the surrounding temple ground for intestinal parasites including *Giardia* which recorded a prevalence rate of 2.5 %. Villagers sampled at the Thai/Myanmar border showed an infection rate of 7.7 % [161], while Myanmar immigrants working in a Thai food factory recorded a rate of 14.1 % [163].

In the **Philippines**, studies on *G. duodenalis* are limited. It is also believed that prevalence rates of this organism in the country are underestimated. In a study among residents living in a slum area in Manila, results showed that 22.05 % of 2,354 stool samples collected contained *Giardia* cysts [164]. Most of the studies conducted on giardiasis were from samples of patients with diarrhoea. Samples from 3,456 patients from all over the islands in 2004–2005 recorded an overall 2.0 % prevalence rate of giardiasis (Luzon, 5.0 %; Mindanao, 4.9 % and Visayas, 2.2 %) [78]. In another study, Mindanao Island recorded the highest rate of giardiasis at 3.6 %. The highest rate is among the 5–9-year-old group, higher in males than females. It was observed that *Giardia* transmission peaks in September with infection being more prevalent in the rainy than dry seasons [78]. Similar observation was noted by Adkins et al. [165] when they isolated *Giardia* from 1,698 diarrhoeic stools and noted 0.1 % positive for cysts. The number of patients with diarrhoea increases in the monsoon season and peaked during maximum rainfall. In a cross-sectional study of stool sample survey (238 children, aged 18 months to 15 years), in a squatter area of Manila, 20 % were positive for *Giardia*, and 84 % of the children showed polyparasitism [166].

In **Cambodia**, about 2.9–3.2 % of school children surveyed were positive for *Giardia* in Kampong Cham and Battambang [167, 168]. Between the years 2006 and 2011, a total of 16,372 faecal samples from symptomatic children, in Siem Reap Hospital, were examined for parasitic infections. A total of 3,121 (19.1 %) of these samples were positive for parasites, and *G. duodenalis* was the most common parasite (8.0 % of samples) [169]. Studies have shown that risk factors include young children, who are most likely to have very close contact with household pets [169].

Prevalence of giardiasis in **Lao PDR** was first reported in children with symptomatic diarrhoea in Vientiane in 1994 [170]. This study which was conducted among outpatients showed a prevalence of 0.5 %. Later, a survey among children in rural Khammouane Province indicated a rate of 8.6 % for giardiasis [171]. Adults showed a lower prevalence rate (i.e. 4.9–5.2 %) in the fishing community on the Nam Gum Reservoir for giardiasis [172].

Giardiasis in **Vietnam** is very rarely reported. Preliminary reports showed that prevalence in adults was 3.1 % [173]; however, that of children was 4.0 % [174]. In the Haham Province, 2 % of the community was infected with *Giardia duodenalis* [175]. In **other Southeast Asian countries**, it is suffice to state that giardiasis is thought to be endemic in Brunei Darussalam, Singapore and Timor-Leste. However, currently, reports of prevalence have not been documented.

The introduction of PCR-based genotyping technology has improved our understanding of the epidemiology of giardiasis in Southeast Asian countries.

Genotyping *G. duodenalis* isolated from Malaysian Orang Asli (indigenous) showed a predominance of assemblage B compared to assemblage A (42 vs. 1) [176]. A recent study in Malaysia genotyped 71 *G. duodenalis* isolates based on three genetic loci (*tpi*, *gdh* and *b*-giardin) and reported an equal distribution of assemblages A and B with high proportion of mixed assemblages A and B. Subtyping analysis showed that all isolates of assemblage A belonged to the anthroponotic subtype AII [177]. The predominance of *G. duodenalis* assemblage B (86.5 %) was also reported in the Philippines [164]. Both assemblages A and B have been reported from Thailand, and subtyping analysis identified assemblage A as AII [158–160]. Based on these findings, it would seem that the dynamic of transmission in Southeast Asian countries is human to human. However, zoonotic transmission should still be considered. *Giardia* isolated from human and dog populations was genotyped in Thailand. Following genotyping, a majority of the *Giardia* isolated from the dog population was found to be of assemblage A, followed by assemblages D, B and C, respectively, while human isolates were of assemblages A and B. Therefore, it was suggested that dogs in temple communities posed a potential zoonotic risk to humans for the transmission of *Giardia* (especially assemblage A genotypes) [162].

5.2.6.2 In Animals

The possibility of zoonotic transmission of giardiasis in **Malaysia** was elucidated when 310 faecal samples from goats from eight different farms in Malaysia were tested for the presence of *Giardia* using a PCR-coupled approach. Of these, 21 (6.8 %) samples were positive. In this study, *Giardia* assemblages A, B and E were identified. The identification of the 'zoonotic' assemblages A and B suggests that *Giardia*-infected goats represent a possible reservoir for human giardiasis in Malaysia [178]. Earlier Muhid et al. [179] isolated 12.5 % of calves that were positive for *Giardia* cysts, and 68.8 % of farms with calves were contaminated with the flagellates. There was also evidence that domestic animals like cat and dogs in **Thailand** may play a role in the transmission of *G. duodenalis* as indicated by the presence of assemblages A and B in these animals through genotyping [160, 162].

In northern **Vietnam**, dairy cattle are mainly managed in small-scale farms, where animals are kept confined and feeding occurs by cut and carry methods. In this study by Geurden et al. [180], the occurrence of parasitic infections was examined in five provinces around Hanoi. A total of 201 farms were visited, and 334 stool samples were collected from calves younger than 3 months, animals between 3 and 24 months and adult cows. Fifty percent of the calves younger than 3 months ($n = 68$) were positive for *Giardia*. Most *Giardia* isolates were identified as the non-zoonotic *G. duodenalis* assemblage E based on the beta-giardin gene. However, one out of 17 samples for genotyping indicated *G. duodenalis* assemblage A. This study demonstrated that parasitic infections occur frequently in dairy cattle around Hanoi although animals are mainly kept confined. It should be noted

that isolating assemblage A from animals is not a conclusive evidence of zoonotic transmission and warrants further subtyping analysis.

5.2.6.3 Contamination in the Environment

In **Cambodia**, water spinach in the lake near Phnom Penh was found to be 56 % positive for parasitic infections [119]. In **Malaysia**, *Giardia* has been detected in recreational water, and genotype analyses indicated the presence of *Giardia duodenalis* assemblage A [181]. Zoo environment at the outskirts of Kuala Lumpur was also screened, and microscopic examination revealed that 17 of 18 water samples taken from the zoo were *Giardia* cyst-positive with concentrations ranging from 1 to 120 cysts/L [182]. In the study, nine (52.9 %) of the 17 cyst-positive samples produced amplicons of which 7 (77.8 %) could be sequenced. *Giardia duodenalis* assemblage A (6 of 7) and assemblage B (1 of 7), both infectious to humans, were identified at all sampling sites at the zoo. The presence of human infectious cysts raises public health issues. Other aquatic environment has also been incriminated to harbour *Giardia* cyst. Furthermore, Lim et al. [115] isolated 18–8,480 cysts of *Giardia*/L in sewage treatment plants, while Azman et al. [117] reported 0.023–0.72/L in recreational river waters in the state of Selangor. These environmental studies highlight the importance of incorporating environmental sampling and monitoring in addition to routine faecal examinations to determine veterinary and public health risks to giardiasis in Malaysia.

5.3 Conclusions and the Way Forward

This chapter highlights that cryptosporidiosis and giardiasis are prevalent in many parts of Southeast Asia. While epidemiological studies in humans are available from most of the Southeast Asian countries, information on these infections in animals can only be gathered from Malaysia, Thailand and Vietnam. In addition, data on contamination of these parasites in the environment could only be found in Malaysia, Thailand and Cambodia. It was also evident that there is a paucity of information on foodborne cryptosporidiosis and giardiasis. It is important that these areas are being looked at as there is a real risk of *Cryptosporidium* and *Giardia* contamination of fruits and vegetables in Southeast Asia as agricultural and manufacturing practices in primary production and hygienic practice during food preparation may be substandard. Further research work is necessary to understand fully the extent of this threat that these protozoan parasites pose to food safety in this region. Understandably, a lack of information and awareness of the burden and transmission of the disease at the regional and global level may be partially explained by the unavailability of quality diagnostic strategy with suitability for field-level application. These factors are further compounded by a scarcity of

validated, simple and sustainable intervention packages as part of integrated control programmes for *Cryptosporidium* and *Giardia*.

The impact of these diseases needs to be assessed and this exercise requires reliable epidemiological datasets acquired from informative, national and regional surveillance systems. The availability of comprehensive information on the impact of diarrhoeal diseases will ensure implementation of effective control measures. Unfortunately, these surveillance systems are not available even in the more developed countries of this region.

Nonetheless, there is a positive and encouraging trend in Southeast Asia in these last couple of years whereby there has been an increase in the effort to utilise more advanced diagnostic and surveillance tools (e.g. real-time PCR and GIS) to study *Cryptosporidium* and *Giardia*. However, as more molecular data is made available in the Genbank, the challenge now is to make sense of this information. In addition, we as regional researchers need to consolidate these data so that they can be relevant and beneficial in our pursuit to address key epidemiology and population genetic questions that underpin surveillance, source-tracking and control of these diseases within and across national and international boundaries.

Given the geographical closeness and similar challenges face by Southeast Asia member countries, it is crucial to put in place a win-win strategy for reducing the burden of cryptosporidiosis and giardiasis in this region. The following collective approaches are recommended (a) establishing national/regional surveillance systems for cryptosporidiosis and giardiasis based on standardised validated microscopic and molecular-diagnostic tools and reporting systems; (b) developing shared regional epidemiological databases for the monitoring of geographical and seasonal variations between the various countries; (c) establishing shared one-stop centres, especially for molecular diagnostic testing to optimise the accessibility to equipment, information and expertise; and (d) providing evidence based data to assist the formulation of effective policies, particularly to incorporate *Cryptosporidium* and *Giardia* detection as a parameter in national drinking water and food quality standards. These highly recommended approaches for the Southeast Asian countries should assist in strengthening collective understanding on the epidemiology, genetic variation, management, control and prevention of these infections [53].

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Chapter 6
Entamoeba histolytica in Southeast Asia

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Abstract Amoebae may be free-living or parasitic. Parasitic amoebae belong to many genera. *Entamoeba histolytica* belongs to the genus *Entamoeba,* and it is an important human pathogen. *E. histolytica* is prevalent worldwide. It causes intestinal and extraintestinal amoebiasis. It is much more common in the tropics including Southeast Asia wherever sanitation is poor. Amoebiasis is a major health problem in Southeast Asia. It is the third leading parasitic cause of mortality after malaria and schistosomiasis in the developing countries. Food and water contaminated by human faeces containing *E. histolytica* cysts are the main sources of infection. The main reservoir of the infection is the human cyst carriers. Transmission is via faecal–oral, mechanical vectors and sexual contact. The life cycle of *E. histolytica* is completed in a single host, human. Trophozoites can invade all tissues of human including the intestinal mucosa and liver, which is most commonly affected, followed by the lung, skin and brain. The parasite produces virulent factors which are responsible for invasion and destruction of the human tissue. The typical manifestation of intestinal amoebiasis is dysentery. Amoebic liver abscess is the most common extraintestinal complication of amoebiasis. Diagnosis consists of stool examination, serodiagnosis, molecular diagnosis and imaging methods. Treatment consists of the use of amoebicides, and no vaccine is yet available against amoebiasis in humans. Control and prevention include personal hygiene, proper sanitation, drinking safe water and treatment of cases.

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6.1 Introduction

Entamoeba histolytica is an anaerobic parasitic protozoan, part of the genus *Entamoeba* [1]. It predominantly infects humans and other primates. *E. histolytica* is estimated to infect about 50 million people worldwide. The parasite is responsible for up to 100,000 deaths per year, placing it second only to malaria in mortality due to protozoan parasites. Previously, it was thought that 10 % of the world population was infected with *E. histolytica*, but with the recognition of the second species *E. dispar*, at least 90 % of these infections were due to this nonpathogenic species [2]. Dogs and cats can become infected transiently, but do not contribute significantly to transmission. Several members of the genus *Entamoeba* infect humans. Among these only *E. histolytica* is considered pathogenic, and it causes amoebiasis or amoebic dysentery. *E. dispar* is morphologically similar to *E. histolytica* and was previously considered to be the same species. However, molecular and biochemical data clearly showed that the nonpathogenic *E. dispar* is a distinct species. The two species are distributed worldwide, but they are more common in tropical countries or areas with poor sanitation. High prevalence of amoebiasis occurs in the Indian subcontinent, the Far East, Southeast Asia, western and southern Africa and parts of South and Central America. In Southeast Asia, amoebiasis is commonly found in aborigines, communities with poor sanitation, population with low socioeconomic status both rural and urban and immigrants from endemic areas. Normally the parasite resides in the large intestine, and the trophozoites occasionally penetrate the intestinal mucosa and may disseminate to other organs causing extraintestinal amoebiasis. The factors that trigger invasion are unknown. Many people apparently infected with *E. histolytica* are asymptomatic, and the infection subsides spontaneously [2].

6.2 History

Fedor Aleksandrovich Lösch (1875), a physician from Saint Petersburg, found amoebae in faecal samples, and he described amoebic trophozoites in the stool and colonic ulcerations of a farmer with a fatal case of dysentery [3].

Fritz Schaudinn (1903) established the differentiation between *Entamoeba histolytica* and *Entamoeba coli* and decided to call it *E. histolytica* because of its ability to cause tissue lysis [3].

Emile Brumpt [4] pointed out the existence of *E. histolytica* as a species complex, comprising two morphologically indistinguishable species, *E. dysenteríae,* which is the cause of symptomatic infection, and *E. dispar* in asymptomatic carriers. Brumpt's hypothesis was dismissed by other workers. In the 1970s, accumulated data gave support to Brumpt's hypothesis of the existence of two distinct organisms within what was being called *E. histolytica*, and in 1993, a formal redescription of *E. histolytica* was published, separating it from *E. dispar* [2].

Louis Diamond et al. [5] developed an axenic culture medium for *E. histolytica* which allowed *in vivo* and *in vitro* studies.

Sargeaunt and Williams [6] distinguished for the first time *E. histolytica* strains by isoenzyme electrophoresis, confirming *E. histolytica* was indeed a species complex comprising both pathogenic and nonpathogenic species [6].

Diamond and Clark (1993) described again Brumpt's original 1925 hypothesis, concluding that there was enough evidence to support the existence of two morphologically indistinguishable species, a pathogenic and a nonpathogenic one, corresponding to *E. histolytica* and *E. dispar*, respectively. The World Health Organization accepted this hypothesis in 1997 [3].

6.3 Epidemiology of Amoebiasis in Southeast Asia

6.3.1 Singapore

Intestinal parasites are chiefly responsible for parasitic infections in Singapore. It must be appreciated however that while Singapore is a small island, it is composed of many types of racial and cultural communities living under widely different socio-environmental conditions. For this reason, a survey carried out by Desowitz et al. [7] on typical communities of tenement urban village (local term = *kampung*), urban farm, rural farm, labour lines, coastal village communities and medical students was examined. The results of the preliminary survey showed that 9.2 %, 5.3 %, 8.2 %, 1.9 %, 6.1 %, 1.9 % and 3.9 % of tenement area, urban village, urban farm, rural farm, labour lines, coastal villages and medical student were infected with *E. histolytica*, respectively. Amoebiasis does not appear to be a serious medical problem in Singapore as there were only 28 hospital admissions for the infection in 1958. Furthermore, amoeba abscesses were the only abscesses that predominated in the right lobe in the Singapore study [8].

6.3.2 Cambodia

Cambodia is recognised as an endemic area for malaria and schistosomiasis [9, 10], but the infection status of intestinal parasites in Cambodia has not been thoroughly investigated. However, several reports showed that Cambodian children and refugees were infected with intestinal parasites including nematodes, trematodes and protozoa [11–13]. A survey was made to find the extent of intestinal parasite particularly *E. histolytica* infection in Kampong Cham, Cambodia, in February 2002. A total of 251 stool specimens were collected from Tonlebat primary school children and examined by formalin–ether sedimentation technique. The infection rate of *E. histolytica* was found in 0.8 % stool samples. The positive rates of males

and females were similar (0.8 %). *E. histolytica* infection was only found in grade 3 school children [14]. Likewise, study conducted among children in Bat Dambang in 2004 reported the same prevalence of *E. histolytica* (0.8 %). However, male was found to be highly infected (1.2 %) compared to female (0.4 %) [15]. Symptomatic intestinal amoebiasis was highly endemic among the Cambodians living at Green Hill, an evacuation site on the Thai–Cambodian border between June 1987 and May 1989 [16]. Monthly incidence rates of intestinal amoebiasis were determined to be inversely proportional to cumulative monthly rainfall. The highest incidence of amoebic dysentery was 63/1,000 in children aged 12–23 months old. The main route of transmission of *E. histolytica* was not identified, but was most likely via the faecal–oral route.

6.3.3 Lao PDR

Laos People's Democratic Republic (Lao PDR) is a landlocked country situated in the Great Mekong subregion of Southeast Asia, where socioeconomic and eco-epidemiological characteristics vary greatly according to location. In the northern part, similar ecosystems are found as in southern People's Republic of China with mountain and highlands dominating the landscapes. These topological features are natural barriers that might impede social and economic developments, since transportation of commodities, communication and other exchanges are hampered. These issues exacerbate people's access to health care, clean water and adequate sanitation. Indeed, according to the results of the national population and housing census carried out in 2005, less than 20 % and only about half of the population living in these areas had access to clean water and sanitation, respectively [17]. The overall prevalence rate of the intestinal parasites in Lao PDR ranges from 76.8 to 95.7 % depending on living conditions. However, low infection rate of *E. histolytica* has been found as well as low level of anti-amoebic antibodies [18].

6.3.4 Myanmar

Intestinal parasitic infections are an important public health problem worldwide, especially in developing countries. These infections are frequently found in Myanmar and Thailand [19, 20]. According to the growth of labour demand, foreign workers from low-wage countries, mainly Myanmar, have migrated to urban areas of Thailand, particularly Bangkok and Samut Sakhon which are industrialised provinces. Due to their poverty that limits access to health services, Myanmar migrants may become carriers of parasites that contribute to the persistence of parasite transmission. Among the intestinal parasite infections, *E. histolytica*/*dispar* infection (3.8 %) was the predominant intestinal protozoa in these subjects [21]. This may be because Myanmar migrant workers frequently consume fresh

vegetables and raw pork contaminated with the infective stage of this parasite. In addition, they also live in poor personal and community hygiene conditions. A survey on the prevalence of amoebiasis was carried out by Sun et al. [22] in Western China near the China–Myanmar border. It was carried out in Nabang Township, China, and four natural villages in the City of Laiza, Myanmar, near the China–Myanmar border. Cysts of *E. histolytica*/*dispar* were detected microscopically using iodine. Antigens of *E. histolytica* were detected using ELISA. The general rate of infection with *E. histolytica*/*dispar* was 1.77 % (16/903), and the general rate of infection with *E. histolytica* was 15.17 % (137/903). The Myanmar rate of infection with *E. histolytica*/*dispar* and *E. histolytica* was 1.94 % and 16.69 %, respectively. In 2005, Maneeboonyang et al. [23] conducted a study on 701 school children from three primary schools near the Thai–Myanmar border. The results revealed that 2.6 % of the participants were infected with *E. histolytica*.

6.3.5 Thailand

Thailand is one of the tropical countries which is regarded as an endemic area, and a survey conducted by Mahidol University during 1968–1973 found the parasite all over the country. The prevalence rate was between 1.4 and 4.2 % depending on areas and socioeconomic status [24, 25]. Due to the lack of sanitary standard system and health education during that period, the people were exposed to acquiring *E. histolytica* from contaminated food and drinking water. Although the rate of infection is quite low, but it is the cause of potential loss of life. A serological survey was conducted in the villages of Phichit Province, the northern region and in the urban slum communities in Bangkok to determine whether amoebiasis was endemic in the areas and to determine the prevalence rates [26]. Six rural villages ethnically and culturally alike with a population of 3,019 and two urban slums with a population of 1,510 were surveyed. Sera were tested for indirect haemagglutination antibody (IHA) to *E. histolytica* and the stools examined for the parasite by direct smear method. Positive IHA titres (greater than or equal to 1:128) were detected in 482 (11 %) and 176 (20 %) sera and *E. histolytica* found in 639 (2 %) and 208 (3 %) stool specimens of rural and urban slum populations, respectively. Out of a total of 88 persons who showed significant levels of IHA antibodies to *E. histolytica* antigen, 5 had *E. histolytica* cysts in their stool specimens. The survey confirmed serologically and parasitologically that amoebiasis is endemic in the lower socioeconomic areas. Substandard living and sanitary conditions within the areas were considered responsible for the transmission of the disease. The prevalence of intestinal parasitic infection was studied by stool examination in institutionalised and non-institutionalised Thai people with mental handicaps by Sirivichayakul et al. [27]. The common parasite found in institutionalised people was *E. histolytica* (7.1 %). Institutionalised mentally handicapped people should be considered as a high-risk group for intestinal parasitic infection, and a parasitic control measure should be emphasised. Nithikathkul et al. [28] performed a study

among the residents of remote Karen villages in Thailand and found that 1.43 % of the participants were infected with *E. histolytica*. A cross-sectional study of the prevalence of intestinal parasitic infections at eight schools in Bo Klau district and four schools in Chalerm Prakiet district, Nan Province, was carried out by Waikagul et al. [29]. A total of 1,010 faecal samples were examined using the formalin–ether sedimentation technique. Results revealed that the rate of *E. histolytica* infection was 1.4 %. Whereas, a study conducted by Saksirisampant et al. [30] among children in an orphanage in Pathum Thani province revealed that the prevalence of *E. histolytica* was 3.7 %. No history of diarrhoea symptoms was recorded among these orphans. Based on molecular identification, the prevalence of *E. dispar* infection was greater than *E. histolytica* infection in the studied population in Thailand. A previous study revealed 13.3 % of amoebiasis patients are infected with *E. histolytica* and 20 % with *E. dispar* [31].

6.3.6 Philippines

During recent years, parasitological surveys have been conducted by many researchers throughout the Philippines in an attempt to update information on the prevalence and distribution of infectious diseases among rural populations. Included in these studies have been efforts to determine the prevalence and distribution of amoebiasis by stool examinations and by testing sera for antibodies to *E. histolytica*. In 1979, Cross and Basaca-Sevilla conducted a survey among various islands of the Philippines, and the prevalence and distribution of amoebiasis were determined by stool examination and by IHA test for antibodies to *E. histolytica*. Over 13,000 single formalinised stool specimens were examined microscopically by direct and ether concentration methods and 5 % found positive. Males (5 %) and females (5 %) were equally infected with the parasite, and the prevalence tended to increase with age. The lowest seropositivity rates were in areas with a pronounced dry season and the highest in areas where rain occurred most of the year. Conversely, the stool positivity rates were similar in both areas, but in areas with a pronounced dry season, the stool positivity rate was higher than the seropositivity rate [32]. In 1988, a study was performed in a squatter area of Manila. The cross-sectional survey performed among 238 children aged 8 months to 15 years revealed that 21 % of the surveyed children were harbouring *E. histolytica* [33]. Rivera et al. [34] used PCR to study the distribution of *E. histolytica* and *E. dispar* in 1,872 individuals in 14 communities in the northern Philippines. This assay detected 137 stools (7.32 %) containing *E. dispar* and 18 stools (0.90 %) containing *E. histolytica*. The most affected age group for *E. histolytica*/*E. dispar* infections were those 5–14 years of age. There was no significant difference in the sex distribution of *E. histolytica,* while in the case of *E. dispar*, a higher prevalence was observed in females (9.20 %) than in males (5.73 %). An apparent clustering of stool-positive cases of *E. histolytica* and *E. dispar* was also observed in the northern part of the study area. The result of this survey was performed by Baldo et al. [35] to know the infection status of

intestinal parasite in children on the residential institutions and street communities in Metro Manila. A total of 284 stool samples from 11 institutions and 3 street communities were examined by the formalin–ether concentration method. Of the children examined, 2.9 % were found to be harbouring *E. histolytica*. Infection rates for amoebiasis were higher in males (4.5 %) than females (1.2 %). In 2006, Rivera et al. [36] conducted a study on 113 mentally retarded patients residing in a mental institution in Manila. They were screened for the presence of *E. histolytica* based on microscopy and PCR. Anti-*E. histolytica* antibodies were also screened in 97 serum samples collected using IFA test. Parasitological examination showed *E. histolytica*/*E. dispar* in 43 cases (38.05 %), while PCR detected 74 cases (65.48 %) positive for *E. histolytica* and 6 cases (5.30 %) positive for *E. dispar*. Interestingly, these 6 samples were coinfected with *E. histolytica*. IFA test revealed that 80.41 % (78/97) of the respondents possessed significant antibody titres for intestinal infection of *E. histolytica*. Of this number, there were five patients negative in IFA test but positive in PCR.

6.3.7 Indonesia

Although there is an increased number of antiprotozoan drugs available today, protozoan infections are a continuing problem in Indonesia. The most important protozoan parasites in Indonesia are the malarial parasites, *E. histolytica* and *Toxoplasma gondii*. In Indonesia, amoebiasis is endemic throughout the archipelago. The prevalence rates reach up to 18 % [37] and are found higher among the poor, for instance, in Yogyakarta, where the prevalence in hospital personnel was 17.1 and 25.2 % in suburban area, due to poor sanitation [38]. Extraintestinal infection mostly occurs in the liver, but amoebic liver abscess is not very often found. Bintari [39] found 25 cases in 26 years and another 27 cases at autopsy. Nurlela et al. [40] found 15 cases admitted to Cipto Mangunkusumo Hospital in Jakarta from 1971 to 1973. Alisah et al. [41] found 11 cases in Persahabatan Hospital, 8 of which perforating into the pleural cavity of the lung and one into the abdominal cavity. Three other cases were pulmonary amoebiasis. In Yogyakarta, only 14 cases with amoebic liver abscess were found in a 5-year period [42]. Muljono et al. [43] reported a case of amoebic liver abscess perforating into the pleural cavity and lung. Results of a serology survey in April 1972 for *E. histolytica* antibody among 484 inhabitants of the isolated Lake Lindu Valley of Central Sulawesi (Celebes) were presented by Clarke et al. [44]. Indirect haemagglutination antibody titres (IHA) for amoebiasis were found in over 10 % of the population, although only 3.7 % demonstrated significant titres of 1:128 or greater. There appeared to be no relationship between antibody titres and the age and sex of individuals tested, and the frequency distribution of antibody titres indicated a low prevalence of invasive amoebiasis in the population. In 1974, Stafford et al. conducted a survey of intestinal parasites of man on the island of Bali with a total of 270 stool specimens from three villages examined, and only one case was reported positive with *E. histolytica*. In 1975, a

parasitological survey was conducted among the inhabitants of seven villages in three regencies in South Kalimantan Province [45, 46]. A total of 2,169 stool specimens and 1,027 serum specimens were obtained, representing samples from approximately 10 % and 5 % of the population, respectively. *E. histolytica* was found in 12 % of the stool samples. Seroepidemiological studies on amoebiasis were done by use of the IHA test. The frequency distribution of the reciprocal antibody titres showed a bimodal distribution with 34 % of the population demonstrating positive reactions at titres of 1:128 or greater. Sera collected from people living along the slopes of Mount Merapi and Mount Merbabu in Boyolali Regency, Central Java, were tested by IHA for antibodies to *E. histolytica* [45, 46]. A total of 695 sera from 439 males and 256 females, 2–75 years of age, were tested for amoebiasis, and 17.6 % had positive antibody titres of 1:128 or greater. The prevalence of antibodies was the same for males and females and increased with age. Cross et al. [47] also conducted a survey in five villages in North Sumatra, and a total of 2,066 stool specimens and 969 sera were examined. The most common intestinal protozoa found were *E. histolytica* (7 %). Testing sera for *E. histolytica* antibodies by IHA test demonstrated positive reactions in 13 % of the population. Stafford et al. [48, 49] collected 300 stool specimens from indigenous inhabitants of the small isolated Torro Valley in the mountain of Central Sulawesi and found 8 % of the population infected with *E. histolytica*. Intestinal parasitic infections were surveyed in the inhabitants of three coastal and two inland villages of Campalagian district, South Sulawesi, in July 2002 and found that infection rate for *E. histolytica* was 10.9 % [50]. A study was conducted in Jakarta on 903 women workers before going abroad through stool examination by Ritchie's technical method. Of the women workers studied, 640 subjects were found to be infected with intestinal parasites of either helminth, protozoa or combination [51]. Out of these infected, 14.5 % of them were infected with *E. histolytica*. This study revealed that various life style, habits and indiscriminate defecation were the courses of the continuous transmission of intestinal parasitic infections.

6.3.8 Vietnam

The use of wastewater and human animal excreta in agriculture and aquaculture continues to be common in China, South and Southeast Asia as well as various areas in Africa [52] in particular where water scarcity is becoming more severe. The main sources of water for irrigation in Vietnam are fresh water, wastewater and ground water. In Hanoi, about 80 % of vegetable production is from urban and peri-urban areas irrigated with diluted wastewater [53]. The use of household sewage and human and animal excreta in agriculture and aquaculture has a long tradition in Vietnam [54]. Despite the potential health risk for intestinal disease of using excreta and animal waste in agriculture [55], 85 % of farmers in the northern provinces of Vietnam regularly use human excreta in agriculture [56]. A study in Hanoi on the epidemiology and aetiology of diarrhoeal diseases in adults engaged in wastewater-fed agriculture and aquaculture showed that the diarrhoeagenic *Escherichia coli*

and *E. histolytica* were the most common pathogens [57]. To further understand the transmission of *E. histolytica* infection, Pham Duc et al. [58] conducted a case–control study to access the importance of handling practices of human and animal excreta and wastewater use in irrigation in agriculture and aquaculture, in relation to other potential risk factors, including sanitary conditions, drinking water, food consumption and personal hygiene practices. In their study, 31 cases (67.4 %) were found in Nhat Tan and 15 cases (32.6 %) in Hoang Toy commune. Only a few study participants reported gastrointestinal symptoms, 11 cases (23.9 %) and 17 controls (12.3 %). In 2002, the distribution of amoebic liver abscess (ALA) cases was analysed in the province of Thua Thien Hue in Central Vietnam, a region known for its high incidence of invasive amoebiasis [59, 60]. Based on the analysis of hospital charts from April 1990 to April 1998, 2,031 cases of ALA were identified, indicating an ALA incidence of at least 21 per 100,000 inhabitants per year. Incidence varied substantially between the various districts of TT Hue and directly correlated with population density. The risk of ALA was significantly higher in summer and was age and sex dependent because 95 % of the cases were adults, of which more than 80 % were males. There was no clustering of cases within households, and recurrent cases of ALA occurred more frequently than predicted in the study population. Despite the higher incidence of ALA in males, the parasitological and seroepidemiological survey revealed a significantly higher infection rate for intestinal protozoan parasites, including *E. histolytica* in females. In the following year, a longitudinal study was performed by Blessmann et al. [61] over an observation period of 15 months with a group of 383 randomly selected adult individuals living in Central Vietnam. The results indicated an *E. histolytica* prevalence of 11.2 % and an annual new infection rate of 4.1 % in the study population. Follow-up of the 43 individuals who were *E. histolytica* positive at enrolment suggested a regular exponential decline in infection of about 3 % per month and a mean half-life of infection of more than 15 months.

6.3.9 Malaysia

The prevalence of *E. histolytica*/*E. dispar* in Malaysia has been reported by many researchers, as far back from the 1960s; the prevalence ranged from 1 to 83 %. The prevalence varied with the population studied, whether preservatives were used for stool collections, frequency of stool collected for examination and technique used in the detection of the protozoa. In the early 1980s, Noor Hayati et al. [62] and Hamimah et al. [63] reported 3.4 % and 2.3 % of children admitted with gastroenteritis symptoms excreted cysts of *E. histolytica*/*E. dispar*, respectively. The prevalence was in agreement with findings reported earlier by Bolton [64]. A recent study by Sinniah et al. [65] reported 1.3 % of positive case for *E. histolytica*/*E. dispar* among three Orang Asli communities in Perak. A recent study carried out among population living in the tropical highland and mountainous area in Sabah showed a high prevalence of *E. histolytica*/*E. dispar* (21 %) [66]. Only one stool specimen was collected from each subject, and the stools were not fixed. If stools

were collected more than once and fixed in preservative, a higher prevalence of *E. histolytica*/*E. dispar* would be expected in their study. A later study performed among school children in Sabah confirmed that *E. histolytica*/*E. dispar* infection was common, with prevalence of 83.8 %. In their study, formalin–ether concentration method was performed to enhance the detection of cysts [67].

In peninsular Malaysia, many recent studies reported high prevalence of *E. histolytica*/*E. dispar*. Recent study conducted by Shahrul Anuar et al. [68] reported a high prevalence of *E. histolytica*/*E. dispar* infection among three aborigine tribes in peninsular Malaysia which were 8.7 % (Proto-Malay), 29.5 % (Negrito) and 18.5 % (Senoi). Likewise, a study carried out among Pos Piah reported 11.5 % of population studied were infected with *E. histolytica*/*E. dispar* [69]. In their study, stools were collected once and fixed in PVA. A study by Noor Azian et al. [70] among aborigine population also reported high prevalence of *E. histolytica*/*E. dispar* (18.5 %). Hartini and Mohamed Kamel [71] reported 22.5 % of Orang Asli children studied harboured *E. histolytica*/*E. dispar* cysts. On the other hand, the prevalence of *E. histolytica*/*E. dispar* was very low in the urban community of Malaysia. A study by Jamaiah and Rohela [72] among the public in Kuala Lumpur reported a very low prevalence (0.4 %).

In Malaysia, no attempt was made to differentiate cysts and trophozoites of morphologically identical *E. histolytica*, *E. dispar* and *E. moshkovskii* previously. This is because all community and hospital studies were based entirely on microscopic examination of fresh stool samples for parasite identification. Very little information is available on the prevalence of the two or three *Entamoeba* species. To the best of our knowledge, there are currently only three community studies that have been carried out in Malaysia to differentiate the *Entamoeba* species. Anuar et al. [73] reported a high prevalence of *E. dispar* (13.4 %), as compared to *E. histolytica* (3.2 %) and *E. moshkovskii* (1 %), by using single-round PCR assay. In contrast, Ngui et al. [74] and Noor Azian et al. [75] reported a high prevalence of *E. histolytica* which were 75 % and 13.2 %, respectively, among aborigine community by using nested PCR. Their findings contradicted most studies as reports usually documented higher prevalence of *E. dispar* than *E. histolytica*.

The earliest reports on invasive amoebiasis in Malaysia were by Chellappa and Rangabasham [76], Balasengaram [77] and Goh et al. [78]. A case report by Manukaran et al. [78] revealed a rare clinical presentation of intestinal amoebiasis with multiple colonic perforations and ruptured liver abscess in a 43-year-old Indian labourer. Goh et al. [78] in their study had reviewed 204 cases of liver abscess seen between 1970 and 1985 in the University Hospital, Kuala Lumpur; the findings showed 44.1 %, 11.8 % and 0.5 % of liver abscess were classified as amoebic, pyogenic and tuberculosis. The cause of liver abscess in the remaining of 43.6 % was not established. This study also reported fever with chills and rigours; right hypochondrial pain and tender hepatomegaly were the most common clinical presentations of ALA. Almost 87 % of ALA seen in this study is a single abscess and mostly located in the right lobe. The patients were predominantly males, Indians and in the 30–60 age group. A 10-year retrospective study on amoebiasis was also carried out in the University Hospital, Kuala Lumpur, between 1984 and

1994 by Jamaiah and Shekar [80]; of 51 amoebiasis cases traced, 30 (59 %), 20 (39 %) and 1 (2 %) were amoebic dysentery, ALA and combination of amoebic dysentery and ALA, respectively. Most of the cases were reported in Malays, majority in males and unemployed. The most common clinical presentations were diarrhoea and dysentery. Trophozoites of *E. histolytica* were only identified in 13 (43 %) and 9 (30 %) of stool and intestinal biopsy of amoebic dysentery patients. Only one out of 20 ALA cases showed trophozoites in the stool and biopsy [80]. These findings showed the difficulty in isolating trophozoites from clinical specimens; thus, antigen or antibody test and PCR are very useful to confirm the disease. A latest 10-year retrospective study in the same hospital showed a decreasing trend of invasive amoebiasis as compared to the study by Goh et al. [78] and Jamaiah and Shekar [80]; only 34 cases were traced and analysis showed ALA was the commonest presentation with 22 (65 %) cases. Amoebic dysentery was seen in 12 (35 %) patients [81]. The clinical presentations were almost similar with the study carried out by Goh et al. [78].

6.4 Transmission

Humans acquire infections with amoebiasis via ingestion of food or water contaminated with faeces containing *E histolytica* cysts. The main sources of infection are human carriers or cyst passers. Food and drink may become contaminated with cysts of *E. histolytica* by polluted water supply, dirty handling by infected individuals, infected food handlers, contamination by houseflies and cockroaches, use of night soil in vegetable plots and poor personal hygiene in orphanages, prisons and mental hospitals where crowding and problems with faecal contamination are contributing factors. Transmission of *E. histolytica* by water is common when the population depend on untreated wells and rivers.

Unusual modes of transmission among male homosexuals include oral and anal sex. Cases of amoebiasis transmitted via contaminated enema apparatus have also been reported.

Amoebiasis can also spread within families, so household contacts of patients with the disease should have their stools screened for *E. histolytica*. The higher prevalence in areas of lower socioeconomic status is likely due to poor sanitation. *E. histolytica* is rarely the cause of travellers' diarrhoea and is usually associated with long-term (>1 month) stays in an endemic area.

6.5 Morphology

See Figs. 6.1 and 6.2.

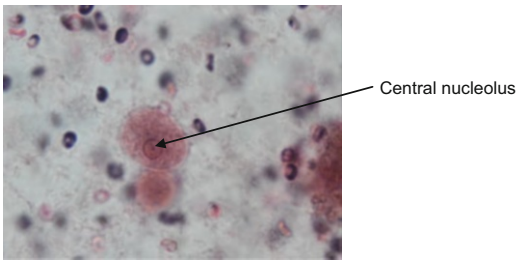


Fig. 6.1 Trophozoite of *Entamoeba histolytica* in stool smear showing central nucleolus (magnification ×100, stained with iron haematoxylin). *Entamoeba histolytica/E. dispar* trophozoites have a single nucleus with a centrally placed karyosome and uniformly distributed peripheral chromatin. The cytoplasm has a granular or "ground-glass" appearance. *E. histolytica/E. dispar* trophozoites measure 15–20 μm (range 10–60 μm). Erythrophagocytosis (ingestion of red blood cells by the trophozoite) is the only morphological characteristic that can be used to differentiate *E. histolytica* from the nonpathogenic *E. dispar*

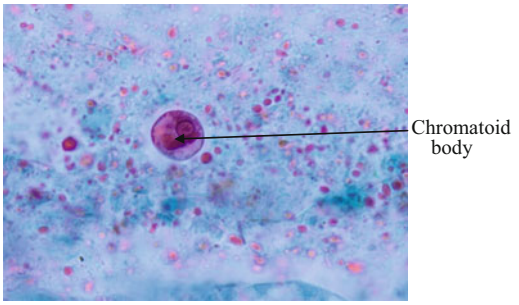


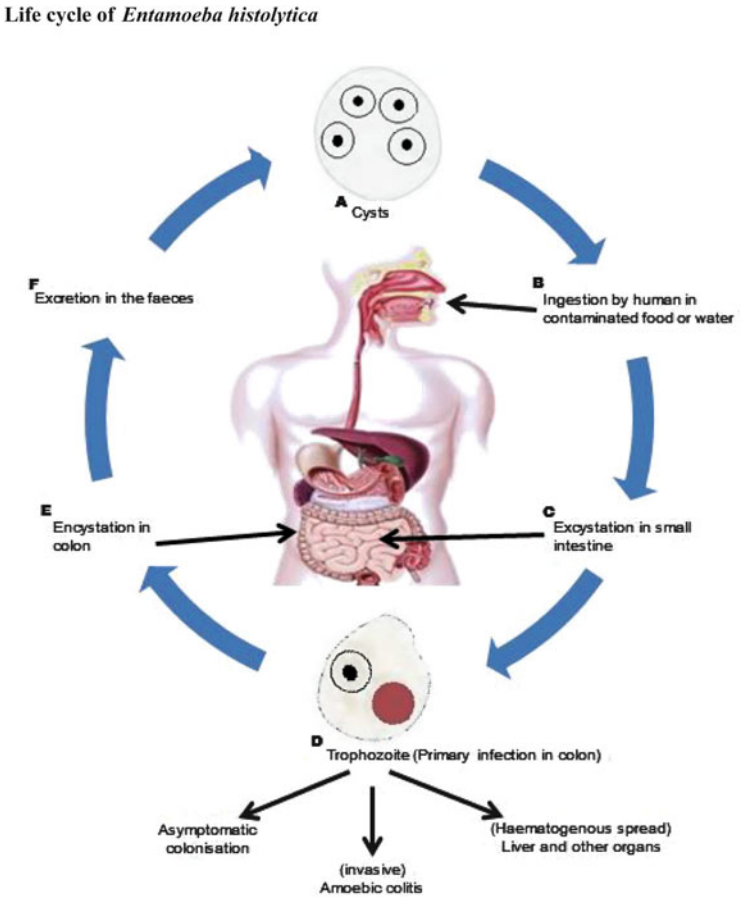
Fig. 6.2 Cysts of *Entamoeba histolytica* showing chromatoid body (magnification ×100, stained with trichrome). A mature *E. histolytica/E. dispar* cyst is round and has a maximum of 4 nuclei with a characteristically centrally located karyosome and fine, uniformly distributed peripheral chromatin. Cyst measures 12–15 μm. A premature cyst has a chromatoid body with blunt, rounded ends (*arrow*). As the cyst matures the chromatoid body disappears

6.6 Culture

Axenic cultivation (bacteria-free culture) of *E. histolytica* was achieved by Diamond in 1961. Later methods of mass cultivation of different strains of *E. histolytica* and *E. histolytica*-like amoebae were developed for studies on physiology, pathogenicity and immunology. Diamond [82] did an axenic cultivation of *E. histolytica*. Since that time many improvements have been made in the culture media and the technical procedures for handling the amoebae. He used a medium that does not require supplementation with a cell-free extract of chick embryo. A clear liquid medium is used for initiation, maintenance and mass cultivation of the amoebae. This medium consists of trypticase, Panmede (ox-liver digest), glucose, cysteine, ascorbic acid and salts supplemented with horse serum and a vitamin mixture [82].

Amoebic cultures are not commonly employed in routine diagnosis. The usefulness of *in vitro* cultivation of *E. histolytica* has been in the study of its metabolism, pathogenicity, testing of anti-amoebic drugs *in vitro* and the production of antigen for use in immunodiagnosis of amoebiasis.

6.7 Life Cycle of *Entamoeba histolytica*



E. histolytica exhibits a life cycle consisting of infective cysts passed in faeces of convalescents and carriers. The cysts remain viable under moist condition for about 10 days. Humans acquire the infection via ingestion of the cysts. The risk factors are similar to other diseases transmitted by the faecal–oral route. The sources of

infection are probably contaminated food and water. Upon ingestion, the cysts pass through the stomach undamaged and excyst in the small intestine. Excystation involves rupturing of the cyst wall, and the quadrinucleate amoeba emerges through the opening. This stage is called the metacyst. The metacyst undergoes nuclear division to produce eight uninucleated trophozoites, sometimes called amoebulae. These trophozoites colonise the large intestine, especially the caecal and sigmoidorectal regions. They feed on bacteria and cellular debris and undergo repeated cycles of binary fission. *Entamoeba* trophozoites are obligate fermenters and lack enzymes of the tricarboxylic acid cycle and proteins of the electron transport chain. In keeping with anaerobic metabolism, *E. histolytica* lacks mitochondria and only has a mitochondrial remnant called a mitosome. The parasite appears to have obtained its metabolic enzymes through lateral gene transfer from bacteria. Some trophozoites undergo encystation and develop into precystic forms and cysts which are passed in faeces to repeat the cycle. The entire life cycle is completed in one host.

6.8 Pathogenesis

The amoebae that live in the lumen of the large intestine do not cause symptoms. They may invade the intestinal mucosa and cause disease. The other most commonly affected tissue of the human body is the liver, but nearly every organ of the body may be affected. Not all strains of *E. histolytica* are pathogenic or invasive. The pathogenic *E. histolytica* produces virulence factors and include:

(i) Amoebic lectin which is a 260 kDa surface protein galactose-inhibitable adherence lectin. It mediates in the adherence of amoebae to the intestinal mucosa.
(ii) Ionophore-like protein which causes leakage of ions from the target cells.
(iii) Hydrolytic enzymes that cause proteolytic destruction of the tissue.
(iv) Toxins and haemolysins.

Based on electrophoretic mobility of five isoenzymes, *E. histolytica* strains can be classified into at least 22 zymodemes, of which only seven are invasive or pathogenic and the rest are noninvasive or nonpathogenic. The zymodemes demonstrate a geographical distribution. Nonpathogenic zymodemes are more common in comparison to pathogenic ones (Ω 10 % of total population) even in endemic areas [83, 84].

Virulence may also be conditioned by bacteria in the colon which may stimulate the invasive process of the amoeba or produce favourable condition for invasion. The outcome of infection may be influenced by host factors such as stress, malnutrition, alcoholism, corticosteroid therapy and immunosuppression.

Invasion of the mucosa happens when trophozoites adhere to mucins of the colon and epithelial cells, and it is mediated by a 260 kDa surface protein galactose-inhibitable adherence lectin. It is responsible for adherence of

trophozoites to galactose receptors found in the intestinal mucosa. After adherence, the trophozoites lyse the target cells via the ionophore-like protein.

The trophozoites penetrate the colonic mucosa facilitated by the tissue lytic substances released by the amoebae. This will cause damage to the mucosal epithelium and is aided by the motility of the trophozoite. It produces discrete ulcers with pinhead centre and raised edges. The mucous membrane between ulcers remains healthy. The lesion is confined to the mucosal epithelium and may spread laterally. Lesions may heal without any effects. Sometimes, amoebae may invade the submucosal layer causing lytic necrosis leading to classic flask-shaped ulcers of amoebiasis which has a crater-form appearance, wide base and narrow opening with overhanging edges. The ulcers are confined to the colon more often seen in the caecal and recto-sigmoid junction where the colonic flow is slow. The amoebae may be found in the base of the ulcer. Erosion of blood vessels may cause haemorrhage. The ulcers may perforate the muscular and serous coats of the colon causing peritonitis.

Occasionally a chronic ulcer may give rise to a granulomatous growth on the intestinal wall causing amoebic granuloma or amoeboma which is sometimes mistaken for a malignant tumour, or tuberculous or actinomycotic granulomas. It may be diagnosed by biopsy, serological tests or by response to anti-amoebic therapy.

From the colon, amoebae are often carried by the portal blood circulation to the liver, the right lobe being more affected. Further dissemination from the liver to other organs such as the lungs, heart and brain may take place.

In the liver the amoebae cause lytic necrosis leading to amoebic liver abscess. Amoebic trophozoites lyse neutrophils and hepatocytes. The centre of the abscess contains thick chocolate-brown pus (anchovy sauce pus). The size of the abscess varies from a few millimetres to several centimetres. It is bacteriologically sterile and free of trophozoites. The amoebae are found in the wall of the abscess. More than 80 % of the abscesses are confined to the upper right lobe of the liver, and more often the abscess is solitary. Males are chiefly affected.

Pulmonary amoebiasis is infrequent and usually occurs as a direct extension of a liver abscess in the lower right lung. Amoebic abscess of the brain is rare and is usually associated with liver and pulmonary amoebiasis.

6.9 Immunity

Invasive amoebiasis induces both humoral and cellular immune responses. The humoral immunity can be demonstrated within a week of invasive amoebiasis by the presence of circulating antibodies in the serum. IgG antibodies are predominantly produced. Cell-mediated responses appear to limit the extent of invasive amoebiasis, protecting the host from recurrence following successful treatment. It is noteworthy that HIV infection has not led to an increase in invasive amoebiasis.

6.10 Clinical Symptoms and Signs

6.10.1 Intestinal Amoebiasis

Infection with intestinal amoebiasis may be asymptomatic, and the infection subsides without any signs of disease. About 4–10 % of asymptomatic individuals may develop disease over a year [85]. Patient with amoebic colitis presents with diarrhoea, abdominal pain and tenderness. The onset is often gradual, with patients reporting several weeks of symptoms. Profuse, watery diarrhoea might be noted. *E. histolytica* may invade the colonic mucosa, and even if no blood is seen, occult blood in stools is almost always positive. White blood cells can be present in the stool, and in severe cases pus can be visible. Fever is unusual (<40 % of patients); weight loss and anorexia can be present. Sometimes, patients may develop fulminant amoebic colitis, with profuse bloody diarrhoea, fever, pronounced leucocytosis and widespread abdominal pain. Paralytic ileus and colonic mucosal sloughing may be seen, but intestinal perforation (in >75 % of individuals with fulminant amoebic colitis) often dominates the clinical picture. Mortality with fulminant amoebic colitis is higher than 40 % in most series. Pregnant women, immunocompromised individuals and patients receiving corticosteroids are especially at risk of fulminant disease, and associations with diabetes and alcohol use have also been reported. Toxic megacolon complicates amoebic colitis. Amoebomas—localised inflammatory annular masses that develop in the caecum or ascending colon—can cause obstructive symptoms and can be misdiagnosed as carcinomas [85].

6.10.2 Amoebic Liver Abscess

Amoebic liver abscess is the most common complication of intestinal amoebiasis. It arises from haematogenous spread of amoebic trophozoites that have invaded the colonic mucosa but at times occurs by direct extension. Some patients presenting with amoebic liver abscess have concurrent amoebic colitis, but more often they have no bowel symptoms. Stool microscopy is usually negative for *E. histolytica* trophozoites and cysts.

Patients can present with amoebic liver abscess months to years after travel or residency in an endemic area. Amoebic liver abscess should be suspected in patients with a history of exposure (residency or travel to an endemic area). Patients present with fever, right upper quadrant pain and hepatic tenderness. Cough may be present, and dullness and rales in the right lung base may be present. Jaundice is uncommon. Symptoms are usually acute (<10 days in duration) but can be chronic, with features of anorexia and weight loss. Leucocytosis without eosinophilia, mild anaemia, raised alkaline phosphatase level and a raised erythrocyte sedimentation rate are common laboratory findings [85].

6.10.3 Other Extraintestinal Amoebiasis

Pulmonary amoebiasis may occur following extension of liver abscess through the diaphragm, and the lower part of the right lung is usually affected. Patients develop cough, pleuritic chest pain and respiratory distress. This complication occurs in 7–20 % of patients with amoebic liver abscess. If a hepatobronchial fistula develops, patients can cough out reddish-brown pus containing necrotic material. In 2–7 % of individuals with amoebic liver abscess, rupture into the peritoneum may cause peritonitis. A rare complication is when the abscess ruptures into the pericardium, which can cause pericarditis, and the mortality is very high. Amoebic brain abscesses are very rare and result from haematogenous spread from amoebic lesions in the colon or other sites. Sudden onset of symptoms (headache, vomiting, seizures and mental status changes) and rapid progression to death have been reported in almost half of the patients with brain abscesses, and metronidazole therapy has improved its outcome [85].

6.11 Diagnosis of Amoebiasis

6.11.1 Microscopy

Amoebiasis diagnosis test is the demonstration of *E. histolytica* trophozoites or cysts in stool or colonic mucosa of patients. For many years, a direct smear examined either as a wet mount or fixed and stained was done by microscopic examination of stool. Repeated stool sample examinations (at least three) may be needed. The presence of haematophagous amoebic trophozoites in a stool sample has always suggested *E. histolytica* infections [86]. Nonetheless, the specificity of this finding was further reduced when it was demonstrated that in some patients, trophozoites of *E. histolytica*/*E. dispar* also contains red blood cells [87]. Also, in view of the high frequency of *E. dispar* in many areas, dysentery due to entities such as shigellosis will probably be misdiagnosed as amoebic colitis if microscopy is the sole diagnostic criteria [85]. However, in the absence of haematophagous trophozoites, the sensitivity of microscopy is limited by its ability to distinguish between samples infected with *E. histolytica* and the morphologically identical *E. dispar* and *E. moshkovskii*. Confusion between *E. histolytica*, other nonpathogenic amoeba and white blood cells such as macrophages and polymorphonuclear cells in stool frequently results in the overdiagnosis of amoebiasis. Delays in the processing of stool samples affect the sensitivity of light microscopy, which under the best circumstances is only 60 % of that of the stool culture method followed by isoenzyme analysis [88].

As *Entamoeba* trophozoites generally degenerate rapidly in unfixed stool samples and refrigeration is not recommended, samples should be preserved with a fixative which prevents the degradation of the morphology of the parasite and allows concentration and permanent smears to be performed. Fixatives used for

the concentration procedure include Schaudinn's fluid, merthiolate–iodine–formalin, sodium acetate–acetic acid–formalin (SAF) or 10 % formalin. The fixatives for the permanently stained smears (trichrome, iron hematoxylin, Ziehl–Neelsen stains) include modified polyvinyl-alcohol (PVA) and SAF.

6.11.2 Culture and Isoenzyme Analysis

Stool culture technique followed by isoenzyme analysis has been considered as the "gold standard" for many years. This method has been used to distinguish between *E. histolytica* and *E. dispar*. Culture of *E. histolytica* can be performed from stool samples, rectal biopsy specimens or liver abscess aspirates. However, the process usually takes between 1 and 4 weeks to perform and requires sophisticated laboratory equipment, making it not feasible as a routine procedure especially in the developing world where *E. histolytica* is rampant. The rate of success of *E. histolytica* culture in reference laboratories has been reported to be between 50 and 70 %. Moreover, isoenzyme analysis is labour intensive and costly and often produces false-negative results for many microscopy-positive stool samples [89].

6.11.3 Antibody Detection Tests

Serological methods may be useful diagnostically to detect infections with *E. histolytica* in developed countries where infections are not as common as in endemic developing nations [90]. In developing countries, individuals are constantly exposed to *E. histolytica* making serological tests unable to definitively distinguish past from current infections [91]. Amoebic serology is highly sensitive and specific for the diagnosis of amoebic liver abscess (ALA) [92]. Conversely, a study of asymptomatic individuals living in an *E. histolytica* endemic area of Vietnam revealed that about 83 % of those infected had detectable anti-amoebic antibodies [59, 60]. Several assays for the detection of antibodies to *E. histolytica* infections have been developed. These include indirect haemagglutination (IHA), latex agglutination, immunoelectrophoresis, counterimmunoelectrophoresis (CIE), the amoebic gel diffusion test, immunodiffusion, complement fixation, indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA). With the exception of ELISA, all the other tests have been either costly to perform (complement fixation), less sensitive and nonspecific (IHA and latex agglutination test), time consuming (immunodiffusion) or requires skills in culture and antigen preparation [87].

ELISA is a reliable, easy to perform and rapid method for the diagnosis of *E. histolytica* infections especially in developing countries. It has been used widely for the study of the epidemiology and diagnosis of symptomatic amoebiasis (intestinal or extraintestinal). An ELISA to detect antibodies to *E. histolytica* has been shown to be 97.9 % sensitive and 94.8 % specific for detection of *E. histolytica*

antibodies in ALA patients in a non-endemic country [93]. Unlike immunoglobulin G (IgG), IgM is short lived and does not remain in the serum for longer periods making it a very useful marker for the detection of present or current *E. histolytica* infections. An ELISA for the detection of serum IgM antibodies to the amoebic, Gal or GalNAc-inhibitable adherence lectin has been reported. A study conducted in Egypt reported that anti-lectin IgM antibodies in the serum were detected in 45 % of patients who had been suffering from acute colitis for <1 week [94]. Since there was no cross-reaction with other non-*E. histolytica* parasites, the use of ELISA thus seems to be an excellent choice for the routine laboratory diagnosis as well as the surveillance and control of amoebiasis in the developing world.

6.11.4 Antigen Detection Tests

Several investigators have developed ELISAs that detect antigens in fresh stool samples with sensitivity closer to that of stool culture methods and polymerase chain reaction (PCR). These ELISAs are usually easy and rapid to perform. Coproantigen-based ELISA kits specific for *E. histolytica* exploit monoclonal antibodies against the Gal/GalNAc-specific lectin of *E. histolytica* (E. histolytica II, TechLab, Blacksburg, VA) or against serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin Diagnostika, Bornheim-Hersel, Germany). Other ELISA kits include the *Entamoeba* CELISA PATH kit (Cellabs, Brookvale, Australia) and the ProSpecT EIA (Remel Inc.; previously manufactured by Alexon-Trend Inc., Sunnyvale, CA) [87]. The early 1990s of the twentieth century have witnessed the introduction by TechLab of an ELISA kit for the specific detection of *E. histolytica* in stool. This antigen detection test captures and detects the parasite's Gal/GalNAc lectin in stool samples. It can also be used for the detection of the lectin antigen in the serum and liver abscess in patients with invasive intestinal amoebiasis and ALA [95]. However, the diagnosis of ALA normally relies on the identification of liver lesions and positive anti-*E. histolytica* serology. Yet, neither provides conclusive results for ALA. The Gal/GalNAc lectin is conserved and highly immunogenic, and because of the epitopic differences in the lectins of *E. histolytica* and *E. dispar*, the test enables specific identification of *E. histolytica* [96]. Because of some disadvantages observed with the TechLab ELISA kit, a newer more sensitive and specific version TechLab *E. histolytica* II kit was produced. This second-generation *E. histolytica* II kit has demonstrated good sensitivities and specificities when compared to real-time PCR [97, 98]. Other studies, however, have reported a lesser sensitivity (14.3 %) and specificity (98.4 %) in comparison to stool culture and isoenzyme analysis [99]. Cross-reactivity is another concern with the use of the assay, since it seems that *E. dispar*-positive samples by means of PCR may sometimes give false-positive outcomes [100]. Accurate detection of *E. histolytica*, *E. dispar* and *E. moshkovskii* could be helpful for diagnostic and epidemiological studies in places where it is impractical and expensive to use molecular assays and where amoebiasis is most prevalent, such as in the developing countries.

6.11.5 DNA-Based Diagnostic Tests

Several PCR-based techniques that amplify and detect *E. histolytica* DNA are currently used for the clinical and epidemiological studies in non-endemic-rich countries (Katzwinkel-Wladarsch et al. 1994; Calderaro et al. 2006) [32]. The sensitivity and specificity of PCR-based methods for the diagnosis of *E. histolytica* infection is as comparable as those of stool culture followed by isoenzyme analysis. PCR methods can be used to detect *E. histolytica* in stool, tissues and liver lesion aspirates. Of all the different gene targets used to identify *E. histolytica*, the small-subunit rRNA gene (18 ssu rRNA) is believed to be more sensitive than the best antigen detection method used and performs equally well compared to stool culture [101].

Several groups have developed a variety of excellent conventional PCR assays, targeting different genes, for the direct detection and differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii* DNA in clinical specimens such as stool and liver abscess samples [102] (Paul et al. 2007). Of all the targeted genes, assays amplifying the 18 ssu rRNA genes are the ones in wide use as they are present in multiple copies on extrachromosomal plasmids, thus making them easily detectable than single-copy genes [103]. Other gene targets used in PCR to study the epidemiology of *E. histolytica* include the serine-rich *E. histolytica* protein (SREPH) gene [104], cysteine proteinases gene and actin genes [105]. The SREHP is also used to study the genotypes of *E. histolytica* in human populations. However, it is now being replaced by the use of PCR amplification of tRNA gene-linked short tandem repeats which, in addition to providing details of the epidemiology of *E. histolytica*, also provides a tool to predict the outcome of the infection.

A nested multiplex PCR was developed by many groups. This method has the added advantage of increasing the sensitivity and specificity of the test while simultaneously detecting and differentiating *E. histolytica* and *E. dispar* from DNA extracted from microscopy-positive stool specimens [106, 107]. A nested PCR method for the identification of *E. moshkovskii* in stool samples was developed as a nested 18 ssu rRNA PCR followed by restriction endonuclease digestion [108]. The method exhibited a high sensitivity and specificity (100 %).

Real-time PCR is another type of PCR which is more sensitive than the conventional PCR. It is faster than the conventional PCR and characterised by the elimination of gel analysis and other post-PCR analysis, thus reducing the risk of contamination and cost [109]. However, its application in developing countries is limited to research only. Real-time PCR allows specific detection of the PCR product by binding to one or two fluorescence-labelled probes during PCR, thereby enabling continuous monitoring of the PCR product formation throughout the reaction. Furthermore, real-time PCR is a quantitative method and allows the determination of the number of parasites in various samples [87]. Despite being used for the successful identification of *E. histolytica*, *E. dispar* and *E. moshkovskii*, the various PCR methods used are still confined to research institutes in the developing world where amoebiasis is endemic. PCR-based method application

in routine clinical diagnostic laboratories in low-income societies is hindered by difficulties such as cost and time to perform the test.

6.11.6 Loop-Mediated Isothermal Amplification

A new platform for the detection of pathogens has been developed known as loop-mediated isothermal amplification (LAMP) and was developed in 2000 by Notomi et al. [110]. This method uses a set of two specifically designed inner primers and two outer primers that recognise six distinct regions of the targeted DNA. The reaction is performed under isothermal conditions, and simple incubators, such as a water bath or heat block, are adequate for the specific amplification of the desired genetic material. Considering these advantages, the LAMP assay could be a useful and valuable diagnostic tool particularly in developing countries where most of the infections are common as well as in hospital laboratories. Recently, this method was developed specifically for the detection of *E. histolytica* [111]. The efficiency of the developed method was compared to that of existing PCR methodology and was similar in terms of sensitivity and specificity.

6.12 Treatment

6.12.1 Intestinal Amoebiasis

The treatment for amoebiasis is the nitroimidazole derivatives (metronidazole, tinidazole, ornidazole). Amoebic colitis is treated with metronidazole (both luminal and tissue amoebicides), followed by a luminal agent (paromomycin, iodoquinol or diloxanide furoate) to eradicate colonisation. Fulminant amoebic colitis, even with perforation, is managed conservatively, with the addition of antibiotics to deal with bowel flora. Asymptomatic patients should be treated with a luminal agent to eradicate infection. *E. dispar* infection does not require treatment. The physician must be aware that the infected person has been exposed to faecally contaminated food or water. The drugs for the treatment of amoebiasis are generally effective with minimal toxicity.

6.12.2 Amoebic Liver Abscess

Amoebic liver abscess can be cured without drainage. Surgical drainage of uncomplicated amoebic liver abscesses is generally unnecessary and should be avoided.

Metronidazole treatment alone can cure amoebic liver abscess. Most patients show a response to treatment (reduced fever and abdominal pain) within 72–96 h [85].

Individuals with amoebic liver abscess should also be treated with a luminal agent to eliminate intestinal colonisation. Aspiration can be carried out if (1) diagnosis is uncertain (where pyogenic abscess or bacterial superinfection of the amoebic liver abscess is a concern), (2) patients have not responded to metronidazole therapy (persistent fever or abdominal pain after 4 days of treatment), (3) large left-lobe abscesses (because of the risk of rupture into the pericardium) and (4) large abscesses are present which make rupture seem imminent. Surgical drainage could be life-saving in the treatment of amoebic pericarditis.

6.13 Prevention and Control

Prevention and control measures are as for all other diseases transmitted by the faecal–oral route. The major difference is that humans are the only host for *E. histolytica* and there is no possibility of zoonotic transmission. Control is based on avoiding the contamination of food or water with human excreta. Health education in inculcating healthy personal habits, sanitary disposal of faeces and hand washing helps in control. Although waterborne transmission of *Entamoeba* is lower than other intestinal protozoa, protecting water supplies from being contaminated will lower endemicity and epidemics. Like *Giardia*, *Entamoeba* cysts are resistant to standard chlorine treatment, but are killed by iodine, boiling, dessication and freezing to below −5 °C. Sedimentation and filtration processes are quite effective at removing *Entamoeba* cysts. Chemoprophylaxis is not recommended.

6.14 Other *Entamoeba* Species Infecting Humans

Several other *Entamoeba* species in addition to *E. histolytica* and *E. dispar* infect humans. *E. hartmanni* and *E. coli* are two relatively common commensals found in human faeces. *E. moshkovskii* and *E. polecki* infections are generally rare. *E. histolytica/dispar*, *E. coli* and *E. hartmanni* can be distinguished by size and minor morphological differences. *E. coli* is the largest and is distinguished by eight nuclei in the mature cyst. The trophozoites of *E. coli* can be difficult to differentiate from *E. histolytica/dispar* since there is some overlap in the size ranges. *E. hartmanni* closely resembles *E. histolytica* and was previously considered a "small race" of the latter. Generally 10 mm is chosen as the difference in size between *E. histolytica* and *E. hartmanni*. *E. moshkovskii* is generally considered to be a free-living amoeba reported in environments ranging from clean riverine sediments to brackish coastal pools and sewers. Morphologically it is indistinguishable in its cyst and trophozoite forms from *E. histolytica* and *E. dispar*, and

phylogenetic analysis indicates that the three species form a clade, thus suggesting a common ancestor. *E. moshkovskii* can be distinguished by its ability to grow at ambient temperatures during *in vitro* culture, whereas *E. histolytica* and *E. dispar* need to be cultivated at 37 °C. *E. moshkovskii* has been isolated from human faecal samples, and in some of these cases, the patients are symptomatic. *E. polecki* is nonpathogenic and is usually associated with pigs and monkeys, but human cases have been occasionally reported. It appears to be geographically restricted to some parts of Southeast Asia particularly in Papua New Guinea where it is a common intestinal commensal. The trophozoites resemble that of *E. coli*, except a little smaller, and the cysts are similar to *E. histolytica* except that the mature cyst has a single nucleus. *E. gingivalis* which has no cyst stage is nonpathogenic and can be recovered from the soft tartar between the teeth, and its trophozoite exhibits a similar morphology to *E. histolytica*. *E. gingivalis* trophozoites will often contain ingested leucocytes which can be used to differentiate it from *E. histolytica*. The trophozoites are often recovered from patients with periodontal disease, but an aetiology between the organism and disease has not been established.

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Chapter 7
Romancing *Blastocystis*: A 20-Year Affair

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Abstract This review captures the surrounding enigmatic aspects of *Blastocystis* sp. perhaps one of the most fascinating organisms seen under the microscope and highlights the pioneering findings from researchers from Southeast Asian countries in almost all aspects of this parasite. The parasite's classification, biochemical properties, life cycle, prevalence, and genotypic and phenotypic characteristics including treatment have received conflicting opinions and suggestions. Like all organisms, the journey has been riddled with questions and even more questions. Our intimate association with the organism for the past 20 years has brought forth crucial information. Throughout this review, these findings along with results from other researchers from this part of the world will be unfolded in an attempt to fill the glaring gaps in all aspects of this parasite in the hope that the organism gains a greater global attention it deserves.

7.1 First Chromist Known to Parasitize Human

Outbreaks of diseases bring its share of health issues and challenges, but its major contribution is often the increased focus on the causative organism. This was the case with *Blastocystis* sp. seen in two respective outbreaks as early as 1849 in Britain [1] and America [2]. However, the identification of the organisms discovered was confined to describing them as annular and cholera bodies, respectively. Hence, like most new organisms, its taxonomy was controversial and remained confusing largely due to the limitation of tools such as a good microscope.

Efforts were made in trying to ascribe the organism to different groups such as from a coccidian [3] to a yeast [4]. Opinions changed and it was reclassified as a cyst form of *Trichomonas intestinalis*, *Chilomastix mesnili*, or *Endolimax nana*

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[4]. Then in the following year, Brumpt isolated the organism from a human and named it *Blastocystis hominis*. However, it got ascribed this time to a harmless yeast possibly in the genus of *Schizosaccharomyces* and *Saccharomyces* [5]. Subsequently, in 1938, it was proposed that *Blastocystis* sp. was an alga [6]. Almost 30 years later, Zierdt reclassified it as a protozoan according to its protozoan characteristic [22] only to be reclassified later as a phycomycete fungus, also a type of yeast [7]. In 1976, *Blastocystis* sp. was seen as a sporozoa, a protozoan without any locomotive body [8, 9].

However, it slipped back to being classified as a protozoan 7 years later when Zierdt proved through a series of experiments that the organism responded to antiprotozoal drugs [10]. It was then classified as a protozoan in the subphylum of Sarcodina [11]. Ten years later it was again reclassified according to the six-kingdom system of life [12]. Since it was devoid of cilia, *Blastocystis* was placed in a newly created class Blastocystea in the subphylum Opalinata, infrakingdom Heterokonta, subkingdom Chromobiota, and kingdom Chromista making the organism the first chromist known to parasitize humans.

Molecular studies by a group of Japanese scientists then recognized this organism as a stramenopile through the small subunit rRNA phylogeny study [13]. Till today it remains as a stramenopile or sometimes known as heterokont, which includes algae, diatoms, and also water molds [14]. It cannot be ascertained if in the future, the organism will be regarded as something else.

We deliberately began this review by highlighting the extent of the controversies and confusion surrounding the taxonomy of this organism let alone a host of other enigmatic issues which constantly seems to enshroud *Blastocystis*. Researchers in Southeast Asian countries in the early years gave rise to some of the most basic information on *Blastocystis* that laid a strong foundation which formed the basis of much of the research in the later years to be unleashed from other parts of the world. This review attempts to capture some of these highlights carried out in this part of the world that has contributed in the elucidation of some of the crucial aspects of this enigma that envelopes one of the most fascinating organisms ever to be seen under a microscope.

7.2 A Highly Polymorphic Unicellular Organism

Blastocystis sp. is a polymorphic organism that exists in four main forms, namely cyst, vacuolar, granular, and amoeboid. Other less frequently encountered forms are avacuolar and multi-vacuolar which have been reported previously [15].

7.2.1 Vacuolar Form

The earlier reference to vacuolar forms was the central body form [16]. This form is usually seen in *in vitro* cultures. Its size varies widely; it can grow from as small as 2 μm to as huge as 200 μm with an average size of 4–15 μm [15]. It contains a large vacuole occupying almost 90 % of the cell and a thin peripheral band of cytoplasm surrounding this large vacuole. The function of the organelle has been reported to be for storage, and often electron-dense materials have been seen within [17]. A previous report has also highlighted that portions of the cytoplasm do invaginate into the central vacuole, which results in the deposition of cytoplasmic material [18]. The vacuole has also been known to be a depository site for materials possibly apoptotic bodies or its reminiscent resulting from programmed cell death [19–21]. Organelles such as nucleus, Golgi apparatus, and mitochondria-like structures are located within the thin rim of cytoplasm. A surface coat of varying thickness, sometimes called a slime layer, is present [22]. These forms are commonly seen in *in vitro* cultures. Unusually small vacuolar forms have been noted in the Indonesian stool samples with sizes varying from 3 to 5 μm. These almost undetectable forms under the light microscopy were only evident when stained with acridine orange and survived at room temperature up to 9 days. The Bangladeshi and Malaysian isolates are generally large (i.e., between 10 and 15 μm) and survived only up to 5 days [23]. Size and shape also appears to be different depending on the site of isolation.

7.2.2 Granular Form

This form in our opinion is the most enigmatic form of all as this life cycle stage is seldom seen in other protozoa. Granular form of *Blastocystis* sp. arising from the vacuolar life cycle stages has been reported to be due to a variety of factors [15] which include keeping cultures for long periods without changing the medium [22] or when cultures are treated [24]. The deposition of granules within the central body confers the name granular form which appears for all purposes morphologically similar to the vacuolar stage. Granules have been suggested to be lipid, metabolic, and reproductive in nature [25]. There have been various other descriptions for the granules including small vesicles, lipid droplets, myelin-like inclusions, as well crystalline granules [26]. All these descriptions could be apt depending on the stains used or when visually viewed under light or phase microscopy. It was our conviction that these granules were reproductive in nature as the high parasite count within a short time could not justify the process of binary fission which was and is the only plausible mode of reproduction thus far accepted. Small grapelike clusters of progeny *Blastocystis* within a large organism under scanning electron microscope justified our claim [27]. Suggestion for progeny formation has been reported earlier when the organism was thought to undergo schizogony and endodyogeny [16,

22]. The many progeny of *Blastocystis* within a large cell of the organism were clearly demonstrated through acridine orange staining [28]. These were some of the earlier findings that pivoted a role for the granular form which in the life cycle described showed that the vacuolar and the granular forms existed predominantly both *in vitro* and *in vivo*. The former was more easily demonstrated and hence reported but the latter hardly. The authors went on to claim that these granular forms could also be the thin-walled cysts responsible for maintaining an infection internally. On hindsight, this could be true as the large numbers of parasites seen in symptomatic patients in infected stools cannot be justified if binary fission was the only mode of reproduction [29].

7.2.3 Amoeboid Form

Amoeboid form of *Blastocystis* sp. is usually not a commonly detected stage, and there is a lack of information on the factors that induce transition to this form. Nonetheless, this form is reported in old or drug-treated cultures and seen when grown in colonies [30, 31]. The distinct morphology for the identification of this form is the presence of one or more non-locomotive pseudopods.

The detection of these forms found occurring in older cultures, cultures treated with antibiotics, and occasionally in fecal samples [31] continues to increase the suspicion that these forms could be accidental occurrence in response to stress. The suggestion that these forms survive despite not having plasma membrane disqualifies it as a life cycle stage but more of an artifact arising from sample preparation [17] compounded the confusion. Hence, these discrepancies in the descriptions made it unclear if these forms were mere artifacts or accidental occurrence. One of the earliest confirmations of the existence of these forms came while observing transformational changes from vacuolar to cystic forms during the *in vitro* encystation experiment [32]. At 12 h in the encystment medium, most of the vacuolar forms were triggered to form the amoebic stage, implying that these forms were intermediate ones between vacuolar and cystic stages. Almost the same time, the observation that these forms could have arisen from avacuolar form was also made [15], and in later years the initial suggestion made was confirmed when amoebic-like forms that arose from colonies of vacuolar forms grown in agar plates were noted [20, 21]. It was reported that using both light microscopy and electron microscopy, these cells were irregular in outline and possessed distinct pseudopod-like extensions [20, 21].

The turning point on conferring a possible role for these forms and truly establishing its existence came from a fortuitous discovery of the predominant presence of amoeboid forms in cultures isolated from symptomatic patients [33]. The consistent presence seen only in cultures of isolates from symptomatic patients does imply a pathogenic role for this organism. Using transmission electron microscopy, two types of amoeboid form, one which contained a large central vacuole completely filled up with tiny electron-dense granules and the other,

instead of a large central vacuole, which had multiple small vacuoles in the cytoplasm, were identified. The cytoplasm showed strands of ribosome structure and a surface coat uneven with some parts five times thicker than the other with bacterial adherence [34]. It was later found that the amoeboid form may undergo plasmotomy [35].

7.2.4 Cyst

This is the most important life cycle stage as it is responsible for transmission. In the initial years when the organism was discovered, many cyst-like structures seen in stools were thought to be the cystic stage of *Blastocystis*. The desperate need to create animal models to study experimental *Blastocystis* infection as well as to elucidate biological properties of cystic stages triggered the need to carry out *in vitro* induction using an encystation medium [36]. This *in vitro*-encysted form could cause experimental infection and was one of the earliest evidences to implicate that the cystic stage was the transmissible form. The idea was extended to induce cystic forms of *B. ratti* [37] by varying serum concentration of culture medium.

The cystic structure for the first time was convincingly demonstrated from a stool sample of a *Blastocystis*-infected patient confirmed positive by the *in vitro* culture method [38]. The 3–5 μm-sized cyst forms are probably the reason for the delay in identifying this structure. The isolation method introduced for the first time is an important contribution that paved the way for other animal infection studies [39]. These ovoid structures have one to four nuclei and are usually surrounded with multilayered wall [15]. It must be noted that the size of the cysts of *Blastocystis* from humans differs from those seen in animals [40]. Cysts were shown not to lyse in distilled water [41] and can survive up to 19 days at room temperature [42]. Transformation during encystation seen *in vitro* [36] and *in vivo* [44] as well as excystation from cystic to vacuolar [37, 39, 43] has been described.

The recent painstaking *in vivo* observation of the encystation process captured in a time course study [44] in an infected human stool sample concurs with the *in vitro* encystation process from the vacuolar to the cystic [36] carried out almost 16 years earlier. The study also confirmed an earlier report [29] of the irregular shedding patterns of the vacuolar and cystic stages of the parasite implying that certain conditions such as diet habits, life style, drugs, and host immune response could have triggered the transformation of the vacuolar forms to the cystic forms of the parasite.

Stools were collected weekly for a period of 36 weeks from the same infected individual. The weeks between 25 and 28 offered the best opportunity to study encysting parasite as they were relatively more homogenous and synchronous to elucidate the transformational stages between vacuolar and cystic unlike previous

in vitro studies on encystation [36, 45], where the stages of *Blastocystis* would be usually not synchronous.

The following step-by-step transformational process was noted in their study:

1. Vacuolar forms: common forms seen in stools and *in vitro* culture.
2. Granular form: the formation of granules in the central body leading to the granular form of the parasite.
3. Amoeboid form: the parasite becomes irregular and transforms to the amoeboid form. This form allows the parasite to phagocytose bacteria that may be needed to provide nutrients for the encystation process.
4. The precystic form: the parasite attains a rounded shape; the cytoplasm becomes filled with ribosome-like particles and undergoes active protein synthesis for the formation of the cyst wall. A homogenous layer of immature cyst wall is formed around the parasite which differentiates into the precystic form.
5. The cystic form: dehydration of the cytoplasm occurs in the precystic form of the parasite, the immature cyst wall thickens and differentiates into three layers, and the fully developed cyst is formed. The cyst possibly contains reproductive granules, which are capable of developing into progeny *Blastocystis* that are released through the pore during excystation.

These observations provide a role for precystic form which should be included in the life cycle as a distinct life cycle stage. Such descriptions have been previously made on life cycle stages in stools [42, 46]; however, this study, based on proportion of the respective life cycle stages seen in stools at different time points of collection, provided a clear transformational sequence between vacuolar and cystic forms.

7.3 Striving to Understand Its Life Cycle

There have been many proposed life cycles for *Blastocystis hominis*, but these still remain inconclusive [15, 16, 28, 31, 47, 48]. Nonetheless, at this juncture, Centers for Disease Control and Prevention (CDC) has adopted the life cycle proposed by Singh et al. [28]. This is probably the only life cycle that incorporates the thick- and thin-walled cysts. The thick-walled cysts excreted in the feces are believed to be responsible for transmission by fecal–oral route, particularly through ingestion of contaminated water or food or possibly due to poor hand hygiene. While the thick-walled cysts have been previously shown, the granular forms suggested in the life cycle are the implicated thin-walled cysts and upon certain trigger releases the reproductive granules to form the vacuolar forms [49]. Vacuolar forms will then differentiate to multi-vacuolar and amoeboid forms. Multi-vacuolar forms develop into precysts that originate thin-walled cysts, which are responsible for autoinfection. Amoeboid forms give rise to precysts that develop into thick-walled cysts which then get excreted in feces [28]. The recent *in vivo* study on encystation [44]

reports of the exact sequence of events seen earlier and hence provides credible evidence for the life cycle proposed by Singh et al. [28].

7.4 Multiple Modes of Reproduction

The groups of researchers from Southeast Asia have been one of the strongest advocators that the organism does multiply other than the established binary fission. It is possible that this arises from their extensive observation over an extended period of time of the *in vitro* cultures they have had the opportunity to maintain for other studies. The suspicion that the parasite does multiply through other modes of reproduction which includes plasmotomy, endodyogeny, schizogony [16], and sporulation [50] was raised but there were no convincing evidence. The grapelike clusters seen as progeny *Blastocystis* under scanning electron microscopy were one of the earliest evidences of a multiple fission-like mode of reproduction [27]. The acridine orange stained form clearly demonstrated small progeny *Blastocystis* within a large vacuole. The evidence of multiple fission-like reproduction was again demonstrated using transmission electron microscopy on *Blastocystis* isolated for the first time from house lizards [51]. Here saclike pouches was formed to cushion these developmental processes. Exogenous protrusion of cytoplasm which appeared to pinch off from the side of the organism was reported as budding [52]. It was even thought that the nucleic acids were poured into the central body housing the granules to form the progeny [43, 53]. These claims were subsequently refuted as there was no substantial evidence provided for the progeny *Blastocystis* to have any genetic material [54]. Given the generation time of 9.3 h seen in isolates obtained from asymptomatic persons as the shortest time possible [55], it is difficult to justify the sharp rise in cultures seen often if binary fission was the only mode of the reproduction. Hence, this debate continues!

7.5 Apoptosis Is Subtype Related

Understanding apoptosis in *Blastocystis* has been one of the major contributions from scientists in Southeast Asian countries. They have been actively investigating programmed cell death (PCD) or apoptosis which has only been seen in two intestinal protozoa, namely *Blastocystis* sp. and *Giardia lamblia* [56]. Apoptosis in *Blastocystis* has demonstrated shrinkage of the cell, preservation of membrane integrity with increasing permeability, nuclear fragmentation, and externalization of plasma membrane phosphatidylserine (PS) residues [57]. Several of these features have been described in *Blastocystis* sp. exposed to cytotoxic drugs such as metronidazole [58] and a surface-reactive cytotoxic monoclonal antibody (1D5) [20, 21]. It was later found that apoptosis in *Blastocystis hominis* occurs independently of caspase and mitochondrial pathways [59].

In a more recent study, the significant elevation in the rate of apoptosis of isolates belonging to subtype 3 (*Blastocystis* ST3) in the treated condition showed that symptomatic isolates are more prone to apoptosis in comparison with asymptomatic isolates. This study has also reported a positive correlation between apoptosis and cell viability, since increased cells undergoing apoptosis show a corresponding increase in viable cells. Haresh et al. [60] showed that drug-treated parasites had a larger number of granular forms. These forms could have been implicated in the possible release of the reproductive granules and thus increases in the number of viable parasites in culture. It is highly probable that pathogenicity could be influenced by cell numbers. Perhaps that is why, as the apoptosis rate increases, cells that are becoming viable in subtype 3 (*Blastocystis* ST3) are significantly greater in comparison with isolates from other subtypes [61]. This further postulates that there is a mechanism involved in *Blastocystis* sp. that actually regulates the apoptotic process to produce a higher number of viable cells of the parasite in response to treatment. Further detailed research at the molecular level may need to be carried out to elucidate apoptosis mechanisms and the cell death pathway in *Blastocystis* sp. Apoptosis in unicellular organisms could be a defense mechanism for the whole population. The type of stimuli used to induce cell death needs to be studied further.

7.6 Diagnosis Is Challenging

The identification of *Blastocystis* sp. has been the greatest challenge as the size and shape has been likened to oil globules, yeast, and sometimes just an artifact when seen under light microscopy. The sensitivity, strength, affordability, and accessibility are some of the criteria involved when selecting the detection methods which currently include direct microscopy, *in vitro* cultivation, serology, and molecular techniques. In many laboratory settings in Southeast Asian countries, direct microscopy and/or *in vitro* cultivation is preferred over molecular methods as cost of service becomes the greatest concern. Staining is yet another technique to confer visibility during detection. Some of the stains used are Giemsa, Field's, and trichrome. Trichrome is by far the most popular stain employed as suggested by Tan [62]. It is also a better detection method as compared with wet mounts with higher sensitivity for detection when *in vitro* cultivation is used as the gold standard. In the same work, it is reported that the 95 % confidence interval of the area under the ROC curve for trichrome staining was significantly higher than 0.5 as compared with wet mounts. Usually wet mount preparation and staining together create better accuracy for detection. However, these methods are tedious and time consuming, and often to an untrained eye, there are possibilities that *Blastocystis* can be missed. Some of the earliest studies to compare the sensitivity of this method with an *in vitro* method using Jones' medium showed a threefold [63] and fourfold [51] difference favoring the *in vitro* method.

For diagnostic purposes, *Blastocystis* sp. from fecal materials are often cultured in Jones' medium [64] or Boeck and Drbohlav Locke-egg-serum medium [65] for the growth of xenic culture in the presence of unknown microorganisms. In most laboratories, Jones' medium has been the preferred medium for the cultivation of *Blastocystis* sp. from human fecal samples for easy identification of the vacuolar form of the parasite [66–71]. However, it was reported that *Blastocystis* sp. isolated from Australian marsupials [67] as well as from dogs and cats (unpublished observation) could not grow in Jones' medium [67]. Hence, it is likely that different culture medium other than Jones' should be attempted.

For axenic culture, a pure culture of *Blastocystis* sp. alone in the absence of any microorganism is grown in media such as Iscove's modified Dulbecco's medium (IMDM), medium essential medium (MEM), Roswell Park Memorial Institute (RPMI) 1640, and basal minimal essential (Eagle's) medium (BME) [72, 73]. According to American Type Culture Collection (ATCC), Stone's modification of Locke's solution (ATCC medium 1671) should be used for the axenic culture of *Blastocystis* sp. In another study, IMDM has been suggested to be the medium of choice for axenic culture of *Blastocystis hominis* [72].

Serology could have been another technique of choice; however, given that currently a total of 13 different subtypes have been reported [74–76], vast antigenic variations for each *Blastocystis* sp. subtype may limit the usage of this technique. Hence, serological kit if developed must take into consideration these variations.

7.7 Frequently Found in Most Fecal Surveys

Blastocystis sp. is one of the most commonly reported organisms in any stool survey. Many studies have been carried out and fecal surveys have been seen in urban and suburban populations with scanty reports on rural communities. The prevalence of *Blastocystis* sp. in developing countries ranges from as low as 1.9 % in China [77] to 60.0 % in Indonesia [78]. PCR-based screening of Danish cohorts showed prevalence as high as 50 % in some cohorts, and it was estimated by extrapolating this information that more than one billion people worldwide could be infected [79]. A baseline study to establish prevalence of *Blastocystis* among asymptomatic high-rise flat dwellers in Kuala Lumpur revealed 14.9 % [80]. A recent stool survey conducted among the aboriginal people (Orang Asli) in Malaysia revealed 20.4 % of 500 individuals infected with *Blastocystis* [81]. To date, there are 29 publications on prevalence of *Blastocystis* sp. and its genotype in surveys carried out among general populations (3.5 %; 1/29), patients (86 %; 25/29), rural communities (7 %; 2/29), and schoolchildren (3.5 %; 1/29). However, these epidemiological studies seem to concentrate on patients. Varying results are probably due to the different detection methods used, namely direct microscopy and *in vitro* culture method. The molecular epidemiological results obtained from various studies further compound the confusion especially when they are obtained from preserved stool samples. Different preservatives affect the DNA integrity.

Hence, it is imperative that when prevalence data is compiled and compared, this factor is taken into serious consideration. In most cases, unless a comprehensive patients' history is captured, it would be a challenge to categorize the severity and type of infection of the respective patient, which is important for the classification of the health condition of the participants in epidemiological surveys.

Besides being prevalent in humans, *Blastocystis* infection is also commonly reported in annelids, arthropods, amphibians, reptiles, birds, and mammals [17, 48]. High prevalence of *Blastocystis* infections has been reported in certain animals such as laboratory rats (60 %; [82]), pigs (70–95 %; [83, 84]), and birds (50–100 %; [83, 85, 86]) and even in house lizards [51]. A recent survey of 236 goats in five different farms in Malaysia revealed a prevalence of 30.9 % [87]. Interestingly, the prevalence of *Blastocystis* in dogs tends to vary greatly among different countries: Brisbane and Cambodia were 2.5 % (2/80) and 1.3 % (1/80), respectively, while dogs in India showed a 24 % (19/80) prevalence [88].

7.8 Most Likely to Be a Pathogen

The greatest controversy contributing to the enigma enshrouding this organism is whether it has a pathogenic potential. The debates in the early years divided a global opinion until more recently incriminating the parasite to be pathogenic. Signs and symptoms attributed to blastocystosis (infection by *Blastocystis*) are often nonspecific. It includes nausea, abdominal pain, bloating, flatulence, and acute or chronic diarrhea. These clinical features are common to other infections. Since the symptoms are nonspecific, many clinicians may overlook blastocystosis and misdiagnose the condition of a patient. Therefore, it may lead to underreporting of the disease.

The most challenging aspect of attributing symptoms in patients to *Blastocystis* is the difficulty in finding the organism alone without any other accompanying bacteria, virus, or parasites. In one comprehensive study which examined 1,041 stool samples over a period of 1 year submitted to a local hospital, 59 patients were found positive for *Blastocystis* after being screened for virus, bacteria, and parasites. Diarrhea was the leading symptom followed by abdominal pain, vomiting, nausea, and heartburn [29]. The results concurred with other studies carried out in more recent years [70, 89, 90]. The severity of diarrhea varies from mild diarrhea [91] to chronic diarrhea [92] to acute gastroenteritis [93]. The identification of such illnesses was based on the number of *Blastocystis* sp. detected per high-power field (400× or 1,000×). It was suggested that observation of five or more parasites in a high-power field is associated with acute presentation of gastrointestinal symptoms [89, 90].

Irritable bowel syndrome (IBS) is a bowel disorder where abdominal pain is associated with a defect or change in bowel habits [94]. Accumulating studies suggested an association of *Blastocystis* sp. infection with IBS [95] though its significance was in question [94, 96–98]. Recent studies also suggested possible

association between cutaneous lesions or chronic urticaria and *Blastocystis* sp. [99–101]. The suggestion that amoeboid forms of *Blastocystis* sp. had a significant role in contributing to the pathogenicity as evidenced by its predominant presence in isolates obtained from symptomatic patients [34] concurred with another study that showed acute urticaria being associated with amoeboid forms of subtype 3 [99]. It is suggested that the amoeboid forms may be prevalent in symptomatic patients since these studies shared the same findings. However, further investigation is needed to validate this observation.

The parasite's association and its possible role in exacerbating colorectal cancer were demonstrated when solubilized antigen of *Blastocystis hominis* was shown to trigger the growth of human colorectal cancer cells [102]. This implies that screening for parasites especially *Blastocystis* must be done for all colorectal cancer patients. Another interesting finding showed the occurrence of *Blastocystis* in colorectal cancer patients who underwent chemotherapy and who previously was shown to be negative for this infection. This is possible due to low immunity as a result of oxidative stress after chemotherapy. The dormant cysts of *Blastocystis* could have reactivated to the vacuolar forms seen in stools [102]. Therefore, this study highlighted the fact that the presence of *Blastocystis* sp. may be a threat to immunocompromised patients.

7.9 Emergence of Drug Resistance

Many treatment regimens had been designed to treat blastocystosis in the past, some dated as far back as more than 20 years ago. In the past, a small quantity of patients had been treated with furazolidone, ciprofloxacin [94], quinacrine [103], tinidazole [104], trimethoprim–sulfamethoxazole [16], co-trimoxazole [105], ketoconazole [106], and entero-vioform [16]. In a most recent case, a Danish symptomatic patient infected with *Blastocystis* sp. subtype 8 has been successfully treated with trimethoprim–sulfamethoxazole [69].

Apart from that, metronidazole and iodoquinol have been prescribed for treating blastocystosis. But iodoquinol was reported to have high toxicity; hence, it had been removed from the shelf and is no longer advisable for administration [15]. However, metronidazole is still the drug of choice though it may not be the best with reported drug resistance incidents [60, 94, 107, 108]. Nevertheless, a recent study revealed a better drug efficacy for paromomycin in eradicating blastocystosis compared to both metronidazole and clioquinol [109].

A previous treatment of tinidazole and ciprofloxacin [110] and metronidazole [111] caused higher cystic counts in stools of infected patients which can be responsible for further transmission. Its recommended dosage for the treatment of blastocystosis is between 250 and 750 mg, three times per day for 5–10 days [112]; 200 mg, four times per day for 7 days [113]; or 2 g/day for 5 days [114]. The treatment of blastocystosis is rather complicated mainly because of the adoption of different dosages and regimens and the occurrence of drug resistance against

metronidazole in a subset of *Blastocystis* [115]. The successful inhibition of *Blastocystis* in *in vitro* using plant extracts from five antidiarrheic Thai medicinal plants clearly showed that alternative treatment approach other than allopathic drugs must be sought to eliminate this robust stubborn infection [116].

As it was previously shown that there are phenotypic differences between subtypes, it is highly possible that drug resistance also depends on subtypes. Hence, to institute a treatment regimen based on some previous developed protocols may not be the right approach. This explains probably why there is still an absence of proper treatment to eliminate blastocystosis since the drug of choice creates resistance. Hence, more drug resistance studies at the molecular level through gene expression and drug interaction must be attempted.

7.10 Extensive Genetic Diversity

In 1996, *Blastocystis* was historically classified as a Stramenopiles based on sequencing of the small subunit ribosomal RNA (SSU rRNA) [117]. Since then, many molecular approaches have been applied to study the genetic heterogeneity of *Blastocystis*. These approaches include polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [118], random amplified polymorphic DNA [119, 120], PCR subtype-specific sequence-tagged-site (STS) primer [121], single conformational polymorphism [122], and pyrosequencing [123]. These various types of molecular approaches have given rise to many different terminologies for *Blastocystis* genotypes such as ribodeme [118], subgroup [124], subtype [121, 125], and clade [121, 126]. These different methodologies and terminologies have made the comparison between studies difficult. Hence, a standardization of the *Blastocystis* nomenclature has been proposed by Stensvold et al. [75], and all *Blastocystis* infecting humans has been reclassified into nine *Blastocystis* sp. subtypes (*Blastocystis* ST1 to ST9). This classification has facilitated future investigation of the epidemiology and clinical importance of *Blastocystis*.

Blastocystis is genetically diverse and to date at least 13 *Blastocystis* subtypes (ST1 to ST13) have been discovered in both humans and animals based on small subunit ribosomal RNA (SSU rRNA) [74, 76]. Till date, only nine STs (ST1 to ST9) have been reported in humans [74, 127, 128]. Across the world, *Blastocystis* ST1 and ST3 remain the most frequently found in humans. Molecular epidemiological studies in the Africas, Central Asia, East Asia, Southeast Asia, and Australia revealed the highest prevalence of *Blastocystis* ST3 followed by ST1 [127]. Meanwhile in Americas, *Blastocystis* ST1 was found to be more prevalent than ST3. In Europe, *Blastocystis* ST3 was the most prevalent followed by ST4 and ST1. It is unknown why *Blastocystis* ST4 is the second most prevalent subtype (ST) in the UK and is commonly found across Europe [127].

Among the Southeast Asia countries, human *Blastocystis* isolates have been genotyped in Singapore, Thailand, Philippines, and Malaysia, whereas no reports from the remaining countries such as Myanmar, Cambodia, Lao PDR, Vietnam,

Brunei Darussalam, Indonesia, and Timor-Leste. In a major hospital of Singapore (National University Hospital), a total of 276 stool samples were examined for the presence of *Blastocystis*. The diagnosis was done by *in vitro* cultivation using Jones' medium and subsequently the isolates were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The prevalence rate was determined to be 3.3 % (9/276). Of these, 78 % (7/9) of the isolates were of ST3, while 22 % (2/9) were ST1 [129]. Meanwhile, a recent study in a major hospital in Thailand (Clinical Microscopic Laboratory and Parasitology Laboratory in the Srinagarind Hospital, Khon Kaen University, Thailand) on 562 fresh fecal samples revealed that *Blastocystis* ST3 was the most dominant subtype in northeastern Thailand, followed by ST1. Interestingly, ST6 and ST7, which are considered as avian subtypes, were found for the first time in humans in Thailand [130].

On the contrary, a cross-sectional study on screening of intestinal parasitic infections in a primary school in Thailand revealed a prevalence of 18.9 % with *Blastocystis* ST1 (77.9 %) and ST7 (22.1 %). It was noted in this particular study that the absence of the ST3 could be due to the limited sensitivity of the molecular approach used [131]. In the Philippines, a total of 12 human fecal samples were obtained from a public health facility in Manila City and subjected to *in vitro* cultivation followed by PCR-RFLP analysis of *Blastocystis* SSU gene. The results revealed ten *Blastocystis* ST1 [132]. A more recent study of five human *Blastocystis* isolates obtained from animal handlers at animal facilities revealed four *Blastocystis* ST3 and one ST1 [133]. In Malaysia, a total of 20 *Blastocystis* isolates were examined and results showed ten *Blastocystis* ST3, nine ST1, and one ST7 [55]. A more recent genotyping analysis of *Blastocystis* isolates obtained from 40 HIV-positive and cancer patients revealed 20 *Blastocystis* ST3, 11 *Blastocystis* ST6, five ST1, and two ST7 [134]. In general, *Blastocystis* ST1 and ST3 are the two common STs reported in Southeast Asia countries with ST3 as the predominant ST.

7.11 *Blastocystis* ST3 Could Be Related to Disease

Many studies have been designed to study the possible link between a certain *Blastocystis* ST with disease; however, the results have been either non-conclusive or conflicting [62, 135]. Nevertheless, studies in the Southeast Asia countries pinpointed the possible link of *Blastocystis* ST3 with disease. In Singapore, *Blastocystis* ST3 was found to be the predominant ST among *Blastocystis* isolates obtained from a major hospital [129]. Meanwhile, a clinical relevance study of *Blastocystis* ST was done in a major hospital of Thailand on 25 symptomatic patients and 31 asymptomatic individuals [130]. The symptomatic group was further subdivided into patients with and without gastrointestinal symptoms. ST3 was found to be significantly more prevalent in patients with gastrointestinal symptoms (16 subjects) than in patients without (4 subjects) ($P < 0.05$). A clinical relevance study in Malaysia involving ten symptomatic patients and ten

asymptomatic individuals indicated that ST3 correlated well with disease, as all ten *Blastocystis* isolates obtained from patients with gastrointestinal symptoms such as diarrhea, abdominal pain, and flatulence belonged to ST3 [55].

7.12 Multiple Routes of Transmission

7.12.1 Human-to-Human Transmission

The evidence for human-to-human *Blastocystis* sp. transmission has not been substantiated extensively other than the one study using molecular evidence to highlight such a transmission in Japan [136]. This transmission apparently took place in a health-care facility where patients who were previously negative became positive. This was attributed to the transmission from positively infected patients from another health-care facility who were shifted into this premise. There is a lack of evidence for this mode of transmission as studies are not being carried out to include family members of positively infected patients admitted to the hospital or those recruited for the study were from different residential or geographical areas.

7.12.2 Zoonotic Transmission

A fecal survey carried out as early as 1999 from a zoo in Malaysia [137] was among the first to highlight the zoonotic potential of *Blastocystis*. The study showed that animal handlers (41 % of 105) were found to be at significantly higher risk of acquiring *Blastocystis* infection as compared to flat dwellers in the city (17 % of 163). Since then, many molecular approaches have been applied to examine the zoonotic transmission of the organism. In Thailand, Thathaisong et al. [138] reported that sequence and phylogenetic analysis of partial SSU rDNA of *Blastocystis* from a pig and a horse were monophyletic and closely related to *B. hominis*, with 92–94 % sequence similarity. In another study, *Blastocystis hominis* was detected in cattle and pigs using PCR-RFLP analysis of SSU rRNA [83]. It was reported that 31.8 % of isolates examined were related to *Blastocystis hominis*. Hence, the study postulated that *Blastocystis* sp. in cattle and pigs were a potential source of human infection. Similar observations were reported in Europe, China, and the Philippines, which suggest possible animal-to-human (zoonotic) and human-to-animal (anthroponotic) transmissions [133, 139, 140]. In the Philippines, a total of 12 *Blastocystis* isolates from animal and animal handlers were analyzed by sequencing the full-length small subunit ribosomal RNA (SSU rRNA) gene [133], and the study revealed 99 % sequence similarity of an isolate collected from an animal handler (isolate H4) in a monkey facility in Rizal with two monkey isolates from the same facility, M2 and M9. Moreover, the four animal handlers in a

pig farm in Batangas (isolates H8, H9, and H11) showed 100 % sequence similarity to SSU rRNA sequence of a previously reported pig isolate, PJ99-162 (GenBank accession no. AB107963; [141]).

In a separate study, nested PCR was employed and PCR product was sequenced in order to characterize *Blastocystis* sp., which exhibited the evidence of zoonotic potential of this parasite in dogs (in Thailand) and possums (in Australia) living in close proximity with humans [67]. A single *Blastocystis* isolate from a Thai human was found to have 100 % sequence similarity with an isolate from a dog living in the same community. Meanwhile, *Blastocystis* sequences from a possum and human in Australia were also shown to be 100 % similar to each other. In an extensive stool survey for *Blastocystis* in Perth Zoo, four zookeepers' isolates showed identical sequence to the isolates from the southern hairy-nosed wombat and five primate species [74]. The molecular study involving SSU rRNA study in Kathmandu (Nepal) suggested that the local rhesus monkeys living nearby a hospital could be a possible reservoir for *Blastocystis* sp. ST2 infections in children admitted to the same hospital [142].

One of the most recent studies to implicate both zoonotic and waterborne transmissions is the finding of the unusual predominance of *Blastocystis* ST4 (84.1 %) in a community in Nepal. The transmission was attributed to the rearing of family-owned animals in barns built close to their houses as well as to almost 81 % of the *Blastocystis* sp.-infected participants drinking unboiled or unfiltered water [143]. These studies highlighted an important point that zoonotic transmission from animals known to be positive for *Blastocystis* can be transmitted to pet lovers, animal house handlers, animal experimenters, and zookeepers. Hence, precaution and public education are necessary.

7.12.3 Waterborne Transmission

To the best of our knowledge, to date, there are 17 publications that implicated possible waterborne transmission of *Blastocystis* sp. across the globe from Argentina [144, 145], Chile [146, 147], Italy [148], and the United States of America [149] in the West to Egypt [150, 151], Jordan [152, 153], and Turkey [154] in the Middle East and China [155] and Thailand [131, 156–158] in Asia. Among these studies, two publications [131, 154] had successfully detected the presence of *Blastocystis* sp. in drinking water using two different molecular techniques. The first was carried out using nested PCR of SSU rRNA gene of *Blastocystis* sp. followed by sequencing, and the second was by employing polymerase chain reaction using sequence-tagged-site (PCR-STS) primers.

The first molecular evidence on the occurrence of waterborne transmission of *Blastocystis* was reported in a cross-sectional study of intestinal parasitic infections in a primary school that consisted of approximately 700 children between 6 and 13 years of age, at Chacherngsao Province, central Thailand, in January 2005. There were 51.9 % (126/243) of the schoolchildren who were infected with

Blastocystis. Meanwhile *Blastocystis* ST1 was found in one of the rainwater storage tanks which was mainly used by the school for drinking without further treatment. More importantly, the nucleotide sequence of the SSU rRNA gene showed 100 % identity to those of ST1 found in stool specimens of schoolchildren [131].

In the second study, *Blastocystis* sp. was detected in humans and animals living within an environment where they coexisted and in tap water in the homes of the infected humans [154]. This study showed that *Blastocystis* sp. subtype 1 was again detected in humans, drinking water supply, and pets. Apart from that, *Blastocystis* sp. subtypes 2 and 3 were also detected in both humans and pets. Therefore, it revealed the possibility of waterborne transmission of *Blastocystis* sp., since the same subtype was detected in both humans and drinking water source.

Viable cysts of *Blastocystis* sp. have been detected in both Scottish and Malaysian sewage samples [159], giving rise to the fact that this parasite may contaminate the environment should the septic tank be mismanaged. The most recent study has successfully used molecular techniques to identify *Blastocystis* sp. isolated from wastewater samples in the Philippines [160]. These studies are very important because it lays the foundation that *Blastocystis* sp. cysts can be detected in sewage and wastewater, which support the fact that there is a possibility that if this parasite survives in water, it may cause infection when ingested.

7.12.4 Risk Factors

Studies on risk factors have been scanty with only strong evidence linking to drinking unboiled water [131, 156, 161, 162], consumption of water plant [162], poor hand hygiene, and poor construction and management of sanitary system [152, 153, 163]. These reports however need validation from other studies.

7.13 Cryoprotection

Long-term *in vitro* maintenance of *Blastocystis* may possibly cause a genetic drift. Cryopreservation is the answer but the organisms are very fragile, and there is a need to develop sensitive cryopreservation protocols. Zierdt [16] used dimethylsulfoxide (DMSO) to cryopreserve, but extreme care was needed especially during the process of cooling and the subsequent maintenance of parasites in liquid nitrogen. DMSO in mannitol and glycerol was shown to have better cryoprotective ability [51]; however, the percentage yield was about 38 % when re-cultured in IMDM medium with 20 % horse serum. DMSO in glycerol and fetal calf serum increased the yield to 94 % when re-cultured in IMDM with 20 % horse serum [52].

7.14 Concluding Remarks

World Health Organization (WHO) states that the deaths from cancer globally are projected to continue increasing, with an estimation of 12 million deaths in 2030. Infections which can result in tissue inflammation such as gastritis, hepatitis, and colitis are estimated to account for 15–20 % of all cancers worldwide. Infections by parasites including intestinal parasites that inhabit the human's gastrointestinal tract can increase the chances of developing certain types of cancers such as colon and biliary tract cancer.

There have been studies associating cancer and *Blastocystis* infections [102, 164]. A study also demonstrated high levels of oxidative stress in rats infected with *Blastocystis* [164]. Generally parasitic infections initiate carcinogenesis through inflammatory processes. When a host's immune system is triggered by parasitic infection, oxidative burst is activated by macrophages which in turn affect the inflammatory system. Inflammatory cells of the host produce free radicals including reactive oxygen species (ROS) primarily to kill the invading parasites by nitration, oxidation, and chlorination reactions. Excess amounts of ROS can cause injury to host cells and may stimulate DNA mutations. Prolonged accumulation of this process could lead to carcinogenesis. Emerging evidences continue to point out that *Blastocystis* should not be studied in isolation but in relation to other diseases such as cancer. The physiological and biochemical changes in the body due to such diseases can influence the way the parasite behaves if such patients also happen to harbor *Blastocystis*.

It is obvious that the extensive genetic heterogeneity occurring in *Blastocystis* and the established accompanying phenotypic differences make it dangerous to generalize a treatment regimen for all *Blastocystis*-infected patients, more so when studies have shown that isolates obtained from different countries also express such variation. This is an important point to consider especially when it comes to dealing with *Blastocystis*-infected migrant. Screening generally does not include this parasite as clinicians and government health authorities still do not give importance to this organism. A general parasitology diagnostic laboratory routinely screens for *Entamoeba histolytica* and *Giardia lamblia* as well as helminths such as *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm and tends to ignore this parasite. Hence, there is a need to shift the important scientific contribution arising from the laboratories to the rooms of policy makers. The relevance of all these information is the bridge that will make this parasite more visible and be seriously considered.

The low host specificity of the organism together with the multiple modes of transmission does pose potential health hazard to the human population globally. The high prevalence of *Blastocystis* in various animals must warn pet lovers to be careful as the intimacy between man and animals is an opportunity for zoonotic transmission to take place. The scenario is worsened with the emergence of drug-resistant strains.

The recent report on the whole genome sequence of *Blastocystis* subtype 7 [165] has set a whole new platform of research opportunities particularly on the nuclear

genome which may lead to better understanding on its host–parasite interaction and fundamental molecular mechanisms. An extensive comparative study between different subtypes may shed light on the pathogenesis of the organism.

The journey for the past 20 years has been challenging. With the efforts of researchers and more than 100 students at graduate and postgraduate levels passing through our laboratory all these years, we have been able to generate information in almost all major biological aspects of the parasite. Thus far, significant available data on *Blastocystis* has come from Malaysia, Singapore, and Thailand, while limited data from Indonesia, Philippines, and Cambodia. It is crucial that information is made available from other Southeast Asian countries as well. As we peel every layer of the enigma enshrouding the parasite, more awaits grabbing our attention, provoking our curiosity, and invoking our passion which constantly becomes a continuing fuel to sustain this romance.

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Chapter 8
Toxoplasma gondii: The Parasite in Trend

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Abstract *Toxoplasma gondii* (*T. gondii*), an enigmatic coccidian and an intracellular obligate protozoan parasite, causes high infection rate and disease burden in humans worldwide. Toxoplasmosis is an important food- and waterborne parasitic disease. The seroprevalence of chronic toxoplasmosis is estimated to vary from <2 % up to 70 % among people living in Southeast Asia. Contact with cats and consumption of uncooked meat are the most common risk factors in the transmission of *Toxoplasma* infection. Interestingly, a similar prevalence rate of toxoplasmosis is also reported among infected animals. In view of this clinical scenario, toxoplasmosis is an etiological factor in pregnant women related to abortion, stillbirth, and bad obstetric history. It requires consideration in differential diagnosis of patients with unexplained lymphadenopathy. Moreover, toxoplasmosis is found to be a common cause in patients with posterior uveitis. With the concurrent HIV/AIDS pandemic, toxoplasmosis is shown to be highly prevalent in HIV-infected patients with substantial incidence of AIDS-related toxoplasmic encephalitis (TE) being reported mainly from Malaysia, Singapore, and Thailand. Majority of active TE patients presented with typical neurological manifestations with CD4 count of less than 100 cells/cumm. Diagnosis of TE is based on positive *Toxoplasma* serostatus and typical ring-enhancing lesions in the brain on CT scan finding. Despite an effective anti-*Toxoplasma* therapy, cases of relapsing TE are still reported. So far, there is no outbreak of toxoplasmosis related to animals or humans documented in Southeast Asia.

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8.1 Introduction

The coccidian *Toxoplasma gondii* (*T. gondii*) is a ubiquitous and an intracellular protozoan parasite that causes toxoplasmosis. Toxoplasmosis is prevalent worldwide and is a public health problem capable of causing a broad spectrum of diseases in different groups of population in Southeast Asia [1–4].

Since 1900, the first observation of *Toxoplasma* was found in a section of the spleen and bone marrow of Java sparrows and was given its definitive description by Nicolle and Manceaux in 1908. However, the first case of congenital toxoplasmosis in human was diagnosed in 1928. Later, Sabin and Feldman developed the first reliable serological assay "the dye test" to detect *Toxoplasma* antibodies in 1948. From these historical events, all related data of this human pathogen for this chapter were obtained from the initial phases of its literature during the late 1950s (i.e., 1959) up to the new era (i.e., 2012). However, no such study on human toxoplasmosis has been reported in Brunei Darussalam, a small but a rich country. Other nine Southeast Asian countries are alphabetically included: Cambodia (2001–2003), Indonesia (1965–2013), Lao PDR (1992), Malaysia (1973–2013), Myanmar (1977), Philippines (2000–2008), Singapore (1967–2011), Thailand (1967–2013), and Vietnam (1959–2008). This chapter focuses on the epidemiological surveillances, clinical perspectives, and diagnostic challenges on toxoplasmosis in Southeast Asia. Toxoplasmosis is definitely a public health challenge and still relevant in the field of infectious diseases. More efforts are needed in its initial control and prevention, and further steps should also be taken in eradicating this enigmatic parasite from this well-known "Sub-Asia" region.

8.2 What Is *Toxoplasma gondii*?

Toxoplasma gondii is a coccidian, ubiquitous, and an obligate intracellular parasite where felids are the definitive hosts with complex life cycles. There are three infective stages of *T. gondii* which exist in the environment. Tachyzoites, crescent to oval shape, are seen in acute infection and are transmitted through the placenta from mother to fetus, blood transfusion, or organs transplantation. Tissue cysts, containing thousands of bradyzoites, are transmitted by consumption of infected raw/undercooked meats. Tissue cyst is associated with latent (chronic) infection and is reactivated in persons who lose their immunity. Bradyzoites are less susceptible to chemotherapy, and the presence of this infective stage in host tissues is of clinical significance, particularly in immunosuppressed individuals. The oocyst stage, excreted in the feces of cats, is the most tolerant form of *T. gondii*. It is ubiquitous in nature, is highly resistant to disinfectants and environmental influences, and plays a key role in the transmission through fecal–oral route.

In 1970, the life cycle of *T. gondii* was described. Members of the family Felidae, including domestic cats, are the definitive hosts, and various warm-

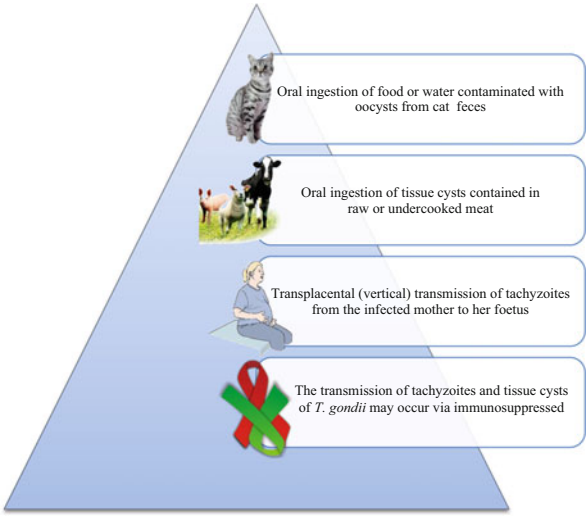

Fig. 8.1 The different routes of *T. gondii* transmission (modified from V. Nissapatorn, Toxoplasmosis in HIV/AIDS patients: a living legacy. InTech, 2011 [5] with permission)

blooded animals including humans serve as intermediate hosts. *Toxoplasma gondii* is transmitted via the ingestion of food or water contaminated with oocysts from cat feces, the ingestion of tissue cysts in undercooked meat, contamination through organ transplantation, and transplacentally (vertical or congenital) from the mother to fetus, as shown in Fig. 8.1.

In definitive hosts, the infection with *T. gondii* occurs following not only ingestion of tissue cysts in undercooked meat but also via ingestion of the tachyzoite forms or the oocysts shed in feces. The cyst wall of *T. gondii* tissue cyst is dissolved by the proteolytic enzymes in both the stomach and small intestine, releasing the slow-multiplying bradyzoite forms. Asexual cycle begins after the invasion of *T. gondii* in the epithelial cells of the small intestine, while sexual cycle is very specific and occurs only in the gut epithelial cells of feline species. The oocysts are produced by gamete fusion and are then shed in the feces of the definitive hosts. These oocysts are highly infectious to the definitive and other intermediate hosts.

In intermediate hosts, the infective stages (oocyst-releasing sporozoites, tissue cyst-releasing bradyzoites) transform into tachyzoites following infection of the intestinal epithelial cells. These tachyzoites multiply rapidly by endodyogeny in the intracellular parasitophorous vacuole. When the tissue cells are filled with tachyzoites, the host-cell plasma membrane ruptures and tachyzoites are released into the extracellular compartment. The free tachyzoites then infect any nucleated cells and intracellular replication continues. This invasion process spreads throughout the host tissues. Figure 8.1 shows the life cycle of *T. gondii*, the modes of transmission, and the association between the route of transmission and clinical presentations of toxoplasmosis.

Results from various genotypic analyses revealed that *T. gondii* isolated from humans and animals in North America and Europe can be assigned into three main

clonal lineages, referred to as types I, II, and III [6–9]. A fourth clonal lineage was recently identified in the wildlife of North America [10]. Isolates from South America are found to be more diverse [11–15]. Type I is rarely isolated (10 % of strains in Europe and the USA) and is mainly from human origin. Type II is the most commonly isolated and it constitutes 80 % of isolates in Europe and the USA. Type II strains are most commonly associated with human toxoplasmosis, both in congenital infections and in patients with AIDS [6, 7]. Most of the agricultural animal isolates are also type II, including pigs and sheep [12, 16, 17]. Type III is rare among isolates originating from Europe and the USA, and it is only found in isolates from wild animals, from remote areas, and from unusual human disease.

Despite the extensive work on the molecular epidemiology of *T. gondii* in the Western countries, yet very little of such work has been carried out in Southeast Asia. The first genetic characterization of *T. gondii* in Southeast Asia was performed on isolates from free-range ducks in Malaysia, in which types I and II were detected [18]. Another study by the same research group found only type I in wild boars in Peninsular Malaysia [18, 19]. In Myanmar, genotyping of insectivorous bats revealed they were closely related to or belong to type I [20, 21]. Free-range chickens from Indonesia and Vietnam were found to be nonclonal, i.e., not assigned to any specific type [11, 12, 14]. Dog isolates from rural Vietnam have genotypes related to those from the dog isolates in Colombia, suggesting their South American origin [22]. As in the case of free-range chickens, the genotypes of dog isolates in Vietnam were different from types I, II, and III lineages that are widely spread in North America and Europe.

It is thus surprising to note that despite the high prevalence of human toxoplasmosis, genotyping of *T. gondii* strains has never been reported from human isolates in Southeast Asia. The level of genetic diversity in this region needs to be properly estimated. It is moreover through proper understanding of genetic diversity and molecular epidemiology that the risk of spread through the food chain and the potential for zoonotic infection can be predicted [9]. Defining the population structure of *T. gondii* based on genetic data can contribute to the better understanding of transmission, immunogenicity, and pathogenesis of this parasite.

8.3 Zoonotic Toxoplasmosis

Toxoplasmosis, a cosmopolitan parasitic disease, can be transmitted through consuming raw/uncooked meat products or contacting soil contaminated with oocysts of *T. gondii*. There are a number of studies in Southeast Asia reporting about *Toxoplasma* infection among animals. Cat, a definitive host of *T. gondii*, is the prime source of *Toxoplasma* infection. Infected stray cats with *T. gondii* have been detected from 3.2 to 42.8 % [23–27]. A recent study from Thailand showed that 11 % of *Toxoplasma* infection was also found among pet cats [28]. For dogs, the seroprevalence was 9.4–50 % [22, 26, 29]. For other mammals, the prevalent rate was 61 % in goats [24] and 9–25.7 % in cattle [30–32]. Interestingly, a recent study

observed that infected cattle with virulent strains of *T. gondii* can lead to maternal toxoplasmosis which could be a cause of abortion and also congenital toxoplasmosis [33]. In addition, the infection rate with *T. gondii* was found to be 3 % in water buffalo [30], 9.17 % in dairy cows [34], 25.7 % in beef cattle [32], 27.9–61 % in domestic goats [31, 35], 3–71.43 % in pigs [36–38], 4.6–51 % in rodents [39, 40], 25.6–45.5 % in elephants [41], 24.4 % in free-range chickens [11, 12, 14], and 29.3 % in insectivorous bats [20, 21]. Based on the data reported, it strongly supported the hypothesis that mammals are definitely reservoir hosts for *T. gondii*.

8.4 Waterborne Toxoplasmosis

There are reports on toxoplasmosis that can be transmitted through drinking contaminated water with oocysts of *T. gondii* worldwide. Based on this hypothesis, a recent first-ever study in Southeast Asia among pregnant women attending antenatal clinic (ANC) in southern Thailand showed that cases seropositive for *Toxoplasma* infection were significantly associated with drinking uncleaned/unboiled water [4]. This study is another stepping stone to further investigate whether other groups of population acquire *T. gondii* through drinking contaminated water. Also, this study was primarily done through serological screening for *Toxoplasma* infection. Therefore, molecular detection is recommended to be performed to confirm the presence of *T. gondii* DNA from various water sources in this region.

8.5 Outbreaks of Toxoplasmosis

Food- and waterborne outbreaks of toxoplasmosis have consistently been reported from various parts of the world. However, it is surprising that these incidences have never been documented in Southeast Asia. It could generally be explained based on the fact that natives in this region generally still adhered to primary behavioral practices, particularly eating well-cooked meats and most importantly drinking cleaned/boiled water. Other reasons may include cases not properly diagnosed, clinicians are not aware of toxoplasmosis as the cause of the symptoms, and lastly the symptoms may be mild, subsiding without treatment.

8.6 Surveillance for Toxoplasmosis in the Region

As far as seroepidemiological study is concerned, the increasing prevalent rate of toxoplasmosis was particularly observed in Indonesia as being 2–63 % in 1964–1980 [24, 42–54], 3.1–60 % from 1981 to 1994 [55, 56], and 58–70 % during

1995 till 2003 [57–59]. Based on the above data, the largest country in SEA clearly showed the highest prevalence of *Toxoplasma* infection compared to its neighboring countries in this region. Furthermore, only one study was reported in Lao PDR, where 15.3 % of *Toxoplasma* seroprevalence was shown in a group of inhabitants with a prevalence increasing with age [60]. However, in Malaysia, the seroprevalence of toxoplasmosis varied from 13.9 to 31 % in healthy persons. The Malays showed the highest prevalence when compared to other ethnic groups and *Toxoplasma* seropositivity tends to increase with age. Moreover, a higher prevalence was found in males and unemployed individuals, whereas a lower rate was observed in people with higher income. The risk behaviors such as contact with cat and consumption of uncooked meat were found to be the main sources of *Toxoplasma* infection [61–69]. Two cases of human acquired toxoplasmosis were reported in the early period [70]. Toxoplasmosis was also suggested to be given priority in the investigation of pyrexia of unknown origin (PUO) cases [71].

Similarly, from 2000 to 2008, studies in the Philippines showed varying results of *Toxoplasma* seroprevalence from <2 to 61.2 % in different settings. The prevalence tended to increase with age and a significantly higher rate was found in rural than urban areas [72–74]. Subsequently in Singapore, the first report on *Toxoplasma* seroprevalence was 41.3 % in the sera of clinically suspected cases and 17.2 % in healthy individuals [75]. Taking into account the comparison between different races, the highest *Toxoplasma* seropositivity was found among the Malays, with their living habits and sanitary conditions greatly attributing to this finding [76]. During the 1980s, *Toxoplasma* seroprevalence was 42.5 % in clinically suspected cases [77]. From 1991 to date, 18.8 % of *Toxoplasma* seroprevalence was shown in healthy individuals, and the epidemiology and clinical profiles of patients presenting with asymptomatic cervical lymphadenopathy were indicative of acute toxoplasmosis [78, 79].

The first study in Thailand showed that 4 out of 265 abattoir workers were seropositive for *Toxoplasma* [80], while three fatal cases of human toxoplasmosis were reported in the late 1970s [81]. *Toxoplasma* seroprevalence was between 2.8 and 18.5 % in healthy persons, and the major risk factors were consumption of raw meat and contact with cats [82–90]. Interestingly, a recent study revealed that traditional lifestyles and climatic changes in environments might contribute to the low prevalence (2.6 %) of toxoplasmosis among nonpregnant women in northeastern Thailand [91]. Vietnam, the first Southeast Asian country to be actively involved with epidemiological study on toxoplasmosis, had an incidence of 2.9 % using toxoplasmin skin test as shown in the earliest study [92]. *Toxoplasma* seroprevalence rates of 15.7 and 24.3 % were found in two different groups of population [93]; while 7.7 % in intravenous drug users in one recent study [94].

In Malaysia, 23–49 % of *Toxoplasma* prevalence was found among pregnant women [95–99], and it was shown to be the highest prevalence rate found in this region as compared to 1.4–28.3 % in Thailand [4, 83, 86, 89, 100–106], up to 17.2 % in Singapore [78, 107], and 11.2 % in Vietnam [94], as shown in Fig. 8.2. Surprisingly, few studies have reported on *Toxoplasma* seroprevalence in children, newborn, or stillbirths in this region. In Malaysia, 33.3 % [108] and 2 % [109] of

Fig. 8.2 Regional status of *Toxoplasma gondii* seroprevalence in pregnant women and congenital toxoplasmosis reported in Southeast Asia. Overall seroprevalence of latent and recently acquired *Toxoplasma* infection are 39 % and 4 %, respectively. Malaysia had the highest *Toxoplasma* prevalent rate; followed by Thailand, Singapore, Indonesia and Vietnam. Clinically confirmed cases of congenital toxoplasmosis have also been reported from these respective countries

Toxoplasma prevalence in children and 45.8 % in women with stillbirths [110] were reported.

Rates of 43.8 and 28.4 % *Toxoplasma* prevalence were found in two different groups of school children in Myanmar [111]. In addition, 7.18–13.14 % were found in the cord blood [103, 106] and up to 21.05 % in the newborn [89] in Thailand. Interestingly, clinical cases with evidence of congenital toxoplasmosis have been reported in this region [112–118], as shown in Fig. 8.2. Based on the data obtained, toxoplasmosis has gained the attention on its importance and the problems associated with pregnant women and their newborns [119–121].

In patients with ocular diseases, few studies showed that 12.5–31.1 % IgG and 3.1–19.3 % IgM of *Toxoplasma* prevalence were found in Malaysia and Thailand [90, 1114]. Interestingly, cases with evidence of congenitally acquired ocular toxoplasmosis, a vision-threatening disease, and the most common which is primary retinochoroiditis were mainly reported from Indonesia, as shown in Table 8.1 [3, 115, 122, 123]. Also, toxoplasmosis is regarded as one of the top five most common specific diagnoses associated with uveitis [124]. The critical stage of the disease most commonly occurs in these patients [125]. A number of cases of ocular manifestations of congenital toxoplasmosis presented with fetal wastage [78, 79]. In addition, ocular manifestations and *Toxoplasma* serodiagnosis were investigated and reported in this region [114, 126, 127]. In other groups of patients,

Table 8.1 Seroprevalence of *Toxoplasma* infection and cases of clinically confirmed toxoplasmosis in different groups of population in Southeast Asia

Country origin	Year	*Toxoplasma* seroprevalence			Clinically confirmed diagnosis of toxoplasmosis (year)
		Healthy persons	Pregnant women	Children	
Brunei	NR	NR	NR	NR	NR
Cambodia	NR	NR	NR	NR	NR
Indonesia	1964–1980	2–63 %	NR	NR	Congenital toxoplasmosis (1976)
	1981–1994	3.1–60 %	NR	NR	Congenital toxoplasmosis (1989)
	1995–2003	58–70 %	NR	NR	NR
Lao PDR	1992	5.30 %	NR	NR	NR
Malaysia	1973–2005	13.9–30.2 %	23–49 %	2–33.3 %	Acquired toxoplasmosis (1976) Congenital toxoplasmosis (2005)
Myanmar	1977	NR	NR	28.4–43.8 %	NR
Philippines	1977–2008	<2–61.2 %	23.8	NR	NR
Singapore	1968–1990	17.20 %	NR	NR	Congenital toxoplasmosis (1967,1971, 1982 and 1989)
	1991–2003	18.80 %	Up to 17.2 %	NR	Acute toxoplasmic lymphadenitis (1991a and b)
Thailand	1967–2003	2.8–18.5 %	1.4–21.7 %	NR	Fatal human toxoplasmosis (1978)
Vietnam	1959–2003	7.7–24.3 %	11.20 %	NR	NR

NR no report

it is interesting to note that a high infection rate with *T. gondii* was also found in patients with schizophrenia [128].

The first case of HIV/AIDS was reported in 1984. Since then, the epidemiological and clinical relevance of toxoplasmosis has been studied in a few dominant countries. No study regarding this has been conducted in Indonesia, Lao PDR, Myanmar, Philippines, and Vietnam (Table 8.2). The first report on seroprevalence of toxoplasmosis in HIV patients was conducted in Thailand [90]. Since then, the varying *Toxoplasma* prevalence from 21 to 53.7 % has been consistently reported from different settings in Malaysia [64, 114, 129–134] and Thailand [90, 105, 135–137]. In Vietnam, screening of toxoplasmosis was recommended in HIV/AIDS patients [94]. After the HIV/AIDS epidemic, toxoplasmic encephalitis (TE) was first reported in clinical practice in Thailand [138]. The incidence of this opportunistic infection varies from place to place, mostly occurring at a time when the patient has a very low CD4 cell count (<100 cells/mm^3). TE is one of the most common opportunistic diseases in patients with AIDS [4]. Reactivation of latent *Toxoplasma* infection and relapse of TE cases have been subsequently reported in AIDS patients [1, 2, 114, 129–132, 134, 139–150]. Interestingly, cutaneous toxoplasmosis was also reported as a rare/unusual manifestation found in HIV patients

Table 8.2 Summary on seroprevalence of latent toxoplasmosis and reported cases of clinically confirmed toxoplasmosis in ocular patients and immunosuppressed (organ transplant recipients and HIV/AIDS) patients

		Seroprevalence of toxoplasmosis (IgG/IgM) antibodies			Cases of clinically confirmed toxoplasmosis		
						HIV/AIDS	
Country origin	Year	Ocular	Organ transplant recipients	HIV/AIDS	Ocular toxoplasmosis	1st case of HIV	Toxoplasmic encephalitis (TE)
Cambodia	2003	NR	NR	NR	NR	1991	Yes but rare
Indonesia	1976–2013	NR	NR	78 %	Present (1976, 1982, 1988, 1991 and 2003)	1984	Yes
Lao PDR	NR	NR	NR	NR	NR	1990s	NR
Malaysia	1974–2011	31.1 %/19.3 %	28 %	21–44.8 %	Present (1974, 1983, 2000, 2005 and 2011)	1986	Yes/relapse
Myanmar	NR	NR	NR	NR	NR	1988	NR
Philippines	NR	NR	NR	NR	NR	1984	NR
Singapore	1991	NR	NR	NR	Present (1991a and b)	1985	Yes
Thailand	1995–2011	12.5 %/3.1 %	11 %	22.4–3.6 %	NR	1984	Yes/relapse
Vietnam	NR	NR	NR	NR	NR	1990	NR

NR no report

[151]. Looking at other groups of patients with immunosuppression, seroprevalence of toxoplasmosis was 11–28 % in organ transplant recipients [4, 152] as shown in Table 8.2 and 67.6 % in patients with different malignancies [153].

8.7 Clinical Impact of Toxoplasmosis Is Linked to the Regional Surveillance and Laboratory Diagnosis

Toxoplasmosis is one of the most important infectious diseases, which causes epidemiological and clinical impacts in humans. Studies conducted over the past years have greatly helped in the better understanding of this parasitic infection in this region. However, there are certain predisposing factors that play important roles in the acquisition of *Toxoplasma* infection.

Indonesia and Philippines are good examples of countries sharing striking similarities in their geographical distributions based on over a thousand islands and volcanic mountains; moreover, both the countries show high *Toxoplasma* seroprevalence (up to 70 %). In this context, geographical variations (mountainous or volcanic vs. plain areas) could be one of the hypotheses to explain the contribution to the spread of *Toxoplasma* infection. On a broader aspect, most countries have shown no difference in their lifestyles, particularly in the consumption of raw or half-cooked meat, which is most probably infected with *Toxoplasma* cyst. However, these studies were mainly conducted in the cities or the urban areas. Therefore, it would be more interesting if future studies could be carried out in the suburbs or rural places, including tribal areas and in certain groups of inhabitants in the remote areas. One such study recently reported among indigenous communities in peninsular Malaysia recorded high seroprevalence of *Toxoplasma gondii* (i.e., 37.0 % with 31.0 % immunoglobulin IgG, 1.8 % IgM, and 4.2 % seropositivity for both anti-*Toxoplasma* antibodies) [63]. It is evident from the living conditions of different regions that certain groups are more exposed to *Toxoplasma* infection than others. Many studies found that Malays have the highest prevalent rate, which was due to the fact of their close contact with cats kept as pets particularly in two neighboring countries of Malaysia and Singapore. Toxoplasmosis is considered as one of the blood-borne diseases either via blood products or organ transplant. However, there are very few data on epidemiological aspects and none is documented on clinical evidence of toxoplasmosis. The importance of *Toxoplasma* screening is very much needed to promote the awareness among blood donors, which would further prevent passive seroconversion particularly during the posttransfusional period. However, thus far, this factor has not posed a major public health problem in this region.

In line with clinical implication of toxoplasmosis, laboratory investigation has since played an important role in the diagnosis. Of this, serological investigation is the first screening tool used to detect anti-*Toxoplasma* (IgG) antibodies such as the Sabin–Feldman dye test [154] or IgG and IgM antibodies based on indirect

fluorescent assay (IFA), enzyme immunoassays (EIA) including enzyme-linked immunosorbent assay (ELISA) and enzyme-linked fluorescent immunoassay (ELFA), and immunosorbent agglutination assay (IAA) are available in this region. Serological diagnosis has thus far played a crucial role in primary screening for anti-*Toxoplasma* (IgG and/or IgM) antibodies in different target groups in Southeast Asia. In recent years, advanced molecular-based polymerase chain reaction (PCR) approach has been increasingly used to detect the presence of *T. gondii* DNA from clinical samples which showed promising results of both sensitivity and specificity [155]. In addition, loop-mediated isothermal amplification (LAMP) is known as a very sensitive, easy, and less time-consuming molecular method. Based on the merits of this technique, three LAMP assays were successfully developed targeting the B1, SAG1, and SAG2 genes for the detection of *T. gondii* infection in human blood samples. The SAG2-based LAMP (SAG2-LAMP) was shown to have greater sensitivity (87.5 %) than the SAG1-LAMP (80 %), B1-LAMP (80 %), and nested PCR (62.5 %) [156]. In addition, there is triplex PCR which is a rapid, sensitive, and specific conventional PCR method for the detection of the B1 gene and ITS1 region of *T. gondii* using newly designed primers [157].

In pregnant women, the clinical implications of *Toxoplasma* infection during pregnancy are extremely dangerous resulting in spontaneous abortion, stillbirths, or premature delivery with various fetal malformations. The studies in Malaysia showed the highest rate of *Toxoplasma* infection compared to other parts in this subcontinent, but the incidence of congenital toxoplasmosis is surprisingly scarce or the rate is very low. Overall, the evidence of congenital toxoplasmosis is not well documented in this region due generally to the fact that either no study has been properly conducted or the incidence is actually low. Even though toxoplasmosis is relatively low in certain countries, a large proportion of antenatal women are still susceptible to this infection. This aspect strongly necessitates imparting health education, including general guidelines of primary prevention to childbearing seronegative women and pregnant women so as to prevent primary *Toxoplasma* infection during pregnancy. Mass or routine antenatal toxoplasmosis screening is cost effective but not appropriate to be practiced in limited resource settings or in areas of low incidence of congenital toxoplasmosis. However, it could be justified in areas with high prevalence of *Toxoplasma* infection among mothers and high prevalence of congenital toxoplasmosis and in countries where TORCH screening is an applicable tool. In addition, women with bad obstetric history such as abortion (spontaneous or repeated), stillbirth, or fetal malformations should be tested for *Toxoplasma* serological status.

Serological diagnosis has played a crucial role in primary screening for anti-*Toxoplasma* (IgG and IgM) antibodies in pregnant women and their newborns. There are a number of standard commercial *Toxoplasma* serological tests based on indirect fluorescent assay (IFA), enzyme immunoassays (EIA) including enzyme-linked immunosorbent assay (ELISA) and enzyme-linked fluorescent immunoassay (ELFA), and immunosorbent agglutination assay (IAA) available in this region. Recently, IgG avidity has been introduced as a reliable and additional serological diagnosis in differentiating chronic (latent/remote) from acute (recent/active)

acquired *Toxoplasma* infection in Malaysia. This standard commercial test has been routinely used in a case–control study to detect anti-*Toxoplasma* antibodies among pregnant women [4] and has also been used for *Toxoplasma* serodiagnosis in healthy persons and patients with psychiatric disorders and ocular diseases [63, 158, 159]. Also, a recent study interestingly showed that the in-house IgG avidity Western blot using *T. gondii* Rgra-7 cloned from nucleotides 39–111 might assist in the laboratory serodiagnosis of acute toxoplasmosis [160].

The existence of clinically diagnosed toxoplasmosis in patients with ocular diseases has been periodically reported in different studies from Indonesia, Malaysia, and Singapore. Whether ocular toxoplasmosis is either congenital or acquired is still controversial; nonetheless, toxoplasmosis is not only the most common cause of posterior uveitis in a majority of cases, but it should also be primarily considered in the differential diagnosis in any suspected patients with ocular diseases. Ocular presentations and *Toxoplasma* serodiagnosis are the primary sources of investigation; in addition, improvement of clinical and fundoscopic conditions after the introduction of anti-*Toxoplasma* therapy should be the clue for the confirmation of toxoplasmosis.

Polymerase chain reaction (PCR)-based molecular technique has been used to detect *T. gondii* DNA from various biological samples such as amniotic fluid, fetal tissue, blood, cerebrospinal fluid (CSF), and other clinical specimens. This approach was successfully used in 42 patients presenting with ocular or psychiatric diseases after heat treatment using a microwave oven on whole-blood samples [158]. Recently, there was a report on the usefulness of an advanced DNA-chip technology in the diagnosis of ocular toxoplasmosis on multiple foci at a single time. However, the diagnostic properties (sensitivities, specificity, accuracy, predictive value, etc.) as well as cost-effectiveness of the DNA-chip approach need further validation, particularly before incorporating into routine laboratory investigation [161].

HIV/AIDS is the subject of great interest and of utmost concern particularly in Southeast Asia, being a region of the fastest-growing HIV epidemic in the world. Toxoplasmosis is still reported in clinical practices in coexistence with HIV/AIDS patients. *Toxoplasma* parasite is the most common cause of intracerebral lesions and one of the leading opportunistic pathogens and causes of death in AIDS patients. Due to its noteworthy significance, toxoplasmosis has been included in the Communicable Diseases Control (CDC), Atlanta, for AIDS defining illness till date. The incidence of toxoplasmic encephalitis (TE) is directly proportionate to the prevalence of *Toxoplasma* infection and the number of AIDS patients. The empirical diagnosis of TE is based on a few criteria. The neurological presentations mimic other brain diseases and make the diagnosis difficult. Results from the brain involvement in either neuroimaging finding, computed tomography (CT), or magnetic resonance image (MRI) are useful tools for the presumptive/empirical diagnosis of TE. TE usually causes unifocal, more frequently multifocal, lesions and less likely diffuse encephalitis. These findings are however not pathognomonic of TE. The typical radiological diagnosis shows in a majority of cases as typical findings, hypodense lesions with ring-enhancing and perilesional edema. CT scan

seems to be a sensitive diagnostic method for patients with focal neurological deficits, as also seen in reported cases of TE from advanced HIV patients in Southeast Asia. MRI is recommended to be performed in patients with neurological symptoms and positive serology to anti-*Toxoplasma* antibodies whose CT scans show no or only a single abnormality, or persistent or worsening focal neurological deficits of disease if results of the initial procedure were negative. However, MRI may not be feasible in this region where a majority of patients with HIV/AIDS are in marginalized conditions and are also in limited resource settings. From these radio-imaging techniques, a complete resolution of cerebral lesions may vary from 3 weeks to 6 months of initiation of therapy in these patients. In addition, stereotactic brain biopsy [162] has also been used in AIDS patients with cerebral lesions to confirm the etiology in a majority of cases.

Serological (IgG and/or IgM antibodies) diagnosis is generally used to detect the evidence of *Toxoplasma* serostatus whether TE is due to primary infection, which is less common, or secondary reactivation, which occurs in more than 95 % of these patients. However, this method can be limited in AIDS patients because of depressed antibody responses. Interestingly, a previous study showed that *Toxoplasma* seropositive in HIV patients recognized the antigenic component, the 32 kDa antigenic band through immunoblotting and enzyme-linked immunosorbent assay which might represent a specific marker for the diagnosis of *Toxoplasma* infection in these patients [137]. Real-time PCR (RT-PCR), a quantitative, sensitive, specific, and less time-consuming technique, enables rapid detection of amplification products as well as hybridization of amplicon-specific probes. This molecular technique has recently been shown to be a promising alternative diagnostic tool for TE in patients with AIDS, particularly in resource-limited settings [163].

Responding to anti-*Toxoplasma* therapy is the key to confirm the diagnosis of TE. Based on a clinical trial, a combination of pyrimethamine (50 mg/day) and sulfadiazine (4 g/day) provides the best primary outcome in treating TE patients [164]. A recent study also showed that a combination of spiramycin and metronidazole yielded promising results, causing a significant reduction of *T. gondii* brain cysts in a mouse model with chronic toxoplasmosis [165]. Moreover, the occurrence of TE was significantly related to a very low level of CD4 count, and one study suggested that the correlation of imaging findings with CD4 counts is especially useful in obtaining a working diagnosis [166]. Primary chemoprophylaxis should be compulsorily given to all new HIV-infected patients particularly in poor resource settings, where they might not be able to access HAART. However, this is not an absolute hypothesis to explain the relationship between the occurrence of TE and its chemoprophylaxis in these patients. In due course, one study found that the mounting medical care cost for adult AIDS patients has become critical [167] in certain countries where the number of HIV/AIDS patients is still increasing. In the era of HAART, it should be considered as the most effective approach in reducing the incidence of TE. However, it is still questionable whether it is the ideal option in case management with regard to this parasite in future.

TE has thus far been mainly diagnosed among immunosuppressed such as patients with AIDS. However, TE has recently been diagnosed in systemic lupus erythematosus (SLE) following intravenous methylprednisolone. This is the first case report in the literature [168].

8.8 Conclusion

Toxoplasmosis remains highly prevalent in Southeast Asia. A few recognized, known, and accepted risk behaviors to *Toxoplasma* infection have consistently been identified. Clinical toxoplasmosis in different groups of population has been periodically reported in this region. However, few recommendations could be conducive to the completeness of this literature review: firstly, more studies on toxoplasmosis in various aspects should be carried out in Southeast Asia in general and the area of Mae Khong region (i.e., Cambodia, Lao PDR, and Vietnam) and Brunei Darussalam in particular. With the spread of the HIV/AIDS epidemic, toxoplasmosis should be given more attention due to the fact that suspected cases might be misdiagnosed and subsequently lead to life-threatening or fatal outcomes. This is also an opportunity for researchers to focus on this pathogen in these patients and extend more work in other vulnerable groups; secondly, there are many other risk factors involved which are undiscovered and need to be investigated to further clarify the pathogenesis of this parasite; thirdly, novel drugs, including herbal medicine, should be given serious consideration in clinical trials, particularly in tackling the cystic stage of this parasite and reducing the treatment burden through new effective medicines of both private and government hospitals; and lastly, multicenter study on toxoplasmosis should be established, which could serve as smart partnerships to strengthen the regional collaborations and also enhance the existing activities in terms of prevention and control measures, thereby logically conducting assessments according to their own feasibilities for the benefit of the affected individuals. It is therefore hoped that a new chapter of managing health issues regarding toxoplasmosis would be successful in the future.

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Chapter 9
Sarcocystis spp. and Pentastomes in Southeast Asia

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Abstract *Sarcocystis* and pentastomes infections are uncommon zoonoses of worldwide distribution. In most of the cases, they were detected as an incidental finding at autopsy or necropsy. Recently, serious attention has been given to *Sarcocystis* when large human outbreaks have been reported in Malaysia. Similarly, the detection of a recent human pentastomiasis case in East Malaysia since nearly four decades ago has alerted that pentastomes should not be ignored by the medical and laboratory personnel. In addition, understanding the routes of infection is crucial in preventing further cases. This chapter will focus on sarcocystosis and pentastomiasis cases among humans and animals.

9.1 *Sarcocystis*

9.1.1 Introduction

Sarcocystis spp. are intracellular protozoa belonging to the family Sarcocystidae. The protozoan was first detected in striated muscles of a house mouse as white threadlike cysts by Miescher in 1843 [1]. It was initially referred as Miescher's tubules for more than 50 years until the name *Sarcocystis meisheriana* was suggested for similar structures found in swine muscle [2]. *Sarcocystis* spp. are the etiological agents of sarcocystosis, an uncommon zoonosis of worldwide distribution. Majority of the human and animal cases were reported from Southeast Asian countries particularly Malaysia and Thailand [3, 4]. The life cycle of *Sarcocystis* is complex, involving asexual and sexual reproduction in intermediate (e.g. herbivores) and definitive (e.g. carnivores and omnivores) hosts respectively.

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Currently more than 130 *Sarcocystis* spp. have been identified from a variety of hosts including swine, birds, snake, rats, cattle, goats and monkeys. Interestingly humans may serve as both the definitive and intermediate (dead end) hosts [1]. Infection by *Sarcocystis* may cause be either a self-limiting intestinal infection or prolonged muscular infection in humans and animals [3, 5]. In most of the reported cases, sarcocystosis was detected as an incidental finding at autopsy or necropsy [6, 7].

9.1.2 Morphology

In general, there are three important *Sarcocystis* stages, namely the oocyst, sporocyst and sarcocyst. The oocysts are about 12–15 μm by 19–20 μm in size, containing a pair of sporocysts which are enclosed by a thin wall. Each of the sporocyst measures approximately 10 by 15 μm and contains four sporozoites and a granular residual body [1]. Both oocysts and sporocysts can be detected in the faeces of infected hosts but the oocysts are less commonly seen as their wall is very fragile and easily ruptured [1]. Sporocysts excreted from different hosts are usually morphologically indistinguishable [1]. Sarcocysts are usually found in striated muscles of the body including the tongue, oesophagus, diaphragm and cardiac muscle [8, 9]. They are spindle in shape with either a thin or thick wall surrounding them. The size of sarcocysts may vary depending on their age and range approximately 140–250 μm by 50–66 μm [8] (Fig. 9.1). Numerous infectious crescent-shaped bodies termed as bradyzoites are contained in each of the sarcocyst [8].

9.1.3 Life Cycle

The life cycle of *Sarcocystis* is based on a prey–predator (intermediate–definitive) host relationship [1]. Asexual reproduction occurs in an intermediate host (e.g. cattle, pigs and goats) following ingestion of oocysts or free sporocysts from food or water contaminated with faeces from the infected definitive host (e.g. monkeys, baboons, dogs and reticulated pythons). In the small intestine, sporozoites are released from the sporocysts and migrate through the gut epithelium to the small arteries throughout the body. The first asexual generation begins at these sites, generating a large number of merozoites. Subsequent generations of merozoites develop in the direction of blood flow to arterioles, capillaries, venules and veins throughout the body and terminate with the formation of sarcocysts in muscles [10]. The sarcocysts would remain non-infectious for at least 2 months until the bradyzoites within the sarcocysts have matured and appeared as crescent shaped [1, 3].

Sexual reproduction occurs when meat containing sarcocysts from an infected intermediate host is eaten by a susceptible definitive host. In the intestine, the

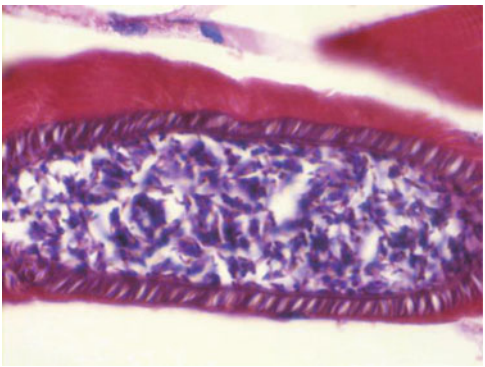

Fig. 9.1 A thick wall sarcocyst with numerous bradyzoites stained with haematoxylin and eosin (×1,000) (adapted from [8] with permission from IeJSME)

sarcocysts are ruptured, releasing the infectious bradyzoites. Male and female gametes are subsequently developed from each bradyzoite and fertilisation occurs. This results in the production of oocysts which are then being excreted in the faeces of the definitive host. Humans can become both the intermediate and definitive hosts. However, humans are usually the accidental intermediate hosts as we are unlikely to be eaten by other definitive hosts [1].

9.1.4 Disease Manifestations and Sarcocystis Cases in Southeast Asia

Infection in humans can result in intestinal and muscular sarcocystosis [1, 5]. Intestinal sarcocystosis can be acquired following consumption of raw or undercooked meat containing mature sarcocysts. The infection is usually self-limiting with symptoms such as nausea, loss of appetite, vomiting, stomach ache and diarrhoea. On the other hand, muscular sarcocystosis can be acquired from ingestion of water or food that has been contaminated with faeces from infected definitive hosts. Individuals with muscular sarcocystosis usually suffer from musculoskeletal pain, fever, rash and subcutaneous swelling, and these symptoms may last from months to years [5].

Muscular sarcocystosis in humans is rare and mostly reported from Southeast Asian countries particularly Malaysia [3, 11]. In Malaysia, the first human muscular sarcocystosis cases were reported in 1975 following incidental findings of sarcocysts in a laryngeal biopsy of a patient with hoarseness and an oropharyngeal biopsy of an Orang Asli girl [6, 12]. Subsequent human cases have been reported by Pathmanathan and colleagues in females and males from different ethnic groups (e.g. Malays, Indians, Chinese and Orang Asli) by examinations of autopsy or biopsy samples [3, 13]. Interestingly, more than 50 % of the infected individuals were associated with malignancies [13]. In a seroprevalence study done in 1978, almost 20 % of 243 individuals from Peninsular Malaysia were positive for *Sarcocystis* antibodies [14]. Prevalence of *Sarcocystis* infection was highest in

the Orang Asli population followed by Malays, Indians and Chinese [14]. It is to be noted that Orang Asli are aboriginal peoples of Peninsular Malaysia and constitute almost 0.5 % of Malaysia's total population [66]. Most of them live in poverty with poor environmental conditions and they hunt wild boar, deer and lizard for meat sources [67]. Hence, they are at a higher risk of getting the infection via consumption of water or food contaminated with sporocysts or eating raw meat of infected definitive hosts. In a separate prevalence study, Wong and Pathmanathan reported the detection of sarcocysts in 21 out of 100 tongues (21 %) collected during autopsies of individuals aged 16–57 years old [9].

In Malaysia, the first large outbreak was published in 1999 involving 7 of 15 US soldiers who had illness 1–3 weeks after returning from field operations in a remote area in 1993 [5]. All but one of them showed symptoms including fever, myalgias, bronchospasm, subcutaneous nodules, eosinophilia and elevated levels of muscle enzymes. Sarcocysts were also detected in muscle biopsies of one of the soldiers [5]. Albendazole was given to this index case patient to reduce the chronic sarcocystosis symptoms [5]. Subsequently a decade later, another 100 suspected muscular sarcocystosis cases were recognised in travellers returning from vacation in Tioman Island, East Coast of Peninsular Malaysia during the summer months of 2011 and 2012. Thirty-five cases were initially identified in travellers from Europe (i.e. Germany, France, Netherlands, Switzerland, Belgium and Spain) and Asia (i.e. Singapore); all had prolonged fever and muscle pain. Another 65 cases were recognised about 6–8 months later with two being asymptomatic [15, 16]. It is presumed that there may be more unreported cases from the outbreak especially from those individuals without any apparent symptoms. In 2012, there was also an unpublished outbreak in an island situated at the north-west of Peninsular Malaysia. In this outbreak, more than 90 suspected muscular sarcocystosis were identified, and sarcocysts were detected in muscle biopsies of three of the infected individuals (personal communication with Prof. Rohela Mahmud, Head of Parasitology Department, University of Malaya, Malaysia). It has been suggested that human muscular cases in Malaysia could be due to ingestion of food or water contaminated with sporocysts excreted from definitive hosts such as dogs, cats or pythons [3].

Besides muscular infection in humans, *Sarcocystis* sarcocysts have also been detected in muscle tissues of wild and domestic animals in Malaysia including rodents [8, 17–19], zoo animals [7], water buffaloes [20, 21], cattle [21], ovine [22], slow lories [23] and monkeys [24, 25]. Most of the infected animals were asymptomatic but pathological changes including haemorrhage and oedema of organs and muscle atrophy have been observed at necropsy [7]. Ambu and colleagues have reported the presence of sarcocysts in 73 out of 146 wild and peri-urban rodents collected in Peninsular Malaysia [8]. The number of sarcocysts detected per histological section varied from 1 to 136 and none could be seen by gross examination [8]. In another study involving 40 dead animals, sarcocysts were detected in three captive mammals and five birds of two zoos in Peninsular Malaysia [7]. Sarcocysts were detected mainly in the skeletal muscles (50 %) followed by the tongue and heart (37.5 %), diaphragm (25 %) and oesophagus (12.5 %) [7]. Thus far, no human intestinal sarcocystosis have been identified or published

in Malaysia [3]. However, it is believed that most of the cases might be missed or misdiagnosed rather than absent as human intestinal infection is usually self-limiting and its clinical symptoms may mimic those of other diseases [1, 26].

In Thailand, consumption of raw infected beef and pork has been identified as the main sources of human intestinal sarcocystosis [4, 27]. This is supported by the detection of bradyzoites in 100 % of 300 swine cardiac muscle specimens collected from three markets in Samut Prakan Province [28]. Besides swine, *Sarcocystis* sarcocysts have also been identified in sections of tongues and muscles from a variety of other animals including cattle and water buffaloes [27]. In a prevalence study among 362 asymptomatic Thai labourers who were going abroad for work, 83.3 % were positive for *Sarcocystis* by stool examinations and most of them were from the northeastern Thailand [29]. It has been reported that Thai labourers particularly those from the northeastern are at higher risk of being infected with *Sarcocystis* due to their habit of eating raw or undercooked pork and poor hygiene practices [28, 29]. Recently, the first large prevalence study of human intestinal sarcocystosis was performed among 15,555 Thais, and sporocysts were detected in stool samples of 233 individuals [4]. Intestinal sarcocystosis have also been reported in 1.65 % of primary school children in Chiang Mai and 45 % of enterocolitis patients in Thailand [30, 31]. To date, only limited data is available regarding human muscular sarcocystosis in Thailand. In the late 1970s, a single study has reported the detection of sarcocysts in skeletal, laryngeal and cardiac muscle biopsies from 15 autopsy cases. Since then, no reports on human muscular sarcocystosis were published until 2011, when a single positive case was identified during histopathological review of 1,063 laryngeal biopsies obtained from 2000 to 2009 [32]. The *Sarcocystis*-positive biopsy belonged to a 66-year-old man who presented with voice hoarseness for 6 months. The patient was initially diagnosed with laryngeal carcinoma and underwent partial laryngectomy. Nonkeratinising squamous cell carcinoma with *Sarcocystis* sarcocysts were detected in his laryngeal biopsy. The patient was not given any antiprotozoal treatment, but he did not show any signs of recurrence during a 3-year follow-up [32].

In Singapore, there is a single species of *Sarcocystis* named after the country (i.e. *S. singaporensis*) following the detection of *Sarcocystis* from naturally infected pythons (*Python reticulatus*). As the life cycle of this species involves alternating sexual and asexual cycles in pythons and rodents, respectively, *S. singaporensis* has been currently used as an agent for biological control of rodents in agricultural habitats of Southeast Asia [33, 34]. Basically, rodents are artificially infected using pelleted bait containing high dosage of *S. singaporensis* sporocysts and a mixture of wheat flour, broken corn, oil and fish or coconut extracts as rodents attractants. Following consumption of the pelleted bait, the infected rodents will suffer from pneumonia and eventually die when infection with sporocysts exceeds a certain threshold [33, 34]. To date, at least three human muscular *Sarcocystis* cases have been reported in Singapore [11, 16].

In Indonesia, a *Sarcocystis* species named *S. sulawesiensis* has been detected in skeletal muscles of seven rodents from three different species (i.e. *Bunomyschrysocomus*, *Bunomysfratrorum* and *Paruromys dominator*)

[35]. *Sarcocystis sulawesiensis* has thin-walled sarcocysts and their primary walls exhibit many hair-like structures [35]. What was unique about this species was it can only be detected in rodents collected in North Sulawesi but not in West Java, thus suggesting its limited geographical distribution [35]. In the same study, *S. singaporensis* was also detected in 13 rodents of six different species [35].

In the Philippines, many of the *Sarcocystis* cases were reported in animals. As early as 1916, *Sarcocystis* was first identified in meat of a water buffalo in the Luzon island [36]. In a separate study, sarcocysts have been identified in muscle tissues of 92 out of 142 (~65 %) water buffaloes. Some of the sarcocysts, identified as *S. fusiformis*, could be detected by gross examination and appeared as milk-white spindle-shaped structures in between of muscles [37]. *Sarcocystis* spp. have also been reported in beef sold in Manila [38], muscle tissues of *Rattus* spp. [39] and livestock animals including hogs, goats and chicken [40–42].

Thus far, only a single report is available on human intestinal sarcocystosis in Vietnam [68]. The study was not performed in Vietnam but Central Slovakia among 1,228 Vietnamese trainees who came to work in that republic in 1987–1989. Sporocysts were detected in 14 of the Vietnamese trainees but none of them reported any signs of gastrointestinal infection [68]. In Vietnam, muscular sarcocystosis was commonly reported in livestock animals particularly water buffaloes [43]. By using molecular approaches (i.e. PCR and DNA sequencing), *S. fusiformis*, *S. cruzi*, *S. hominis* and *S. hirsuta* were able to be identified in meat samples of water buffaloes slaughtered in the Son La Province [43]. In Ho Chi Minh, Vietnam, sarcocysts were detected in 396 out of 502 adult water buffaloes by gross and histological examinations. Higher sarcocystosis prevalence was reported among older water buffaloes (6–7 years old; 93 %) compared to the young animals (2–3 years old; 57 %) [44]. In addition, higher prevalence was also shown among water buffaloes originating from the northern part (89 %) than those from the southern part (69 %) of Vietnam [44].

To date, limited information is available on *Sarcocystis* infection in other Southeast Asia countries such as Myanmar and Laos. In Myanmar, a *Sarcocystis* infection has been diagnosed in a kitten with symptoms of depression and lethargy. *Sarcocystis* stages including the merozoites were found in the spinal cord of the kitten at necropsy [45]. In Lao PDR, the prevalence of human intestinal sarcocystosis among 1,008 individuals screened for intestinal parasites was approximately 10 % in group of individuals aged 20 years and above. Currently, there is no report on the presence of *Sarcocystis* spp. or human/animal sarcocystosis cases in Brunei Darussalam, Cambodia and Timor-Leste.

9.1.5 Diagnosis

Intestinal sarcocystosis should be suspected if individuals have a history of recent consumption of any raw or undercooked meat and exhibit symptoms such as nausea, stomach ache, vomiting and diarrhoea. Definitive diagnosis is usually

based on the presence of oocysts or sporocysts in stool samples. For this, stool samples are initially concentrated by a floatation technique using combinations of high-density solutions (e.g. sodium chloride, cesium chloride, sucrose and Percoll) prior to viewing under a microscope. However, stool examination does not allow identification to species level due to morphological similarity of sporocysts of different *Sarcocystis* species [1].

For muscular sarcocystosis, the final diagnosis is usually made by microscopic detection of sarcocysts in muscle biopsies (e.g. tongue, oesophagus, diaphragm, heart and skeletal muscles) [44]. In most of the human cases, *Sarcocystis* was detected as incidental findings at autopsy [6, 9]. Tissue sections is commonly stained to facilitate visualisation and to differentiate *Sarcocystis* from other protozoa which have similar morphological structures [32, 46]. For example, periodic acid Schiff (PAS) can be used to differentiate bradyzoites of *Sarcocystis* (PAS negative) from those of *Toxoplasma gondii* (PAS positive except the nucleus) [32]. For ultrastructural studies of *Sarcocystis* sarcocysts and species identification, transmission electron microscope is regularly used [35]. In some *Sarcocystis* species, the sarcocysts are large in size and therefore can be detected by gross examination of the infected meat. In addition, the presence of infectious bradyzoites from sarcocysts can be microscopically examined from pellet of meat that has been ground, digested with chemicals (e.g. pepsin and hydrochloric acid) and centrifuged. Serological methods such as enzyme-linked immunosorbent assays and indirect fluorescent antibody test have also been used to determine *Sarcocystis* infection in humans and animals [5, 14, 47, 48]. Recently, polymerase chain reaction assays targeting the 18S rDNA gene and sequencing have been utilised for species identification [43, 49, 50]. In addition, the DNA sequences obtained can also be used for phylogenetic analysis and to determine the possible definitive hosts for the *Sarcocystis* species detected [51].

9.1.6 Conclusion

Sarcocystis was first discovered 170 years ago. However, less attention has been given to this protozoan until recently when large human outbreaks have been reported in Malaysia. Human sarcocystosis can be acquired following the consumption of raw or undercooked meat containing sarcocysts or ingestion of food or water contaminated with faeces from infected definitive hosts. Sarcocystosis may be undetected in asymptomatic cases, or in symptomatic cases, it may be misdiagnosed with other infections. Early identification of the infection is possible if clinicians and medical laboratory personnel are aware with its clinical symptoms, routes of infection, morphology of the organism and the use of suitable samples and diagnostic tools. As sarcocystosis is a zoonotic infection, screening of all pets, livestock animals and meat at abattoirs should be regularly performed. Prevention can also be done by adequate cooking of meat, freezing meat at −4 and −20 °C for 48 and 24 h, respectively, and boiling of water prior to use. In addition, adequate

sanitation is important to avoid contamination of the environment and infection to susceptible intermediate hosts. For communities (e.g. aborigines) who have low hygiene practices and individuals who are going to Southeast Asian countries for vacation, public education should be given to increase their awareness on *Sarcocystis* infection.

9.2 Pentastomes

9.2.1 Introduction

Pentastomes are arthropods, considered to be related to crustaceans [52]. They are commonly known as tongue worms and are adapted for an endoparasite existence in the respiratory tract, especially the lungs of a variety of animals including amphisbaena serpents, lizards, crocodiles, birds, mammals and turtles. Adult *Linguatula serrata* inhabits the nasal passages of mammals. Pentastomes are blood feeders and several species have been seen in the nymphal stage in humans in several countries. Immature pentastomes have been reported from frogs, fishes, serpents, mammals and insects [53]. Pentastomes are included in two orders, namely the Cephalobaenida and the Porocephalida. The more evolved is the order Porocephalida which contains species of medical importance such as *L. serrate*, *Armillifer armillatus*, *Armillifer grandis*, *Armillifer moniliformis*, *Armillifer agkistrodontis*, *Leiperia cincinnalis* and *Porocephalus crotali* [54, 55]. The Cephalobaenida is a more primitive group and one of its genera, *Raillietiella*, is common in lizards and serpents in Malaysia [53], and one species *Raillietiella hemidactyli* has been reported in humans in Indochina. Dollfus and Canet [56] have documented cases of subcutaneous parasitism with this parasite. This resulted from the swallowing of small, live geckos as a folk remedy for conditions such as asthma and emphysema. In the Porocephalida where the life cycles are known, vertebrates are utilised both as definitive and intermediate hosts [57]. In the Cephalobaenida, *Raillietiella* spp. from house geckos, domiciliary cockroaches (*Periplaneta americana*, *Periplaneta australasiae*, *Neostylopyga rhombifolia* and *Supella longipalpa*) act as intermediate hosts [58, 59].

9.2.2 Morphology

In Malaysia, nymphal *Armillifer moniliformis* have been reported from humans. This is the only pentastome reported in humans from Malaysia [64]. Adult *P. moniliformis* is an elongated parasite. The parasite possesses a cephalothorax with two pairs of hooks situated lateral to the oral cadre and a cylindrical vermiform body with annular thickening. The anterior end is broad and wedge shaped. The

posterior end tapers into a blunt-pointed cone. The annuli are characteristically thick and ring shaped. Nymphs from humans measure 10 mm in length and have 30 annuli while those from animals measure 15–20 mm in length and 1.5–1.6 mm in width and have 31–34 annuli. Males measure 24–30 mm in length and 2 mm in width and have 29–35 annuli. Females measure 60–72 mm in length and 3 mm in width and have 30–34 annuli [53].

9.2.3 Human Cases in Malaysia

Man is a dead-end host and acquires the condition through ingestion of contaminated water or vegetables eaten raw or by eating infected serpent poorly cleaned and cooked. Most of the cases reported throughout the world have been discovered at surgery and autopsy.

Prathap et al. [60, 61] found a 45.5 % incidence in a series of 30 consecutive autopsies on unclaimed bodies of Malaysian aborigines while a case each in a Dayak boy and a European woman was reported by Rail [62]. Subsequently, Ong [63] reported a case of pentastomiasis of the fallopian tube in an aborigine. After nearly a lapse of 40 years, a third case was reported in a 70-year-old aborigine farmer from rural Malaysian Borneo [64].

9.2.4 General Considerations

Although asymptomatic, symptomatic cases have been reported; serious illness that includes pneumonitis, peritonitis, meningitis, pericarditis, intestinal obstruction, nephritis and obstructive jaundice has been ascribed to heavy pentastome infection [53]. Adult *A. moniliformis* in Malaysia have been seen in pythons, *Python reticulatus* and *Python curtus*. The nymphs have been seen encapsulated in the liver, the liver mesenteries and omentum of a variety of vertebrates, including lower primates, Insectivora, carnivores, Rodentia and Artiodactyla [53]. Adults have also been collected from *Python molurus* in India and *Python sebae* in West Africa [65]. No cases are known to have been reported from other SEA countries except Malaysia.

9.2.5 Conclusion

Given that the nymphs of the parasite are very common in rodents and other mammals, the uncommon occurrence of human cases is fortuitous. However, extreme care should be exercised by those dealing with snakes. They should wash their hands properly after handling snakes. Those who consume python flesh should cook it properly.

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Chapter 10
Free-Living Amoebae in Southeast Asia

Init Ithoi and Arine F. Ahmad

Abstract In Southeast Asia (SEA), human infections caused by free-living amoebae (FLA) such as species of *Acanthamoeba*, *Naegleria*, *Balamuthia*, *Vahlkampfia* and *Hartmannella* were occasionally reported. To date, human cases or research work on these FLA have only been detected or performed in five countries which were Thailand, Malaysia, Singapore, Vietnam and the Philippines. *Acanthamoeba* keratitis (AK) has been increasingly recognised and diagnosed, along with the spread of contact lens use, and most cases were reported from Thailand, Malaysia and Singapore. As for granulomatous amoebic encephalitis (GAE) and *Balamuthia* amoebic encephalitis (BAE) cases, the only available reports were from Thailand. At least 11 GAE cases (8 were fatal and 3 were cured) have been reported sporadically since the first highlighted case in 1992. While for BAE, only a single fatal case was reported in a 23-year-old healthy male after falling into a swamp during a motorbike accident in 2004. For primary amoebic meningoencephalitis (PAM), an acute fulminant necrotising meningoencephalitis caused by *Naegleria fowleri* has only been reported in two countries; 12 (10 were fatal and 2 were cured) and 2 fatal cases were from Thailand and Vietnam, respectively. Almost all of the individuals with PAM cases had a history of water-related activities including swimming in canals, rivers, community pools and exposure to contaminated water during a traditional Thailand festival called 'Songkran', where people splash water at each other. All of the lethal PAM cases reported in SEA were misdiagnosed as microbial meningitis due to the inability to detect the amoebic trophozoite in the CSF. In cases where amoebae were detected early, a good treatment outcome was achieved and three GAE and two PAM cases in Thailand were cured. The delay in the diagnosis of AK cases may result in severe visual outcomes and require surgical treatment. Finally, the absence of reports or cases from other countries in SEA region does not imply that there were absolutely no

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human infections. In fact, there might be cases which were undiagnosed, misdiagnosed or overlooked. As most of the FLA diseases have a rapid progression and may also cause death, awareness of the diseases by clinician and laboratory personnel together with the availability of suitable diagnostic tools are crucial in successful treatments of infected individuals. In addition, FLA ecological studies should be regularly performed in order to understand their geographical distribution, environment niche and risk to humans.

10.1 Introduction

Free-living amoebae (FLA) are commonly found in all environments and cosmopolitan in distribution. They live as phagotrophs in aquatic environments (e.g. ponds, rivers, stream, lakes, etc.), feed on bacteria and are protozoal fauna of soil. Several species of free-living amoebae (FLA) belonging to the genera *Acanthamoeba, Naegleria*, *Balamuthia* and *Sappinia* have been recognised as being responsible for causing a fatal syndrome of the central nervous system (CNS) in humans and animals [1]. Additionally, several species of *Acanthamoeba* may cause localised extra-CNS infection in immunocompetent hosts or disseminated infection in immunocompromised hosts. Furthermore, *Acanthamoeba* is also a well-known causative agent of eye keratitis in humans. Other genera such as *Hartmannella* and *Vahlkampfia* were occasionally reported to be associated with keratitis [2]. All of these amoebae have a free-living existence and do not have a human carrier state (which is important in disease transmission). They also have limited association with poor sanitation and involve no insect vector. FLA have environmentally stable cyst forms and can take advantage of the tropical conditions to parasitise humans in their outdoor pursuits. To date, there are either limited or no reports of FLA infection as well as research work from Southeast Asian countries such as Indonesia, East Timor, Cambodia, Laos and Myanmar. However, species of *Acanthamoeba*, *Naegleria*, *Balamuthia*, *Vahlkampfia* and *Hartmannella* were occasionally reported as infection cases or in research work from Thailand, Malaysia, Singapore, Vietnam and the Philippines.

10.2 Morphological Characteristics

Except *Naegleria* that has an extra transition flagellate stage from its trophozoite, all of the mentioned FLA consist of two morphological forms in their life cycle, namely the trophozoite and the cyst. Trophozoite is the feeding stage which will turn to a dormant cyst stage in unfavourable conditions such as lack of food, crowdedness and desiccation. All of the three stages may enter the host but trophozoite is the most commonly stage to infect the host. Under microscopy, the trophozoites are characterised as a single nucleus which consists of a prominent

central nucleolus with the specific characteristic of a spike-like acanthopodia (*Acanthamoeba*), rapidly slug-like movement by using broad pseudopodia (*Naegleria*) and spider-like movement using broad or radiating pseudopodia (*Balamuthia*). While the cysts are characterised as thick walled, consist of two-wrinkle layers and variable in shape (*Acanthamoeba*), two smooth layers that are only presented in a rounded form (*Naegleria*) and rounded with a triple-layered cell wall (*Balamuthia*) [3]. The flagellate of *Naegleria fowleri* is a temporary stage and is transformed from the trophozoites when there is a change in ionic concentration. The flagellates exhibit pear-shaped morphology with typically two flagella at the anterior end and have a diameter between 10 and 16 μm. In the laboratory, transformation can be performed by incubating the trophozoites in distilled water or buffer such as Page's amoebic saline and 2 mM Tris [4]. The morphology for all stages of *Acanthamoeba*, *Naegleria* [5] and *Balamuthia* are shown in Figs. 10.1, 10.2 and 10.3.

10.3 Clinical Manifestations and Diseases

Two distinct CNS diseases are granulomatous amoebic encephalitis (GAE) and primary amoebic meningoencephalitis (PAM). GAE is a subacute-to-chronic disease caused by several species of *Acanthamoeba* or *Balamuthia mandrillaris*. Some reports preferred to use the term *Balamuthia* amoebic encephalitis (BAE) in cases that are due to *B. mandrillaris* infection. Both of these amoebae can enter the skin through a cut, wound or through the nostrils. Once inside the body, the amoebae travel through the bloodstream to other parts of the body, especially the lungs, spinal cord and brain. In the case of nasal passages, the amoebae trophozoites may also migrate directly to the brain through olfactory nerves without needing to enter the lower respiratory tract [6]. GAE due to *Acanthamoeba* infection is commonly reported in the elderly and immunocompromised (including those with neoplasia, systemic lupus erythematosus, human immunodeficiency virus and tuberculosis), while *B. mandrillaris* is reported in both healthy and immunosuppressed patients. Alcoholism, drug abuse, chemotherapy, corticosteroids and organ transplantation are also possible risk factors for these amoebae infections. GAE can present with focal paralysis, seizures, brainstem symptoms and other neurological problems, some of which may mimic glioma (especially brainstem glioma) or other brain diseases. These symptoms are caused by inflammatory necrosis of the brain tissue brought on by amoebic infiltrates. The infected person may suffer headaches, stiff neck, nausea and vomiting, tiredness, confusion, lack of attention to people and surroundings, loss of balance and bodily control, seizures and hallucinations. The incubation period is unknown but is estimated at several weeks to several months. Symptoms progress over several weeks and death usually occurs.

Primary amoebic meningoencephalitis (PAM) is an acute fulminant necrotising meningoencephalitis caused by *N. fowleri*. Currently only this species has been associated with human disease and the route of entry into a healthy human body is

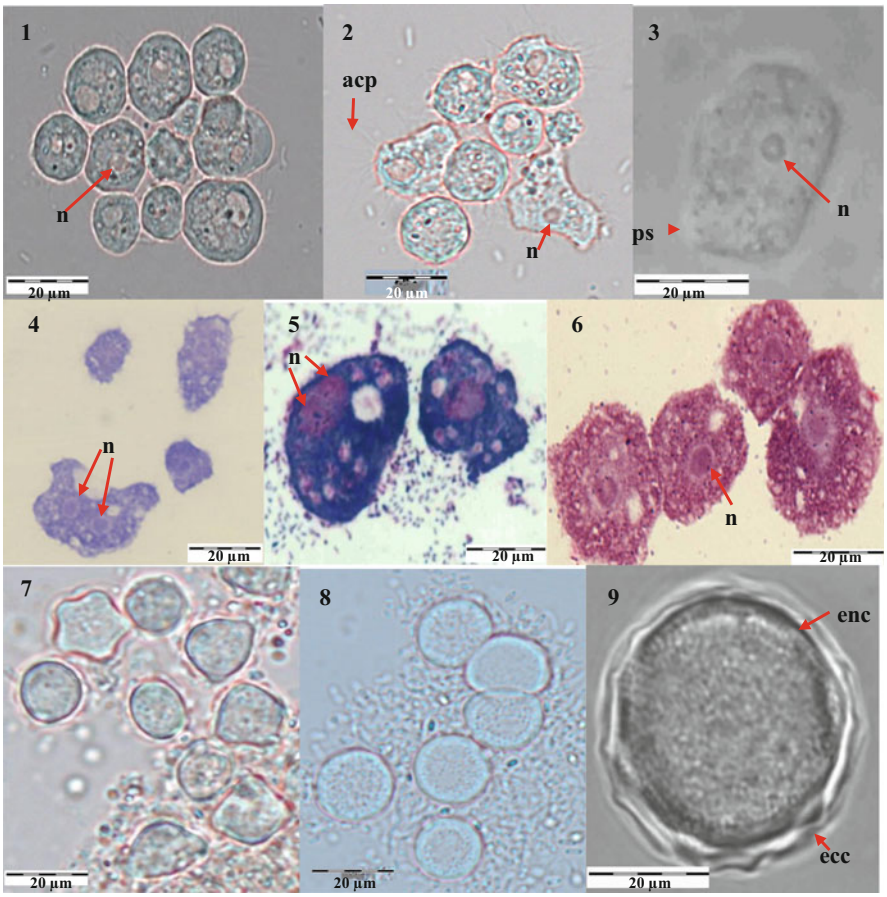

Fig. 10.1 Trophozoite and cyst stage of *Acanthamoeba* observed under light microscope ([5], with permission from Southeast Asian Journal of Tropical Medicine and Public Health) (*1*) Rounded trophozoites in cold buffer after being detached from agar surface, (*2*) various shapes of trophozoites showing acanthopodia, (*3*) a single trophozoite showing pseudopodia and nucleus, (*4*) Giemsa stain shown some trophozoite exhibiting double nuclei, (*5*) trophozoites stained with modified Field's stain, (*6*) trophozoites stained with modified acid fast bacilli stain kit (Merck), (*7*) several cysts shape (*round*, *triangle* and *square*) cultured from environmental sample, (*8*) rounded cysts cultured from cat's cornea swab and (*9*) a rounded cyst exhibiting wrinkled ectocyst and endocyst: nucleus (n), acanthopodia (acp), pseudopodia (ps), ectocyst (ecc) and endocyst (enc)

by inhalation of trophozoites, flagellates or cysts through the nasal passages especially during water-related activities (e.g. swimming, diving and Muslims' ritual ablution). The amoebae invade the nasal mucosa and migrate along the olfactory nerves through the cribriform plate and eventually invade the brain. The term meningoencephalitis denotes the severity of the disease, in which both the brain and membranes (referred to as 'meninges' in Greek, which means membranes) that cover the central nervous system are infected [7]. An infected patient may show

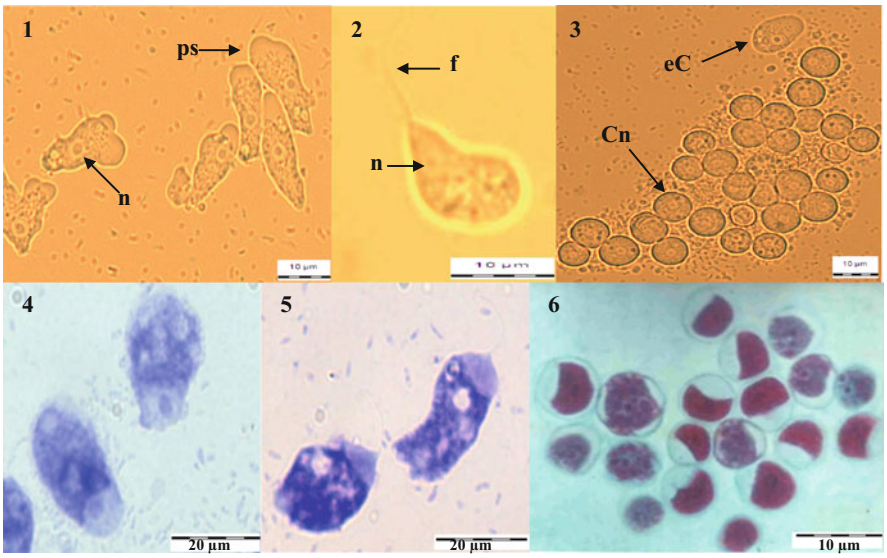

Fig. 10.2 Morphological observation of *Naegleria* stages under a light microscope ([5], with permission from Southeast Asian Journal of Tropical Medicine and Public Health). (*1*) Trophozoites shown a nucleus, (*2*) flagellate stage, (*3*) rounded cysts, (*4*) Giemsa stain of trophozoites, (*5*) Giemsa stain of flagellate stage and (*6*) trichrome-eosin stain of cysts: nucleus (n), eruptive lobopodia/pseudopodia (ps), flagella (f), cyst with nucleus (Cn) and early cyst stage (eC)

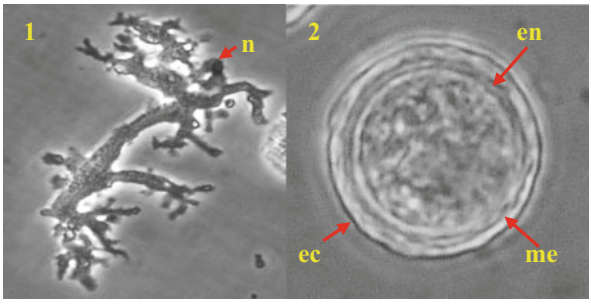

Fig. 10.3 Trophozoite and cyst of *Balamuthia mandrillaris* observed under inverted microscope. (*1*) Trophozoites (×400) with nucleus (n) and radiating pseudopodia and (*2*) cyst (×400) with three layers walled of ectocyst (ec), mesocyst (me) and endocyst (en)

initial symptoms including (but not limited to) changes in taste and smell, headache, fever, nausea, vomiting and stiff neck. This would be followed by confusion, hallucination, lack of attention, ataxia and seizures. The symptoms typically begin between day 3 and day 8 of the incubation period. Patients usually die within 7–10 days after onset of symptoms.

In addition, *Acanthamoeba* has the capability to infect the eye and cause keratitis. Risk factors include contact lens wear, corneal trauma and low levels of

anti-*Acanthamoeba* IgA in tears [8] followed by exposure to the organism (often through contaminated water). *Acanthamoeba* keratitis (AK) patients may complain of pain, decreased vision, redness, foreign body sensation, photophobia, tearing and discharge. Possible early signs are epithelial irregularities, epithelial or subepithelial infiltrates and pseudodendrites. Later signs include stromal infiltrates (ring shaped, disciform or nummular), satellite lesions, epithelial defects, radial keratoneuritis, scleritis and anterior uveitis (with possible hypopyon). Advanced signs include stromal thinning and corneal perforation. Cases of corneal perforation may need to be managed by surgical interventions or else can lead to vision loss.

Infections caused by these FLA are still unfamiliar to clinicians, pathologists and laboratorians in most of the Southeast Asian countries. Moreover, the infections are difficult to diagnose unless suspected. Each laboratory should have an experienced personnel that is familiar with the morphological characteristics of the amoebae and knows the suitable samples and diagnostic tools that need to be used for definite diagnosis of the infection.

10.4 *Acanthamoeba* Species

Only several species of *Acanthamoeba* such as *A. castellanii*, *A. polyphaga*, *A. culbertsoni*, *A. palestinensis*, *A. astronyxis*, *A. hatchetti*, *A. rhysodes*, *A. divionensis*, *A. quina*, *A. lugdunensis* and *A. griffini* are human pathogens [1]. Its life cycle consists of two stages: a trophozoite (which is 8–40 μm in diameter) and a cyst (which has a double-layered wall with a diameter of 8–26 μm). Although *Acanthamoeba* species are commonly present in most environments, human contact with the organism rarely leads to infection.

Earlier in history, *Acanthamoeba* keratitis was associated with corneal trauma, which is the main portal of entry, followed by exposure to contaminated water. Mild antecedent trauma was the most important predisposing factor for *Acanthamoeba* keratitis [9, 10]. It is mostly seen in patients who are agricultural workers with a history of eye injury [11, 12]. Concurrently, the majority of Southeast Asian population is dependent on agriculture to make a living, and injury to the eye is common during their daily activities, thus *Acanthamoeba* keratitis is believed to occur more frequently than the reported cases in this community. In addition, it is difficult to arrive at the right diagnosis for most of the cases even though the patients are presented early. Very often, the patients are first seen by the general practitioners or family physicians, who may not be aware of such infections. Subsequently, the patients might be wrongly treated for other causes of keratitis which might not be effective for *Acanthamoeba* infection. They would only be referred to the ophthalmologist later, when their conditions have deteriorated. Hence, due to such a relatively rare condition of the disease and its legendary resistance to treatment, permanent and severe visual loss is still a common threat in many cases.

Recently, *Acanthamoeba* keratitis has been increasingly recognised and diagnosed, along with the spread of contact lens use. In Malaysia, although not reported, more cases were diagnosed after the first reported case which involved a female contact lens wearer [13]. Currently, diagnosis of this organism is also being introduced to be carried out in patients who are noncontact lens wearers with keratitis symptoms. In Thailand, a review from 1996 to 2006 at Siriraj Hospital identified 8 and 14 cases of noncontact lens (nCL) and contact lens (CL) wearers, respectively. The nCL group had consulted the ophthalmologist much later (nearly 1 month) compared to the CL group (within 1 week). Delayed diagnosis in nCL patients showed more severe clinical features and poorer prognosis improvement in visual outcomes. In Singapore, 42 acanthamoebic keratitis patients (affecting 43 eyes) who were treated between 2000 and 2007 were all contact lens wearers [14]. Each of the cases was identified from the recorded data of diagnosis by microbiologic culture, microbiologic and histological analysis and based on the clinical features and response to treatment with the contact lens solution data that were available. Analysis of related data indicated that multipurpose solution (MPS) was implicated in the outbreaks of AK in Singapore at 2007, when eight local patients were treated [14]. MPS was later found out to induce *Acanthamoeba* cyst [15] which would become resistant and in turn make any infection very difficult to treat. Early diagnosis to detect this organism has led to the good outcome of treatment regimen with anti-*Acanthamoeba* eye drop (e.g. chlorhexidine, polyhexamethylene biguanide, hexamidine and propamidine isethionate) with the duration of therapy ranging from 15 to 283 days. Combination with antibiotic (e.g. cefazolin, gentamicin and levofloxacin) and antifungal eye drops showed an increased in prognosis, since in many cases, *Acanthamoeba* was found coinfecting with several other bacteria such as *Pseudomonas aeruginosa*, nonfermentative gram-negative rods, *Stenotrophomonas maltophilia*, *Serratia marcescens*, *Klebsiella oxytoca*, *Escherichia coli* and alpha haemolytic *Streptococcus*. Delay in diagnosis may yield severe visual outcomes and require surgical treatment [14, 16].

As for GAE cases, they have only been reported in Thailand, out of all Southeast Asian countries. GAE cases have been sporadic after the first case was highlighted by Jariya et al. [17]. To date, at least 11 cases (8 were fatal and 3 were cured) have been reported in Thailand [17–20]. The patients were from two groups: seven patients who had underlying diseases causing impaired immunity and four patients without underlying diseases. Of these, only one patient had a history of exposure to water. The rest were most likely common foci of the pathogenic protozoa in the lung and skin reaching the terminal in CNS, usually owing to the haematogenous spreading process [21]. All of these cases showed symptoms and signs of impairment of consciousness, high fever, headache and stiff neck. Laboratory investigation reported that there was no *Acanthamoeba* in the cerebrospinal fluid (CFS) except in three nonlethal cases, despite the numerous amount of this organism (trophozoites and cysts) detected from autopsy specimens. Most cases presented mononuclear pleomorphic in the CSF profile and were misdiagnosed as microbial meningitis. Inability to detect the amoebic trophozoite can be another cause of delayed specific treatment [22]. However, early detection in CFS of all three cured

cases yielded a good outcome of treatment regiments with a combination of intravenous amphotericin B and oral rifampicin for 1–2 months [19].

Subsequently, *Acanthamoeba* has been increasingly studied especially in Thailand, Malaysia and the Philippines. This organism is isolated from feline corneas, contact lens equipments, various aquatic environments (swimming pool, recreational water, household water tanks, open water storage tanks, natural water sources, etc.), dust and soil [23–31]. Currently, molecular identification has been established to determine the diagnosis fragment 3 (DF3) sequence of 18S ribosomal DNA gene region in order to identify the genus, genotype and pathogenic potential of *Acanthamoeba* [32]. Of all 17 genotypes (T1–T17) identified up to date, T4 was determined to be predominant among both clinical specimens and environmental sources [33]. In Malaysia, several genotypes of *Acanthamoeba* which showed 99–100 % homology with Genbank reference isolates of *A. castellanii* (T4), *A. culbertsoni* (T4), *A. griffini* (T3), *A. hatchetti* (T4), *A. lenticulata* (T5), *A. triangularis* (T4) and *A. quina* (T4) were isolated from the Malaysian indoor dust [24]. In the Philippines, *Acanthamoeba* genotypes T4 and T5 were commonly detected in contact lens storage cases, soil and water, while T3 and T15 were less common [29, 34]. A more interesting finding is from Thailand, where novel T17 genotypes were detected from environmental isolates and T10 genotype in a Thai female keratitis patient [35]. *Acanthamoeba* genotype T4 assemblage *A. castellanii* CDC:0184:V014 (U07401) originating from human keratitis was also detected in naturally infected feline corneas with keratitis symptoms [26]. On the other hand, *Acanthamoeba* was documented to be associated with several environmental bacterial species as endosymbionts or hosts and vehicles for pathogenic bacteria [36]. Concerning endosymbionts, they were documented in xenic and axenic isolates of *Acanthamoeba* but none of the pathogenic *Legionella* spp. was detected [37, 38].

10.5 *Naegleria* Species

Little is known about the presence of *Naegleria* species in Southeast Asia. To date, detection of the pathogenic *Naegleria* species, *Naegleria fowleri*, has only been reported in two countries: Thailand and Vietnam. In Thailand, the first fatal human case of PAM was reported in 1983. It happened to a 5-year-old boy with a history of swimming in the pond along a ricefield. As prompt treatment was not given to the child, he died 3 days after admission [39]. About nine fatal PAM cases involving six males and three females were subsequently reported during the summer months of 1987 to 2001. Almost all of these individuals had a history of water-related activities including swimming in canals, rivers, community pools and exposure to contaminated water during a traditional Thailand festival called ‘Songkran’, where people splash water at each other [40–42]. In addition, two nonfatal PAM cases were reported in a 61-year-old male and an 18-year-old female, respectively. Both of these patients were treated with a combination of drugs containing

amphotericin B, rifampicin and ketoconazole [43, 44]. In Vietnam, two fatal human cases due to PAM were described in 2012. The first case was reported in a 25-year-old male with a history of fishing for oysters and snails underwater at a lake [45]. The second PAM victim was a 6-year-old male without any history of swimming or contact with natural water sources [46].

In a prevalence study involving water samples, *Naegleria* species was detected up to 31 % in natural water as well as hot springs in the central and southern part of Thailand [27, 30]. Besides in water samples, thermophilic *Naegleria* species have also been detected in soil samples collected from Thai paddy fields [31, 47]. *Naegleria fowleri* occurred in 10 % of the total *Naegleria* species detected from thermally polluted water [48]. The pathogenicity of *N. fowleri* was also reported based on its morphological characteristics and pathology results after nasal inoculation in laboratory mice [48].

In Malaysia, the number of studies on the existence of *Naegleria* species in the environment is very limited. In a single study, *Naegleria* species were isolated from 39 environmental samples including 14 swimming pools, 10 recreational lakes, 5 streams, 4 water tanks and 6 air-conditioner units from Klang Valley. *N. philippinensis* was the most common species detected, and none of the samples were positive for *N. fowleri* by PCR and DNA sequencing. In addition, a new species of *Naegleria* was detected in the Malaysian environment based on the differences in DNA sequences after being blasted with the reference DNA sequences available from the Genbank [49].

Currently, there is only a single species of *Naegleria* from Indonesia which was isolated from Bali called *N. indonesiensis* strain NG945. The maximum growth temperature for this strain is between 37 and 39 °C. Interestingly, unlike other *Naegleria* species, the *N. indonesiensis* strain NG945 was unable to transform into the temporary flagellate stage even after using several enflagellation assays including incubation with 2 mM Tris buffer at different temperatures (20 and 30 °C) with or without agitation [4]. In the Philippines, a nonpathogenic strain of *Naegleria* termed as *N. philippinensis* strain RJTM was isolated from a CSF sample obtained from a patient diagnosed with encephalitis. The findings which demonstrated that RJTM strain could only grow at temperatures of up to 40 °C and did not cause death to experimental mice support that it is not a pathogenic species [50].

10.6 *Balamuthia mandrillaris* and Other Possible Free-Living Amoebae

To date, the only available report on the presence of *B. mandrillaris* in Southeast Asia is from Thailand. A fatal case of *Balamuthia* amoebic encephalitis was reported in a 23-year-old healthy male. He was suspected to be infected with *B. mandrillaris* through a lesion on the nose after falling into a swamp during a motorbike accident. *Balamuthia mandrillaris* was not detected in the patient's CSF

but haematoxylin and eosin staining of brain sections following autopsy revealed large numbers of *B. mandrillaris* trophozoites with a few cysts [51].

Other FLA such as *Vahlkampfia* and *Hartmannella* species have also been reported in Thailand and Malaysia, but only from environmental samples [47, 49].

10.7 Diagnosis of *Acanthamoeba* Species, *N. fowleri* and *B. mandrillaris*

Due to the rapid disease progression, absence of amoebae in cerebrospinal fluid (CSF), misdiagnosis or unfamiliarity, majority of GAE and PAM cases were diagnosed at autopsy. In Southeast Asia, all cases were confirmed by the morphological observation of amoebae in the CSF or brain biopsy. Direct microscopic examination is performed by placing one or two drops of CSF (or brain biopsy smear if available) specimen on a glass slide which is then observed for the presence of motile trophozoites or cysts under a light microscope. CSF and biopsy of the brain tissue can also be cultured on 1.5 % non-nutrient agar seeded with gram-negative bacteria such as *Escherichia coli* [52]. The cultures are then incubated at room temperature or 37 °C for at least 24 h before being observed for the presence of the trophozoite stage. Morphological observation needs experienced diagnostic staff and the used of suitable clinical samples. All clinical specimens should be kept and transported at room temperature to the laboratory since the trophozoite stage is very fragile and could be destroyed at refrigerator temperature. Many cases have failed to detect the presence of amoebae in the CSF. This could be due to several factors including very few amount of amoeba, amoeba that was destroyed during transportation or absolutely no amoeba in the CSF. The amoeba may also be accumulated in the membranes that cover the central nervous system and did not extract in the cerebrospinal fluid, thus showing no amoeba in the CSF specimen.

Immunodiagnostic methods, particularly indirect immunofluorescence (IIF), have also been increasingly applied for the confirmation of pathogenic *N. fowleri* in clinical samples. Briefly, CSF or brain sections are fixed and incubated with either anti-*N. fowleri* monoclonal or polyclonal antibodies. Following these, the specimens are incubated with FITC-conjugated secondary antibody and examined with an immunofluorescence microscope [53, 54]. Recently, an enzyme-linked immunosorbent assay for a rapid detection of *N. fowleri* from clinical and environmental samples has been developed and made commercially available in a kit format. The kit is based on the use of a monoclonal antibody (5D12) that recognises a glycosylated epitope on *N. fowleri* [55].

Molecular approaches, particularly polymerase chain reaction (PCR) and DNA sequencing, have recently been preferred for the diagnosis of *Acanthamoeba* spp. and *N. fowleri* infections. A PCR assay which amplifies the diagnostic fragment 3 (DF3) sequence of 18S ribosomal DNA gene region could identify the genotype

of *Acanthamoeba* species [32]. However, the ribosomal internal transcribed spacer (ITS) region has been developed and used for differentiating species within *Naegleria* genus [56, 57]. A multiplex real-time PCR targeting the nuclear small subunit ribosomal genes (18S rRNA gene) was developed in 2006 for a simultaneous detection of *N. fowleri*, *B. mandrillaris* and *Acanthamoeba* spp. in clinical samples [58].

As for *B. mandrillaris* infection, the gold standard for laboratory diagnosis is indirect immunofluorescent (IIF) staining of clinical tissue sections of the brain, kidney, lung or skin using polyclonal antibodies raised in rabbits [59]. In addition, serum titres (concentrations) of *Balamuthia* antibodies in BAE patients can also be revealed using the IIF [60]. Other immunological methods used include enzyme-linked immunosorbent assay (ELISA) [61]. The PCR targeting the mitochondrial 16S rRNA gene has been developed and successfully used to detect *B. mandrillaris* in clinical and environmental samples [62]. The mitochondrial primers (5′ Balspec 16S and 3′ Balspec 16S) are genus specific for *Balamuthia* with a PCR product of 1,075 bp [63, 64].

Unlike *Acanthamoeba* and *Naegleria*, *Balamuthia* does not feed on bacteria and therefore is not suitable to be cultured on non-nutrient agar-*Escherichia coli* plates [65]. The best way to culture *B. mandrillaris* is by using mammalian cell monolayers, such as green monkey kidney cells and human brain microvascular endothelial cells (HBMEC) at 37 °C [3, 66]. Clinical samples, including macerated brain tissue or CSF, can be cultured using this technique [59, 67]. Amoebae may emerge after several weeks following inoculation of the mammalian monolayer. As the amoebae feed on the monolayer, patches of plaques will be formed and the areas are filled with *Balamuthia* trophozoites [59]. In addition, *Balamuthia* can also be cultured on non-nutrient agar plates coated with smaller protozoa including *Acanthamoeba* and *Naegleria* [68].

10.8 Conclusion and Future Task

Concerning the CNS infections reported in Thailand and Vietnam, all of the lethal cases were misdiagnosed as microbial meningitis due to the inability to detect the amoebic trophozoite in the CSF. On the other hand, in cases where amoebae in the CFS was detected early, a good outcome of treatment regiments was achieved, which cured 3 GAE and 2 PAM cases in Thailand. However, an efficient diagnosis depends on the practitioners' familiarity with the symptomatology and the use of appropriate clinical material and tools for a fast and definitive diagnosis. In addition, early consultation and appropriate antimicrobial therapy that may improve chances of survival should be considered for any individual with clinical signs of early meningoencephalitis, CSF findings which are consistent with meningitis, a CSF gram stain showing no organisms and a history of water exposure which involves inhalation of water through the nostrils.

Acanthamoebic keratitis (AK) cases have increased dramatically with the popularity of contact lens wear, and the disease is becoming more recognised among Southeast Asian countries. AK can occur in patients of any age, sex or race, but mostly manifests itself in young and healthy adults. Other than wearing contaminated contact lenses, risk factors of AK also include corneal foreign body, contact with non-sterile water, bullous keratopathy, neurotrophic keratopathy, herpes simplex keratitis, radial keratotomy, swimming and scuba diving, basement membrane dystrophy and bacterial keratitis. Cases sometimes arise even with no identifiable risk factors. Essentially, any event that disrupts the corneal epithelium is a potential risk factor for AK. The common symptoms include watery eyes, eye pain with photophobia, blurred vision and irritation. However, the presence of co-infection with bacteria usually causes difficulty in diagnosing *Acanthamoeba*. AK reported among patients who do not use contact lenses are due to delayed diagnosis, which resulted in more severe ocular manifestations and poorer prognosis. Physicians should be aware of *Acanthamoeba* infection as a cause of keratitis in any patient and not only contact lens wearers. AK should be considered as part of the differential diagnosis of most cases of presumed microbial keratitis, as early diagnosis and prompt treatment are associated with improved clinical outcome. However, long period of follow-up are recommended to observe recurrent episodes and proper management of AK patients [14, 16].

Although none of the FLA cases have been reported in Indonesia, East Timor, Cambodia, Laos, Myanmar and the Philippines, it does not imply that there were absolutely no infected patients. Perhaps the cases could either be undiagnosed, misdiagnosed or overlooked. In addition, the rapid disease progression and limited awareness among the clinicians and diagnostic staff make the diagnosis of FLA diseases a challenging task. It is also important to detect the presence of pathogenic FLA in the environment such as in water and soil samples in order to introduce and recognise their occurrences in particular areas. Not to be forgotten are rare cases such as cutaneous acanthamoebiasis [69] caused by both *Acanthamoeba* spp. and *B. mandrillaris* which are usually present in GAE patients and *Sappinia* amoebic encephalitis [70] caused by *S. pedata*, although they have not been reported in the Southeast Asian region.

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Chapter 11
Soil-Transmitted Helminths: The Neglected Parasites

Hesham M. Al-Mekhlafi, Yvonne A.L. Lim, Norhayati Moktar, and Romano Ngui

Abstract Soil-transmitted helminth (STH) infections are still considered to be the most prevalent infections of humankind. STH, traditionally endemic in rural areas, are increasingly becoming a public health concern in urban slums of cities in tropical and subtropical developing countries in Southeast Asia, sub-Saharan Africa and Central and South America. These helminths, *Ascaris lumbricoides*, hookworm (*Ancylostoma duodenale* and *Necator americanus*), *Trichuris trichiura* and *Strongyloides stercoralis*, can live in silence as chronic infections with prominent morbidity amongst children and mothers of childbearing age. The main impact of STH infections is their associations with malnutrition, vitamin A deficiency (VAD), iron-deficiency anaemia (IDA), intellectual retardation and cognitive and educational deficits. The devastating consequences of these helminths during childhood may continue into adulthood with effects on the economic productivity which trap the communities at risk of infections in a cycle of poverty, underdevelopment and disease. Hence, the WHO regarded the control of STH amongst the top five health priorities within the global massive effort to eradicate poverty. Moreover, controlling STH infections has significant positive impacts on the nutritional status and educational performance of the vulnerable children in endemic communities.

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11.1 Introduction

Soil-transmitted helminths (STH) (or geohelminths) are nematodes with round, non-segment elongated cylindrical bodies. The species of medical importance include *Ascaris lumbricoides* (roundworms), *Trichuris trichiura* (whipworms), *Ancylostoma duodenale* and *Necator americanus* (hookworms) and *Strongyloides stercoralis* (threadworm). They are classified as 'soil-transmitted helminths' because the eggs/larvae passed in faeces need about 3 weeks to mature in the soil before they become infective.

Infections by STH or soil-transmitted helminthiasis are the most common infections of humankind. Recent global estimates suggest that *A. lumbricoides* infects 1.2 billion people, *T. trichiura* infects 800 million, hookworms infect 750 million and *S. stercoralis* infects 100 million people [1]. Moreover, an estimated 5.3 billion people, including 1 billion school-aged children are at risk of infection with at least one STH species, with 69 % of them living in Asia, 22 % in Africa and the Middle East and 9 % in Latin America and the Caribbean [2]. Together with schistosomiasis, STH infections represent more than 40 % of the disease burden caused by all tropical diseases except malaria [3]. By using a metric measurement known as DALY (disability-adjusted life year, i.e. the numbers of years lost from premature death or disability), STH results in 40 million DALYs lost annually which accounts for about 20 % of DALYs lost due to infectious diseases globally [4]. The most important reasons for high DALY values due to STH were based on the association of hookworm with anaemia, ascariasis with growth stunting and trichuriasis with decreased school performance [5]. Although STH infections rarely cause death, recent estimates attributed 65,000 annual deaths to hookworm infections, 60,000 annual deaths to ascariasis and 10,000 annual deaths to trichuriasis [6]. However, the morbidity caused by STH is most commonly associated with infections of moderate to heavy intensities [7]. Certain groups of people including preschool children, school-aged children and women of childbearing age are more susceptible to higher morbidity and mortality rates than others [8]. Hence, STH infections are amongst the greatest challenges to health and economic development of developing countries.

Despite the fact that STH infections are highly prevalent, disability-inducing and poverty-promoting, they are classified amongst the neglected tropical diseases (NTDs) [1]. These diseases are classified as 'neglected' because they persist exclusively in the poorest populations often living in remote, rural areas, in urban slums or in conflict zones and have been largely eliminated in developed countries and thus are often forgotten.

11.2 Aetiology and Burden of STH Infections

11.2.1 Ascaris *Infections*

Ascaris lumbricoides is probably the first etiologic agent of infection ever described in humans with descriptions of the parasite going back to ancient times and the first scientific description dating back to 1683 [9]. The adult stage of *A. lumbricoides* is a cylindrical pink- or cream-coloured worm of the family Ascarididae and the superfamily Ascaridoidea. *Ascaris* is the largest intestinal nematodes infecting humans with male being smaller (120–250 mm in length and 3–4 mm in width) than the female (200–400 mm in length and 5–6 mm in width). The adult worm preferentially resides in the jejunum where it orients with the head facing the direction of the intestinal flow [10]. Mature female *A. lumbricoides* worms produce 100,000–200,000 fertilised or unfertilised eggs per day. Eggs excreted in stool require a period of maturation in soil. The period of development in the soil is temperature dependent and may range from 2 weeks to several months. The infective stage is a second stage larva within the egg. The larvae that emerge from ingested eggs in the jejunum penetrate the intestinal wall and migrate through hepatic venules to the right side of the heart and the pulmonary circulation, where they penetrate into the alveolar spaces and undergo two further moults. From the alveoli, the 1.5 mm long larvae ascend to the trachea and are swallowed, undergo a last moult in the small intestine and develop to adults. From ingestion of infective eggs to the production of eggs by mature adult worms, development takes about 10–12 weeks and the adult worm has a life span of about a year.

During early infections, the invasive larval stages of *Ascaris* will elicit a host eosinophilic inflammatory response in the liver (hepatitis) and lung (Loeffler's pneumonitis). The phase of larval migration is a distinctive type of pneumonitis known as Loeffler's syndrome (simple pulmonary eosinophilia) characterised by mild group of symptoms, a scarcity of physical signs, a blood eosinophilia varying from less than 10–60 %, a benign course and spontaneous healing within a period of 2–3 weeks and transient pulmonary infiltration. There are very few reports of Loeffler's syndrome in Southeast Asia despite the fact that ascariasis is common in this region [11]. Moreover, intestinal and bile duct obstructions are the most common complications associated with ascariasis [12].

11.2.2 Trichuris *Infections*

Trichuris trichiura is a member of the nematode superfamily Trichiuroidea and therefore related to the pathogen *Trichinella spiralis* [13]. The adult worm is approximately 4 cm long; its whip-like shape refers to the wider posterior section and the long, finely attenuated anterior end [13]. Eggs passed in the faeces of an infected individual have a classic barrel shape with a plug at each pole. After

embryonation in the soil, a process that requires 2–4 weeks, a larva develops. Human transmission occurs by the ingestion of the embryonated eggs, which release larvae that moult and burrow into the colonic mucosa upon arrival into the large intestine. The larva buries its entire body in the epithelium of large intestine forming a tunnel. As the worm mature, its posterior end is extruded from the tunnel and hangs freely in the lumen of intestine.

Trichuris causes host injury both through direct effects by invading the colonic mucosa and through the systemic effects of infection. The cecum is the preferential site for invasion although heavy infections will extend throughout the colon and even distally to the rectum. Contact of the anterior end of the adult worm with the mucosa of large intestine causes inflammation resulting in the disruption of the normal colonic architecture [8]. In severe chronic infection, the mucosa becomes oedematous and friable which leads to rectal bleeding (whipworm dysentery) with abdominal pain and rectal tenesmus. Frequent straining as a result of rectal tenesmus causes rectal prolapse. Several investigators have pointed out the clinical similarities between the paediatric colitis caused by *Trichuris* infection and the more established causes of inflammatory bowel disease such as Crohn's disease and ulcerative colitis.

11.2.3 Hookworm Infections

Hookworms are nematodes belonging to the family Ancylostomatidae, a part of the superfamily Strongyloidea. The two major genera that affect humans, *Necator* and *Ancylostoma*, are characterised by the presence of oral cutting organs in the adult stages [14]. The major representative of the genus *Ancylostoma* to infect and complete development in humans is *Ancylostoma duodenale*. In contrast to the major human (anthropophilic) species, *Ancylostoma ceylanicum*, a parasite of dogs and cats, is also infective to humans in some regions of Asia, but it is not considered a major pathogen [14]. Other canine and feline hookworms such as *Ancylostoma caninum* and *Ancylostoma braziliense* cause zoonotic diseases in humans, for example, eosinophilic enteritis and cutaneous larva migrans (CLM), respectively [15, 16].

There are significant pathobiological differences between the two major human hookworms. Unlike *N. americanus*, which can complete its life cycle in humans only after skin penetration by filariform larvae, *A. duodenale* is also transmitted by oral ingestion of the infective larvae. *N. americanus* is smaller than *A. duodenale* and produces fewer eggs and causes less blood loss.

The adult worms live in the small intestine (particularly in jejunum) of man, attaching themselves to the intestinal epithelium by means of their mouth part. Hookworm induces blood loss directly through mechanical rupture of host capillaries and arterioles followed by the release of pharmacologically active polypeptides including anticoagulants, antiplatelet agents and antioxidants [17]. Chronic intestinal bleeding causes hookworm-induced protein loss and iron-deficiency

anaemia. In addition, hookworm-associated iron deficiency during childhood is partly responsible for its physical and mental growth retardation effects [18]. The growth stunting effects of hookworm were well documented by the early part of the twentieth century [19], as were some of the effects of hookworm on intelligence quotient.

Studies of the association of anaemia with hookworm blood loss indicate that there is a disproportionate reduction in plasma haemoglobin concentration after some threshold worm burden is exceeded [20]. Although the adult hookworms elicit most of the pathology attributed to hookworm, the infective larval stages also release macromolecules upon host entry that contribute to morbidity. These include hookworm-derived allergens and tissue invasive enzymes [17]. Some of these molecules contribute to the pathogenesis of dermatitis (ground itch) and hookworm pneumonitis.

11.2.4 Strongyloides *Infections*

Strongyloides stercoralis, known as threadworms, is an opportunistic nematode. Adult worms reside in the intestinal wall of the small intestine. The adult female is parthenogenetic (self-fertilising), rarely seen in stool and is approximately 2 mm in length. It has a short buccal cavity and a long, thin oesophagus. Male worm is shorter and broader than the female [21].

The female is ovoviviparous. The embryonated eggs hatch in the mucosa of the intestine release the noninfective rhabditiform larvae in the host intestine and these are usually detected in stool samples. The rhabditiform larvae passed in stool is approximately 200–400 μm long and 15–20 μm wide and have a short buccal cavity and a prominent genital primordium. While in the lumen of the intestine, the rhabditiform larvae metamorphose into filariform larvae; they may penetrate the intestinal epithelium, causing internal autoinfection, or carried down the intestine and penetrate the perianal and perineal skin, causing external autoinfection. The length of filariform larva is up to 680 μm with a longer oesophagus than the hookworm and a notched, rather than a pointy, tail. The rhabditiform larva may be voided with the faeces and may undergo development in the soil through direct or indirect cycle. In direct cycle, the rhabditiform larva metamorphoses in 3–4 days into filariform larva. In the indirect cycle, also known as the free-living phase, the rhabditiform larvae develop into free-living male and female adults in the course of 24–30 h.

Strongyloidiasis is transmitted by penetration of the skin by the infective filariform larvae in contaminated soil. Although some itching is common during skin penetration, there are few other symptoms associated with this stage of disease and allergic reactions may also occur. Pulmonary symptoms may be present during the migratory phase of the filariform larvae in the lungs. Diarrhoea and abdominal pain caused by the adult worms frequently accompany the intestinal phase of the disease [8].

In immunocompromised individual, autoinfection may lead to the hyperinfection syndrome, which may occur years after the initial infection. Characteristic clinical features of hyperinfection syndrome are larva currens (dermatitis caused by penetration of filariform larvae around the perianal and perineal skin) and dissemination of filariform larvae to the extraintestinal organs. During autopsy, larvae have been found in many organs such as liver, lungs, heart, kidneys and brain. Sepsis and meningitis, often polymicrobial, may develop with the spreading of enteric bacteria from intestine to the circulation [8].

11.3 Epidemiology and Dynamic Transmission of STH Infections in the Southeast Asian Region

Southeast Asia represents one region of the world in which STH are considered to be highly endemic and where these infections constitute a significant public health risk.

Previous studies in **Thailand** demonstrated that STH infections appear to be largely controlled in urban populations, with prevalence amongst urban school children being as low as 0.3 % for hookworms and 0.05 % for *Ascaris* [22]. However, high-risk urban populations do remain, and these include immunocompromised people (with an STH prevalence of 3.8–13.3 % [23]) as well as many patients in institutionalised care (1.1–29.7 % [24]), indicating that the surveillance and control of STH in urban Thai populations is still critical. Most recently, a case of biliary ascariasis-induced acute pancreatitis with cholangitis without imaging support was discovered in a patient coming from an urban area. The investigation results showed no eosinophilia and no *Ascaris* eggs in stool examination; however, the parasite was found when an endoscopic retrograde cholangiopancreatography was performed [25]. In contrast, control efforts have progressed more slowly in Thailand's remote and rural areas, and disease burdens remain high. For example, a study of 1,010 school children in remote communities in northern Thailand (Nan Province) revealed prevalence of 21.7 % for *Ascaris*, 18.5 % for hookworm and 16.3 % for *Trichuris* [26]. Similarly, high prevalence of STH infections (5.7 %, 18.0 % and 28.5 % for *Ascaris*, hookworm and *Trichuris*, respectively) has been reported in rural/remote communities in Nakhon Si Thammarat province in southern Thailand [27]. Recently, molecular tools targeting the internal transcribed spacer 1 (ITS1)-5.8S-ITS2 region of the ribosomal RNA gene identified *N. americanus* as the most common hookworm. However, *A. duodenale* and *A. ceylanicum* were also detected [28].

In **Malaysia**, STH diseases are not notifiable and are considered to be largely controlled [29]. However, foci of high endemicity (with prevalence of 5.5–98.2 % for *Trichuris*, 8.0–67.8 % for *Ascaris* and 3.0–44.7 % for hookworms) still persist in underprivileged or minority communities such as Orang Asli (aborigine) children [30–33], amongst Indians living in estates [34], amongst multiracial communities

living in the squatter areas [35] and in poor Malay living in traditional villages [36, 37] in which sanitation is often poor. However, it is important to note that reinfections are also a major issue, with evidence that prevalence can return to near pretreatment levels just 6 months following deworming [38]. This information emphasises the need for improved integrated control measures (including routine treatment, diagnosis and effective health education) as well as enhanced infrastructure and economic development in underprivileged or disadvantaged communities. Although effective control programmes are imperative, the availability of advanced diagnostics is equally crucial. Recently, a multiplex real-time polymerase chain reaction assay for the detection of various species of STH was developed providing a more specific and sensitive diagnostic tool for the detection of these helminth species in epidemiological studies and monitoring of treatment programmes [39]. Meanwhile, identification of human and animal hookworm species was achieved with the utilisation of the real-time PCR coupled with high-resolution melting (HRM) analysis targeting the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA as the genetic marker providing a rapid and straightforward method for the diagnosis, identification and discrimination of five human hookworms [40]. Molecular tools have enabled confirmation that *N. americanus* and *A. ceylanicum* are found in Malaysia with the former being more common [41] and that *A. ceylanicum* transmission dynamic in endemic areas in Malaysia is heightened by the close contact of human and domestic animal (i.e. dogs and cats) populations [42].

Clinical features of severe STH infections have been described in previous studies carried out in Malaysia. It has been reported that ascariasis was responsible for 42 % and 41 % of all acute abdominal emergencies and intestinal obstruction, respectively, in children 7 years and below admitted to a hospital in Kuala Lumpur [43]. Moreover, previous studies amongst aboriginal children in Selangor and Pahang states strongly indicated that ascariasis is associated with protein-energy malnutrition and vitamin A deficiency [44, 45]. On the other hand, rectal prolapse occurred in 50 % of children with severe trichuriasis which was also identified as the main predictor of stunting and iron-deficiency anaemia amongst Orang Asli children in Malaysia [45–47].

Although *Strongyloides stercoralis* infection is not very commonly reported in Malaysia compared to other parasitic infections, it is common in immunosuppressive patients and may present with hyperinfection. Recently, a case of *S. stercoralis* hyperinfection was reported in a diabetic patient [48], a known case of non-Hodgkin lymphoma (NHL) presenting with recurrent NHL stage IV and had undergone salvage chemotherapy and a case of angioimmunoblastic T-cell lymphoma (AITL) in a patient with lymphadenopathy and bulky neck mass who eventually succumbed following multi-organ failure [49]. Interestingly, *S. stercoralis* rhabditiform larvae were identified in water samples used to wash pegaga, kesum and water spinach, and the number of larvae observed were 152, 9 and 16, respectively. Analysis by real-time PCR confirmed the microscopic observation of this helminth highlighted that vegetables and herbs may be likely sources of *S. stercoralis* infection in Kota Bharu, Kelantan [50]. More recently,

serological and molecular approaches were used to investigate *S. stercoralis* infection amongst an Orang Asli (indigenous) community following a preliminary detection by microscopic examination of faecal samples. Of the 54 samples, 17 samples were positive via enzyme-linked immunosorbent assay (ELISA) and 3 yielded *S. stercoralis* DNA amplification. Given the high ELISA positive results, false positivity is suspected. Hence, PCR method should be considered as an alternative diagnostic tool for the detection of *S. stercoralis* infection [51].

In the **Philippines**, STH infections are still a major human health problem, with the WHO estimating an overall prevalence of >50 % and a regional prevalence of 47.5–92.5 % as of 2004 [29]. Recently, a large-scale study was initiated to evaluate the impact of STH control programmes in endemic regions. The study included 3,373 school children in six provinces and recorded prevalences of 21.0–51.7 % for *Ascaris*, 14.5–59.8 % for *Trichuris* and 0.5–7.5 % for hookworms [52]. A study amongst indigenous individuals reported higher risk of morbidity due to helminth infections in these groups of people [53]. Belizario et al. [52] also recommended a reassessment of existing helminth control strategies to focus on semiannual mass treatment programmes, associated with improved educational campaigns, enhanced environmental sanitation and ongoing surveillance. There is no doubt that the fragmented geography of the Philippines, as a large archipelago, presents major logistical and epidemiological challenges that are likely to have substantial effects on the success of any treatment programmes against STH. Despite this, the WHO currently estimates that the Philippines had reached the 75 % treatment threshold-level for school-aged children established by Resolution 54.19 in 2007 and 2008 [54].

Before and early 2000, studies in **Indonesia** revealed prevalences of 10.0–96.6 % for *Ascaris*, 1.0–98.0 % for *Trichuris* and 0.6–39.7 % for hookworm [55–57]. Since 1975, when the nationwide helminth control programmes were initiated till 1999, control programmes have been inconsistent [58, 59]. Presently, the WHO estimates that, as of 2007 and 2008 data, just 2 % of preschool-aged children and 3 % of school-aged children received regular anthelmintic therapy in Indonesia [54]. Although it is likely that this is, in large part, a consequence of the remote and disparate geography of much of Indonesia, clearly significant focus on expanded national deworming programmes in this region should be considered a major priority. The current status of the efficacy and effectiveness of albendazole and mebendazole for the treatment of *Ascaris lumbricoides* in northwestern Indonesia showed no evidence of drug resistance so far. In addition, although both drugs showed incomplete ovicidal effects, single-dose albendazole is better than mebendazole in sterilising *A. lumbricoides* eggs [60]. There was also a report of the occurrence of an atypical invasive *S. stercoralis* infection of the stomach mucosa in an elderly female patient from Bangka Island, northwestern Indonesia, associated with acute interstitial nephritis. The patient showed rapid improvement after treatment with mebendazole [61].

Data indicated that **Vietnam** has high prevalence of STH (~40.1–44.4 % of the total population is infected with *Ascaris*, 17.5–23.1 % with *Trichuris* and 22.1–28.6 % with hookworm) [29, 62–67]. This may be due to the common practice

of using human excreta as fertiliser in agricultural practices as a recent report has highlighted the presence of *A. lumbricoides* and *T. trichiura* infections associated with wastewater and human excreta use in agriculture in Vietnam [68]. Recent data indicate significant geographic variation in STH prevalence levels: highest (i.e. 75–85 % for *Ascaris*, 38–40 % for *Trichuris* and 27–28 % for hookworm infections) in the north [63] and substantially lower (i.e. <20.0 %), albeit still significant, in the south [69]. These differences in prevalence have been interpreted to be attributable to variation in climate, agricultural practices and/or socioeconomic development [70, 71].

For **Cambodia**, mass anthelmintic treatment has been expanded substantially in recent years. Montresor et al. [72] highlighted Cambodia as one of the few SEA countries with high endemicity of STH to have reached the WHO's target of delivering treatment to 75 % of school-aged children. As of 2006, anthelmintic treatment reached 98 % of school (~2.8 million) and 74 % of preschool (~1.75 million) children [72]. A recent large-scale study [73] showed reductions in helminth prevalence levels in several villages in the provinces of Kratie and Stung Treng from 1997 to 2005, following mass treatment, and reported substantial reductions in the prevalence of *Ascaris* (from 9.5–69.8 % to 0.0–5.4 %), *Trichuris* (from 1.6–9.5 % to 0.0–2.0 %) and hookworm infection (from 45.1–86.0 % to 6.1–26.0 %). Subsequently, a 2006–2011 evaluation on 16,372 faecal samples detected parasites in 3,121 (19.1 %) samples and most common were *Giardia lamblia* (8.0 % of samples; 47.6 % disease episodes), hookworm (5.1 %; 30.3 %) and *S. stercoralis* (2.6 %; 15.6 %). The proportion of infected children increased, and the number of disease episodes effectively treated with a single dose of mebendazole decreased over the 5-year period [74]. However, for *Strongyloides* infection, ivermectin is highly efficacious against *S. stercoralis* but prohibitive costs render the drug inaccessible to most Cambodians [75]. Recently, a sensitive novel real-time PCR assays were developed to detect *Strongyloides* spp. and hookworms providing an alternative in the diagnosis of STH infections in Cambodia [76].

In **Lao PDR**, broadscale deworming programmes have also been highly successfully. A large, national survey conducted in 2003 [77] examined ~30,000 school children. The overall prevalence of STH infections was estimated at 61.9 % (27.2–96.2 % prevalence by province), with a mean prevalence of 34.9 % (1.6–81.9 % by province), 25.8 % (5.4–71.0 % by province) and 19.1 % (3.0–45.1 % by province) for *Ascaris*, *Trichuris* and hookworms, respectively. This information provided the impetus for the initiation of large national deworming programmes [72, 78] yielding one of the most comprehensive datasets with which to evaluate their effectiveness. This deworming programme, targeting school-aged children, were initiated in 2005 and rapidly expanded to reach ~1 million children (~99 % of the school-aged population) by 2007 [29, 72]. A previous study [78] assessed the impact of this programme (which included one to two treatment/s with mebendazole [500 mg] each year, public awareness campaigns and the training of health professionals) and reported substantial decreases in the prevalence of STH (60–20 % for *Ascaris* and 42–31 % for *Trichuris*). These

data are suggestive of substantial reductions in morbidity associated with helminthiasis, in concert with direct decreases in prevalence, as a result of the national deworming programmes. However, a study in Mekong River basin found that multiple species of intestinal parasite infections were common with 86.6 % of 669 studied participants harbouring at least two and up to seven different parasites concurrently. Amongst nematode infections, hookworm was the most prevalent species (76.8 %), followed by *A. lumbricoides* (31.7 %) and *T. trichiura* (25 %) [79]. Another recent study also highlighted that 28.4 % of children studied had monoparasitic infection and 9.3 % had a polyparasitic infection [80]. With regard to hookworm infection, a study found 30 % (61/203 samples) of people infected in Lahanam Village, Savannakhet Province, Lao PDR. Copro-PCR with specific primers for hookworms and *Trichostrongylus* spp. and sequencing discovered *N. americanus, A. duodenale* and also the animal hookworms, *A. caninum*, *A. ceylanicum* and *Trichostrongylus colubriformis* [81]. Another study found that dogs in northern Lao PDR have a role in human hookworm transmission as both *N. americanus* and *A. ceylanicum* were both found in humans and dogs [82].

In **Myanmar**, the majority of clinical manifestations of ascariasis in children responsible for hospital admission were due to intestinal obstruction with the next most common manifestation was intestinal colic [83]. A previous study also highlighted that 7.5 % of laparotomies were due to complications of ascariasis [84]. In 2002–2003, a small-scale survey of children in selected schools (representing the major climatic zones within the country) was conducted by the WHO and the Myanmar Ministry of Health, and estimated mean prevalences of 57.5 % (range from 1.4 to 91.6 %) for *Trichuris*, 48.5 % (18.2–69.1 %) for *Ascaris* and 6.5 % (0.0–17.2 %) for hookworm [85] were reported. Moreover, ~18.2 % (range from 0.9 to 50.3 %) of infected people had 'moderate' to 'heavy' infection intensity, and 22.1 % (range from 9.3 to 34.9 %) of people tested were anaemic [85]. A pilot programme, targeting 25,000 school-aged children, was undertaken to assess mass treatment with albendazole at a dose of 400 mg and cost of ~$0.05 per child [85]. In early 2004, this programme was extended by the Ministry of Health to include a total of 1 million school-aged children in the worst affected regions of Myanmar (representing ~15 % of the countries school-aged children considered 'at high risk' of STH infection). The proportion of children receiving anthelmintic therapy has since expanded to 23 % of school-aged and 19 % of preschool-aged individuals [54].

Currently, no information on STH is available in **Timor-Leste**. However, STH infections are expected to be a significant problem due to poverty and lack of proper infrastructure, especially in the remote communities. Detailed epidemiological surveys of STH in the populations of this country would provide major insights into the burden of disease and would aid in directing deworming programmes, which are currently estimated to reach approximately one quarter of the children in Timor-Leste [54]. Although there are no known STH monitoring or control programmes in **Brunei Darussalam or Singapore** [29], considering the high level of transnational travel (e.g. for employment and tourism) between these countries and their neighbours (in which STH are endemic), the potential for

exposure to these helminths remains a medical health risk, which is worthy of continuous consideration. This is evident as in June 2006; of the 118 Singaporean soldiers who visited Brunei Darussalam for jungle training for 10 days, two soldiers had severe diarrhoea and were diagnosed with severe hookworm infection. An epidemiological investigation and case-control study was then initiated amongst these 118 soldiers. Of 103 soldiers completing both the questionnaire and with all the laboratory tests, 42 soldiers (41 %) had eosinophilia ($>0.6 \times 10^9$/l) and 18 (17 %) had hookworm infection on microscopy. More than 89 % recalled substantial exposure to soil or groundwater, but no exposure was significantly associated with eosinophilia or infection. After adjusting for possible exposures, not wearing footwear during rest periods had a significantly higher odds ratio (2.86) for acquiring hookworm infection or eosinophilia [69].

11.4 Public Health and Economic Consequences of STH

STH infections are global public health problems because of their high prevalence and also because of their consequences. Almost all tropical and subtropical regions are affected by STH infections especially amongst children. Thus, United Nations agencies and other nongovernmental organisations have dedicated their efforts to minimise and eradicate STH amongst the communities at high risk of STH. Despite these efforts and interventions to control STH infections, about 70 % of school-aged children at risk of STH infections are still not protected by deworming treatment [86].

Several studies in different Southeast Asian countries including Malaysia, Thailand, Indonesia and Vietnam have revealed a temporal relationship between STH infections and protein-energy malnutrition, iron-deficiency anaemia (IDA), vitamin A deficiency (VAD), poor cognitive functions and poor school participation amongst schoolchildren [45, 87, 88]. The World Health Organization [3] indicated that STH may have a negative impact on the economic development of communities and nations, resulting from failure to treat school-age children who are infected. These children are often physically and intellectually compromised by anaemia, leading to attention deficits, learning disabilities, school absenteeism and higher dropout rates and this may yield a generation of adults disadvantaged by the irreversible sequelae of infections. Hence, benefits of successful STH control programmes extend well beyond eliminating STH as they improve the growth and physical fitness of children as well as contribute to higher educational attainment, labour force participation, productivity and income amongst the most vulnerable populations [89–91]. Previous studies in Indonesia, Vietnam and Malaysia revealed that the level of serum iron, vitamin A and school performance, respectively, were improved after deworming [7, 87, 88].

The negative consequences of STH infections may continue into adulthood with effects on the economic productivity which trap the communities at risk of infections in a cycle of poverty, underdevelopment and disease [89, 92]. It is well

documented that obtaining more education leads to higher adult income, and thus, the effect of mass deworming on school participation should be central to any reasonable policy analysis for the future development of the individual and society. Moreover, several reports from different parts of the world argue that eradication of the most prevalent intestinal helminth infections is a very high return investment [93]. For instance, the gross national product (GNP) increased in Japan just after the successful control of parasitic diseases including STH, and this means that improved public health conditions preceded economic growth [94].Within this context, the importance of the burden of tropical diseases including STH in impeding economic development of developing nations has received considerable attention in recent years, and there is now broad agreement that they should be a priority for the improved health in large parts of the world population. The WHO regarded the control of schistosomiasis and STH amongst the top five health priorities within the global massive effort against poverty [95].

11.5 Controlling STH Infections

The World Health Organization suggests three main and vital interventions to prevent and control STH infections. These interventions are periodic administration of anthelmintic drug, proper sanitation and effective health education [96]. Anthelmintic drug is aimed at reducing morbidity by decreasing the worm burden. Periodic deworming amongst high-risk groups will reduce the intensity of infection and will frequently result in improvement of child's health, nutrition and development. Adequate proper sanitation is aimed at controlling transmission by reducing the contamination of soil by faeces of infected individuals. Moreover, health education is aimed at reducing transmission and reinfection by increasing people's awareness towards STH and promoting healthy behaviours and hygienic practices including the use of toilets [97]. The combination of these three main interventions is essential for a long-term control and elimination of STH. Indeed, without improvements in sanitation and personal hygiene practices, periodic deworming cannot attain a sustainable reduction in transmission. Similarly, improving sanitation may not attain the desired impact without a parallel improvement in hygiene awareness and health-related behaviours in the population [98].

Generally, the prevalence of STH infections in SEA were high (>50 %) especially in the 1970s. In view of this, national helminth control programmes were initiated in Malaysia in 1974 [99], Indonesia in 1975 [58], Thailand in 1980 [100] and Philippines in 1999 [101]. In addition, school-based anthelmintic (i.e. albendazole or mebendazole) control programmes were also instituted in Vietnam [72, 102], Cambodia [103, 104], Lao PDR [77] and Myanmar [85, 105] by the respective country in collaboration with world agencies and/or foreign developed nations. However, there is no known STH monitoring or control in Brunei Darussalam or Singapore [29].

Key policies in the national governmental control programmes which include surveillance, treatment, improved sanitation, better educational awareness and the provision of safe drinking water have produced successful outcomes in many instances [72, 100]. Until now, STH has been successfully controlled in Brunei Darussalam and Singapore [72]. In Malaysia and Thailand, there are still pockets of populations with high prevalences and these are usually concentrated in rural areas where populations are marginalised with high level of poverty, inadequate clean water supply and improper sanitation facilities coupled with low hygiene and substandard nutrition. However, in East Timor, Indonesia, Myanmar and the Philippines, these infections have remained endemic [106].

11.5.1 Periodic Anthelmintic Drug Distribution

Mass anthelmintic drug administration has been used for generations as the main pillar and the most cost-effective intervention to control STH infections worldwide. It has been considered as the main approach for STH control in areas where infections are heavily transmitted, where resources for disease control are limited and where funding for sanitation is insufficient [107]. In endemic areas, the anthelmintic drug can be distributed to the entire community without prior diagnosis for the infection status (mass treatment) or distributed to certain group of a targeted population, which may be defined by age, sex or any other social characteristics, without prior diagnosis for the infection status (targeted treatment). Moreover, the treatment can only be distributed to the vulnerable people after a diagnosis to detect the most heavily infected people who will be most at risk of long-term consequences of morbidity and mortality. School-based deworming may be particularly a cost-effective approach as it takes advantage of an existing school infrastructure and the fact that schoolchildren are easily accessible through schools. It may also provide an effective way of reaching large portions of an at-risk population, including school personnel, the families of the schoolchildren and other members of the community. Moreover, in highly endemic communities, the periodic distribution of anthelmintic drugs needs to be integrated with programmes which currently deliver health care to children aged between 1 year and school age, such as immunisation programmes, vitamin A capsule distribution programmes and maternal-child health clinics [108].

The WHO recommends four anthelmintic drugs for the control of STH [3]. These drugs are albendazole, mebendazole, levamisole and pyrantel pamoate with albendazole as the drug of choice which is currently used in almost all national control programmes. In addition to these anthelmintic drugs, thiabendazole which has been used for treating strongyloidiasis has been long hampered by low efficacy and high frequency of unpleasant side effects. However, ivermectin, used as a single dose of 150–200 mg/kg against strongyloidiasis, has shown high cure rates [109].

Albendazole is a broad-spectrum anthelmintic agent available in pharmaceutical form as flavoured chewable tablets (200 and 400 mg) and as an oral suspension (100 mg/5 ml) and given in a single dose of 400 mg, reduced to 200 mg for children below 24 months. A single dose of 400 mg is highly effective against ascariasis and hookworm infections. However, strongyloidiasis and heavy trichuriasis may require a 3-day course of treatment [110, 111]. Albendazole, like other benzimidazole derivatives, prevents the formation of microtubules by binding to the nematode β-tubulin and inhibiting the parasite microtubule polymerisation which causes death of adult worms within few days [112, 113]. Moreover, it also interferes with metabolic process by impairing the uptake of glucose, thereby increasing glycogen depletion, and impeding the formation of ATP which is used as the energy source by the helminths [113]. The drug is poorly absorbed by the host and most of its anthelmintic action operates directly in the gastrointestinal tract. However, its absorption can be enhanced several times when ingested with fatty meals.

On the other hand, a low cure rate of a single dose of albendazole drug against trichuriasis has been reported in Malaysia, Thailand, the Philippines, Lao PDR, Vietnam and other countries in the Southeast Asia and Western Pacific regions [38, 72, 114]. Similarly, pyrantel has been extensively used in several STH control programmes, particularly in Southeast Asia. In Malaysia, the national mass deworming programme using a single dose of pyrantel pamoate once or twice a year was discontinued in 1983 due to the low effectiveness of the drug against *Trichuris* and hookworm infections [31].

11.5.2 Sanitation

Sanitation is more important than independence (Mahatma Gandhi, 1923).

Soil-transmitted helminthiasis is a faecal-borne infection, and transmission occurs when the infective stages passed to soil for developmental process and then infect human either directly (hand-to-mouth or skin penetration) or indirectly (through food and water). Hence, sanitation in the context of economic development is the only definitive intervention that eliminates these infections as it plays an important role in protecting the uninfected individuals and reducing the environmental sources of infections. Globally, 1.1 billion people practise open defecation and 2.6 billion people are still lacking access to sanitation, and at any given time, about half of the urban populations of Africa, Asia and Latin America have a disease associated with poor sanitation, hygiene and water [115]. A mathematical model suggests that 1 g of fresh faeces from an infected person can contain about 106 viral pathogens, 106–108 bacterial pathogens, 104 protozoan cysts or oocysts and 10–104 helminth eggs [116]. Hence, open defecation habit plays a major role in contaminating the soil and spreading of STH infections. However, one of the limiting factor is that the cost of sanitation is always higher when compared to other interventions and implementing this strategy is always difficult where

resources are limited [117]. For instance, the STH control in Vietnam, based on regular deworming, latrine construction and health education, has shown that the cost per child for each latrine was very high (US$7.9) when compared with anthelmintic drug treatment which costs pennies. Moreover, the positive impact of improved sanitation is slow and may take few years to achieve desired benefits.

In SEA, the lack of proper sanitation, particularly in rural areas, has been identified as a significant risk factor amongst aboriginal communities in Malaysia [32, 118], southern Thailand [119], central Lao PDR [120], a low-country tea plantation in Sri Lanka [121], underprivileged areas in Indonesia [122] and amongst a community using both wastewater and human excreta in agriculture and aquaculture in Hanoi, Vietnam [123]. A recent systematic review and meta-analysis study investigating on the association of sanitation with STH infections revealed that the introduction of sanitary system reduces the prevalence of STH by about 30 % [124].

11.5.3 Health Education

Health education that is effective, targeted and simple is often recommended as a first option to create the enabling environment for other strategies to thrive, especially in underprivileged communities [125, 126]. Health education can be provided simply and economically, and its benefits go beyond the control of helminth infections [6]. In general, providing information on the disease and the possible adoption of preventive measures frequently results in an increase in knowledge and awareness of the targeted population towards specific health problem but not necessarily in behavioural change which is somehow more difficult and needs longer time [117]. Health educational materials (posters, leaflets, drama, radio and video messages) with some practical activities on hygienic practices have been traditionally used to transmit and disseminate health-related messages.

With regard to STH transmission, reduction in the faecal contamination of soil can be achieved by promoting the use of latrines and promoting personal/family hygiene measures such as washing hands, periodic cutting of nails, proper food preparation and wearing shoes during outdoor activities. The best example here is community-led total sanitation (CLTS), an innovative communications-based approach for mobilising communities to completely eliminate open defecation and achieving ‘open defecation-free’ status [127]. This approach focuses on the behavioural change needed to ensure real and sustainable improvements via community mobilisation rather than helping individual households to acquire toilets. It was developed and introduced in Bangladesh and uses external facilitators and community volunteers to raise community awareness on contamination of open defecation to the environment, water and food ingested by households. Subsequently, CLTS has spread from South Asia to Africa and South America and reported to be highly successful in certain communities.

Previous studies indicated that when health education was used alone without other interventions, it showed minimum reduction rate in the prevalence of STH [55, 128]. However, higher reduction rates were achieved when health education was introduced with sanitation, and this can be considered as the option of choice for sustaining and prolonging the control outcome of other intervention programmes [129]. In Japan, systematic health education programmes were applied alongside various methods of prevention of STH infection like construction of simple latrines, disinfection of vegetables and the use of chemical fertilisers, and all these measures helped in eradicating ascariasis [130].

11.5.4 Can We Deworm This Wormy World? Stories of Success from Asia

Recent estimates suggest that 5.3 billion people are either infected or at risk of STH infections worldwide as they live in areas stable for transmission of at least one STH species [2]. A further 143 million lived in areas of unstable transmission for at least one STH species. These figures make a target of eradication of STH not possible. Thus, WHO programmes and initiatives focus on the elimination of morbidity not parasites. In the eternal battle of humans against worms/helminths, a few success stories in eliminating and reducing the transmission, prevalence and intensity of STH infections have been documented in several Asian countries, notably Japan, South Korea and Taiwan, with mass deworming, proper sanitation and hygiene education being the main components of control programmes [94, 131].

This success was achieved by using a school-health-based approach which was implemented through triangular cooperation amongst government agencies, nongovernmental organisations and scientific experts. Within this context, the Japan International Cooperation Agency (JICA) proposed the Global Parasite Control Initiative in 1998 and established three research and training centres around the world in order to extend the successful experience in controlling parasitic infections to other countries worldwide [132]. One of these centres, Asian Center of International Parasite Control (ACIPAC), was established in 2000 at Mahidol University, Bangkok. This centre has organised several training courses for the school-health-based control of STH for health personnel and educators. Moreover, JICA has supported small-scale pilot projects using the Japanese model in STH control in Cambodia, Lao PDR, Myanmar and Vietnam.

With regard to the WHO strategic plan of eliminating STH infection as a public health problem in children, Cambodia and Lao PDR were amongst the first countries in the world to achieve 75 % national coverage of preventive chemotherapy in school children and maintain high national coverage [86]. Recently, national STH control programmes in Cambodia have achieved a substantial success in reducing the prevalence rates from 80–90 % to less than 15 % [133].

11.6 Future Directions

11.6.1 Vaccination: The Long-Term Prospects for New Control Tools

To date, national deworming programmes through mass drug administration in SEA countries such as Cambodia, Lao PDR, Myanmar, Thailand and Vietnam have had an important impact on reducing the prevalence of STH infections [134]. However, heavy dependence on such drugs alone is a cause of concern. Unlike ascariasis and trichuriasis, in which the highest intensity usually occurs in school-aged children, heavy infection of hookworm can occur in adults [135]. Thus, the deworming programmes which usually target school-aged children are not expected to reduce hookworm infection significantly while they might have some effect on ascariasis and trichuriasis. In addition, such regular mass anthelmintic treatment often fails against hookworm effectively because of the rapid reinfection in endemic areas [136] and diminished efficacy of the anthelmintic drugs with increased and repeated use [137]. Amongst aboriginal schoolchildren in Malaysia, the reinfection rates of STH were reported to be high and by 6 months after completion of deworming, the prevalence and intensity of infections were similar to pretreatment levels [38].

As a potential threat, the rapid increasing mass distribution of these few anthelmintic drugs raises some concerns about a sustained drug efficacy and the potential threat of emerging resistance as a result of drug pressure and widespread use of these anthelmintic drugs [112]. Moreover, some previous studies have reported a low efficacy of single-dose albendazole and mebendazole. Although both drugs are effective against *A. lumbricoides*, mebendazole is not effective against hookworms, while neither of the drugs is effective against *T. trichiura* unless used with large doses for 3 successive days [126]. In addition, anthelmintic drug resistance is already a serious problem in nematode of veterinary importance [138] and this reality should be taken into consideration when implementing drug-based control strategies against STH.

It is thought that even more efficacious and powerful drug compared to albendazole and mebendazole would not be expected to overcome the occurrence of rapid reinfection after treatment. This has prompted efforts to develop an effective vaccine. Because of its simple, single step for disease, infection and transmission interruption, vaccination remains the method of choice to control STH infections. The Human Hookworm Vaccine (HHV) Initiative was initiated in 2000 by the Sabin Vaccine Institute Product Development Partnership (Sabin PDP) in collaboration with the George Washington University, the Oswaldo Cruz Foundation, the Chinese Institute of Parasitic Diseases, the Queensland Institute of Medical Research and the London School of Hygiene and Tropical Medicine [139]. To date, several candidates of vaccine antigens for hookworm have been successfully identified as having potential for vaccine development. For example,

the *Necator americanus-Ancylostoma*-secreted protein-1 (*Na-ASP-2*) vaccine was the first generation of hookworm vaccine that has advanced into clinical development in human [140, 141]. Despite several evidences showing that *Na-ASP-2* are the promising candidate for vaccine development, the trial was discontinued after their Phase I clinical trial in a hookworm endemic area in Brazil when some participants developed allergic reaction to the *Na-ASP-2* vaccine [142].

This has led Sabin PDP to develop new criteria for the selection of helminth antigens for potential vaccine candidate including skin test and seroprevalence study in endemic areas [143]. Currently, two lead candidate antigens, *Necator americanus*-glutathione S-transferase-1 (*Na-GST-1*) [144, 145] and *Necator americanus*-aspartic protease-1 (*Na-APR-1*) [146] are being developed as potential vaccine candidates with Part I of the Phase I clinical trial on *Na-GST-1* being initiated in Brazil. The result indicated that no safety issues were reported from healthy participant (i.e. no history of hookworm infections) [143]. These promising outcomes were sufficient for the researchers to proceed to the next stage of the trial, in which the vaccine candidate will be given to adults who were exposed to hookworm infections. The Human Hookworm Vaccine that is still under development will ultimately incorporate both the *Na-GST-1* and *Na-APR-1* in a bivalent vaccine in making the goal of first-ever human hookworm vaccine a reality.

However, additional research is needed to determine how this vaccine can be incorporated into existing control programmes and how it would be beneficial for vulnerable groups that are currently not targeted for regular deworming programmes. Until these new technologies become available, periodic deworming for high-risk population remains the most practical and substantive means to control STH infections [141]. The coming decade promises to be an exciting one in the history of STH control as new and appropriate technologies are folded together to combat the STH diseases particularly hookworm infection in SEA and other endemic countries around the world. With the establishment of an extensive infrastructure for biomedical research over the last decades in Singapore and other SEA countries and the enthusiasm for seeing helminthological science translated into new interventions, it is believed that this region has major potential for leadership in the development of new alternative and sustainable integrated control tools of STH infections in the years to come.

11.6.2 Using Geographical Information Systems in STH Control

Although it is known that STH is still a major public health problem in many SEA countries, a precise estimate of the total disease burden has not been fully described as collation of systematic information on STH infections is not currently available. Most of the information or record on the prevalence of STH infections is scattered across the literature and not catalogued systematically. These data are seldom

available in an accessible format for policy makers or public health authorities. Hence, previous approach in describing the distribution of STH infections has typically been made at the national level using prevalence data from few available published reports, which are then extrapolated to the country as a whole. Such approach however has limited practical importance to effectively target control efforts. In recent years, there has been renewed interest from the international organisations in the helminth control programme that leads to an increase momentum to attain more comprehensive data, allowing available control resources to be most rational and cost-effectively deployed [147]. As a result of these changes in health priorities, tremendous efforts have been made in the development of methods to map the distribution of diseases, particularly through the use of geographical information system (GIS) and remote sensing (RS) which made data integration and mapping more accessible and reliable [147].

A principal advantage of GIS is that it facilitates regular updating of database and provides a ready basis for mapping and analysis. It also offers us the ability for modelling the spatial distribution of STH infections in relation to the ecological factors which are derived from remote sensed satellite data that are known to influence their distribution pattern, deepening our knowledge and understanding in the biology and epidemiology of the infections [148, 149]. It allows us to predict the distribution of infection and identify endemic areas, thus providing more precise estimates of populations at risk and map their distribution by facilitating the stratification of areas using infection risk probabilities. This can provide basic information on treatment intervention or public health measure delivery systems at broad spatial scale particularly in areas without comprehensive data [148]. Such approach has also the potential in facilitating and assisting the design of sustainable development control programme at realistic scale for national control programme by providing the relevant authorities with relatively low-cost approach for both the upstream (e.g. survey and design) and downstream (e.g. targeting, monitoring and evaluation) control programme, which significantly reduce the cost of practical programme by identifying priority areas or simplifying the monitoring and evaluation processes [148]. Thus, the use of GIS is essential for developing and implementing control measures to those populations in greatest need particularly when the recourses for control programmes are finite and limited.

In addition, the use of remote sensing (RS) satellite data, which provides proxy to environmental data, helps to further enhance the functional capabilities of GIS by predicting the distribution of STH in relation to their ecological limit [147]. The association between the ecological factors such as altitude, climate, temperature and rainfall that influence the distribution pattern of STH has long been acknowledged and observed previously in many studies conducted in SEA. Such association has been observed in several SEA countries including Malaysia [150], Indonesia [151], Myanmar [85], the Philippines [134] and Vietnam [70]. However, such findings were based on the comparative observation prevalence of STH in different ecological zones such as mountain area vs. lowland or northern part of the country vs. southern part.

To date, the GIS and RS tools have been widely used for analysis, mapping and spatial modelling of several parasitic diseases including STH infections [147, 152–159]; however, such approach in STH mapping has been attempted largely in African countries. Only recently, GIS and RS approaches for mapping of STH infections have been extended to Southeast Asia regions including Mekong countries, i.e. Cambodia, Lao PDR, Myanmar, Thailand and Vietnam [156] as well as Indonesia and the Philippines through the support from UNICEF [160]. Findings from the mapping of STH in Mekong countries suggested that *A. lumbricoides* and *T. trichiura* are most unlikely to occur in areas where land surface temperature (LST) exceeds 37 °C particularly in Vietnam where low prevalence of *A. lumbricoides* and *T. trichiura* infections (i.e. less than 10 %) were reported in areas where maximum LST is above 37 °C [156]. Likewise, predictive risk map and disease pattern for the whole Mekong countries have been developed to estimate STH burden in each country at the provincial level. The present examples have also sufficiently proven that if used appropriately, GIS and RS technologies can be used as relevant and important tools to design cost-effective control programme through a more precise and prioritised geographical target population such as school-age children in a high-risk area particularly when the resources for control is finite and limited, using the example and experience in Mekong countries [156].

In order for the potential of GIS and RS to be fully utilised particularly at the identified high-risk areas, it has to be implemented together with the existing appropriate control strategies. One way that this could be achieved is by incorporating and adopting the programme so-called Focus Resources on Effective School Health (FRESH) framework [161]. The FRESH framework was established to provide a consensus approach of good practice for the efficient and successful implementation of health and nutrition services within school-health programme (Anon 2000). Amongst the early international partners together with WHO were UNESCO, UNICEF, World Food Program (WFP), World Bank and Partnership for Child Development (PCD). The framework suggests four core components that have to be considered in designing a cost-effective school-health and nutrition programme which indirectly provides the appropriate mix interventions that can be adopted for STH control programme for the targeted school-age children. However, of course, GIS and FRESH framework do not prescribe the designs of deworming programme in school as such programme is highly variable depending on the specific country. In low-income countries, participation of both public and private sectors is commonly used. For public sector, the Ministry of Health (MoH) can involve in supervising the activity while the Ministry of Education (MoE) assists in the implementation of the activity for intervention programme, particularly through teachers. The participation of public sectors in such approach has been demonstrated to be successful in many low-income countries [148]. As for private sector, the nongovernmental organisation (NGO) bodies can participate and contribute by sponsoring or donating drug for treatment through the MoH and MoE. It has been shown that the involvement of private sector has proven to be sustainable and effective in many middle-income countries such as Indonesia and historically Japan and South Korea by delivering treatment that is paid for the community

[148]. Whatever the design, identifying which populations (i.e. schools and communities) are in greatest need for the treatment is a fundamental part of the process of these GIS and RS approaches.

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Chapter 12
Epidemiology of Cestode and Trematode in Southeast Asian Countries

Zurainee Mohamed-Nor

Abstract Taeniasis/cysticercosis, schistosomiasis, and food-borne trematodiasis have been the major public health problem particularly in Southeast Asia. Data on these diseases for Southeast Asian countries were presented (excluding countries like Brunei Darussalam and Timor-Leste where data was hardly available). Among the countries that indicated high prevalence of such diseases are Lao PDR, Vietnam, Cambodia, Thailand, Indonesia, and the Philippines. Prevalence of taeniasis/cysticercosis (>10 %) was seen in countries like Cambodia, Indonesia, the Philippines, and Vietnam. Schistosomiasis was found highest in Khong Island, Lao PDR (26.8 %). It was also reported in few other countries but with lower prevalence. Vietnam and Thailand demonstrated high prevalence of clonorchiasis, 32.2 % and 23 %, respectively. The prevalence of opisthorchiasis was found very high especially in Lao PDR (85 %) and Thailand (70.8 % in Khon Kaen District and 64 % in central Thailand). Lao PDR was also shown as having the highest prevalence for fascioliasis (13.8 %) and paragonimiasis (51 %) compared to other countries like Vietnam and Thailand.

12.1 Introduction

Parasitic infections (food-borne, water-borne, vector-borne, and soil-borne) are common and widely distributed in the Southeast Asian countries. Basically, their distribution, prevalence, and severity are very much influenced by geography, environment, economic development of the countries, the population's religious and social beliefs, and types of government that rule the country. For example, in countries where increasing economic prosperity and accompanying infrastructure are present, parasitic diseases are almost nonexistent. Religious proscription on

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consumption of certain animals also helped to curb food-borne infection, while culturally deeply rooted habit of eating raw or undercooked foodstuffs, coupled with inadequate hygiene practices and lack of separation between foodstuff and wildlife, have hampered efforts to prevent and control infections effectively.

This chapter provides a brief review on the epidemiology of cestode and trematode infections in Southeast Asian countries.

12.2 Cestode

There are three medically important species of cestode, namely *Taenia saginata* (beef tapeworm), *T. solium* (pork tapeworm), and *T. asiatica* (Asian tapeworm) in Southeast Asia [1–4]. These parasites cause human taeniasis, a disease which is still common and shown as relatively serious public health problem not only in Southeast Asia but worldwide.

Taeniasis in human due to *T. saginata* and *T. asiatica* is caused by eating uncooked or undercooked beef and viscera of swine contaminated with metacestodes of these species, respectively. Metacestodes of *T. asiatica* may develop not only in pigs but also in cattle and goats [5, 6]. *Taenia solium* taeniasis in human is caused by eating uncooked or undercooked pork contaminated with the metacestodes of this species. Persons who do not eat raw or undercooked beef or pork are not likely to get taeniasis. Symptoms are usually mild or nonexistent causing many people with taeniasis to be unaware of the infection.

Taeniasis due to *T. solium* is more prevalent especially in underdeveloped communities due to practice of eating raw or undercooked pork, uncooked vegetables, poor sanitation, and free roaming of pigs due to poor pig husbandry practice. It is therefore widespread in Asia, including China, Indonesia, Nepal, India, South Korea, Thailand, Cambodia, Lao PDR, and Vietnam [1, 2, 7–12].

Taenia solium taeniasis can also lead to a parasitic tissue infection caused by the larval cysts of this tapeworm known as cysticercosis. Cysticercosis is regarded as one of the most important zoonotic diseases in the world affecting approximately 50 million people worldwide. Some 50,000 of those infected die of cysticercosis annually [13, 14] making pork seemed unsafe to consume. It usually occurs when sanitation is poor, meat inspection is not performed or inadequate, and proper pig farming is not implemented.

When eggs that are released from *T. solium* are ingested by humans, pigs, or dogs, the hatched oncospheres develop into cysticerci in many tissues and organs and cause various types of cysticercosis. This larval cyst may form in the brain causing neurocysticercosis leading to seizures, epilepsy, neurological sequelae, or death [15–17]. Another relatively serious type of cysticercosis is ophthalmic cysticercosis which often causes a high degree of visual impairment. An asymptomatic type of cysticercosis is subcutaneous or muscle cysticercosis.

Table 12.1 Prevalence data of taeniasis/cysticercosis in Southeast Asian countries

Country	Prevalence of taeniasis/cysticercosis	Year reported	Reference
Cambodia	21.7 %	2009	[24]
Indonesia	8–51 % (Papua)	2003	[25]
	23.5–56.9 % (central highlands of Papua)	2009	[26]
Lao PDR	0–14 %	2008	[18]
	1.1 % (Northern Lao PDR)	2009	[24]
	2.2 % (cysticercosis), 8.4 % (taeniasis)	2011	[27]
Malaysia	2.2 % (Ranau, East Malaysia)	2006	[28]
Myanmar	6 %	1981	[29]
Philippines	24.6 %	2011	[27]
Thailand	0.2–7.0 %	2007	[1, 2]
Vietnam	5.7 %	2000	[30]
	2.2–7.2 % (cysticercosis), 1.0–12.6 % (taeniasis) (in Bac Kan and Bac Ninh)	2002	[31]
	15.8 % (cysticercosis), 30 % (taeniasis) (in Hanoi)	2003	[12]

Cysticercosis is not only confined to people who raise or consume pork since people are also at risk if they ingest *T. solium* eggs after coming into direct or indirect contact with tapeworm carriers.

Together, taeniasis and cysticercosis occur globally. Many studies have been conducted in the Asian countries, which clearly indicated that the disease is spreading widely in the region [7, 9, 12, 18–23]. Wide variation in the prevalence was observed in association with poverty, pork consumption, and poor pig husbandry. Cysticercosis therefore has been confirmed as a serious threat to human health in many areas of the Southeast Asia region. Table 12.1 showed the prevalence data of taeniasis/cysticercosis in Southeast Asian countries.

12.3 Cambodia

Based on a survey performed from 2007 to 2008, the prevalence of taeniasis in Cambodia was 21.7 % [24].

12.4 Indonesia

Indonesia is one of the countries in Southeast Asia that is endemic with all three species of human *Taenia* tapeworms [32]. *Taenia solium* taeniasis/cysticercosis has been found in several areas of Indonesia mainly in Bali, Papua, and North Sumatra.

Bali is also found to be endemic for *T. saginata* taeniasis [33–35]. Other areas reported of having *T. solium* cysticercosis were Lampung, Jakarta, East Java, West Kalimantan, East Kalimantan, North Sulawesi, South Sulawesi, and Southeast Sulawesi [36–39]. There were also reports that indicated *T. asiatica* endemicity in North Sumatra [21, 22, 34, 35, 40].

Although Indonesia is known for its largest number of Muslim population (88 %) in the world, it was in the regions where most people are Christians or Hindus; hence, higher incidence of *T. solium* taeniasis and cysticercosis were detected [15, 16, 34, 35, 39]. Here, improper cooking of infected pork and reliance upon traditional sanitary are practiced. People generally defecate in their house yards and garden and allow free roaming of pigs to clear the excrement at night time, the use of human feces for fertilizer, inadequate meat inspection coupled with poor control of infected carcasses, and poor hygiene expose human populations to these infections.

In Bali, although the transmission and prevalence have been reported as decreasing dramatically over the years [21, 22, 33–35], recent study showed that taeniasis is still endemic based on a survey conducted from 2002 to 2009 that demonstrated sizable number of *T. saginata* taeniasis cases, dual (*T. saginata*/*T. solium*) infections with *T. solium* metacestodes in the brain, and a dozen of neurocysticercosis (NCC) cases, detected at Sanglah Hospital, Denpasar [41]. A case of ocular cysticercosis was also reported [42]. Here, the traditional dish "lavar," made of minced raw pork mixed with coconut and spices, is commonly consumed.

Cysticercosis and taeniasis in Papua could still be considered as one of the highest in the world. With earlier studies by various workers showed intestinal *T. solium* infection was found varied from 8 to 51 % [25, 34, 35, 43, 44], later study still demonstrated high prevalence of cysticercosis (23.5–56.9 %) in the central highlands of Papua [26]. In fact, these researchers believed that based on the data they observed, the prevalence of cysticercosis and taeniasis here was unchanged from that reported nearly 35 years ago at the beginning of cysticercosis/taeniasis epidemics in Papua.

As far as North Sumatra is concerned, *T. asiatica* is the only species reported specifically from Samosir Island in Lake Toba [45]. Survey conducted 30 years later showed a dramatic reduction of *T. asiatica* taeniasis cases and the reason for that significant reduction was the change of practice in the preparation of pork-based dishes as part of public health education where pork was no longer consumed uncooked. Pigs were also kept indoor, distant away from human feces [20]. Based on latest studies, *T. asiatica* is still found endemic in North Sumatra [21, 22, 34, 35].

12.5 Lao PDR

Despite of limited data available, there is a high degree of spatial and some evidence of temporal variation in taeniasis prevalence in Lao PDR based on several studies conducted between 1989 and 2004 [46–49]. The prevalence was found to

vary from 0 to 14 % [18]. These studies however did not provide enough information to determine the cause of infection although old data ([50] cited by Dorny et al. [7]) indicated *T. solium* and *T. saginata* as the common causes. During the nationwide survey conducted between 2000 and 2008, *T. solium* infection was revealed as 1.1 % in Luangprabang province in northern Lao PDR [24]. In the same year, 15 specimens from Savannakhet and Khammouane were confirmed as being *T. saginata* analyzed by Cox1 sequence and multiplex PCR [40].

There was hardly any data on prevalence and incidence of human cysticercosis apart from a case report on a 43-year-old lady from Xiengkhouang Province seen at Mahosot Hospital in Vientiane Capital which was confirmed positive for cysticercosis after brain CT scan demonstrated radiological features compatible with neurocysticercosis [51]. Another case of a male patient with neurocysticercosis was reported from Oudomxay in the northern territory [24].

Several other patients presenting similar neurological condition could not be confirmed since serological confirmation were not performed to rule out tuberculosis since these patients live in *M. tuberculosis* endemic area [52]. Only recently, by the utilization of antigen capture ELISA, cysticercosis prevalence in Lao PDR was determined to be 2.2 %. They also estimated the prevalence of taeniasis to be 8.4 % with *T. saginata* as the dominant species (94 %) detected using PCR method [27].

Taenia solium has only been reported in the northern part of Lao PDR, whereas *T. saginata* was reported in central Lao PDR [53]. *Taenia asiatica* taeniasis has not yet been detected in Lao PDR. Nonetheless, with *T. asiatica* known to be present in the neighboring countries (Thailand and Vietnam and Yunnan province of China), *T. asiatica* is suspected to exist in Lao PDR [24], and detection should be possible if more sensitive method such as molecular method is employed.

12.6 Malaysia

Cysticercosis has been said to be rare in Malaysia since most Malaysians are Muslim. There was however considerable consumption of pork among Malaysian particularly among the Chinese population [54].

Few cysticercosis cases detected in Malaysia were among the migrants [55, 56]. There was however a case of neurocysticercosis involving a Malaysian Muslim who denied ever eating or in contact with pork [57]. Such infection could be attributed to the transmission of *T. solium* eggs via infected immigrant workers employed as food handlers. A case of Malaysian lady having subretinal cysticercosis was also reported and most likely infection was obtained during her visit to China [58].

A survey done on Malaysian population in Ranau, Sabah demonstrated 2.2 % positive for cysticercosis antibody out of 135 samples tested [28]. According to

Conlan et al. [27], however, this study may have underestimated the seroprevalence in Ranau which was believed to be greater than 10 %.

12.7 Myanmar

The only published study could be found pertaining to cysticercosis in Myanmar was dated more than 20 years ago based on serological study in a local population. Six percent of the population was found positive for cysticercosis antibodies [29].

12.8 Philippines

Not much data on human cysticercosis was available from the Philippines. A seroprevalence survey in the Macanip community indicated a presence of 24.6 % cysticercosis antibody [27].

12.9 Singapore

There has been no documented data found on taeniasis/cysticercosis in Singapore thus far. Nonetheless, due to the influx and efflux of tourists, Singapore is considered "at risk" for taeniasis/cysticercosis. Interestingly, its economic prosperity and up-to-date infrastructure seem to have made not only cysticercosis/taeniasis but other parasitic diseases almost nonexistent in this country.

12.10 Thailand

In Thailand, taeniasis caused by *T. saginata* and *T. solium* is common with infection rate as shown by the national data to be between 0.2 and 7.0 % [1, 2]. High infection rates came from the north [59–61] and the northeast areas [60, 62, 63] of the country where consumption of raw and undercooked meat is a custom in these regions. According to the data, infection rates in other regions (the central and the south) were found to be relatively low compared to the north and the northeast regions.

Cases of cysticercosis have also been reported in Thailand with the number of neurocysticercosis cases being higher compared to the subcutaneous cysticercosis [11]. Studies also showed, like taeniasis cases, cysticercosis cases were also mainly from the northern provinces, followed by central, northeast, east, and the south regions [64, 65].

Later study on taeniasis and cysticercosis performed in Kanchanaburi Province showed that not only *T. solium* and *T. saginata* were causing the infection but for the first time, *T. asiatica* was also detected. The study confirmed sympatrically occurring of *T. solium*, *T. saginata*, and *T. asiatica* in the study area [1, 2, 66]. *Taenia asiatica* has been reported earlier in other Asian countries such as Taiwan, the Republic of Korea, China, the Philippines, Indonesia, and Vietnam [15, 16, 20, 34–36].

12.11 Vietnam

Cysticercosis is widespread in North Vietnam and was concluded to have become a serious health problem in the country [12]. The disease is often seen in male adults but none in children [12, 67]. Serological study performed in North Vietnam showed 5.7 % of its population was positive against cysticercosis [30].

Survey conducted based on fecal and serology in the Bac Kan and Bac Ninh provinces showed 1.0–12.6 % of taeniasis and 2.2–7.2 % of cysticercosis [31].

CT scan of brain on the other hand indicated higher percentage of infection (15.8 %) in Hanoi alone with 30 % of those infected had taeniasis suggesting high rate of autoinfection [12]. In Vietnam, the disease was found to be caused by all *Taenia* species with *T. asiatica* being the highest (55.4 %), followed by T. *saginata* (38.5 %) and *T. solium* (6.2 %) [68].

12.12 Trematode

12.12.1 Schistosome spp.

In Southeast Asia, there are three schistosome species recognized as infecting humans. They are *Schistosoma japonicum*, *S. mekongi*, and *S. malayensis*. The disease, schistosomiasis, poses a public health problem in several Southeast Asian countries. *Schistosoma japonicum* affects millions of people in the Philippines [69] and has also been reported in Indonesia [70, 71]. *Schistosoma mekongi* is endemic in Lao PDR and Cambodia with 60,000 people estimated to be still at risk of infection in Lao PDR and about 80,000 people in Cambodia. Despite satisfactory control measures implemented in both countries, transmission still occurs with prevalence reaching rates of more than 15 % [72]. *Schistosoma malayensis* was first described in 1988 in Peninsular Malaysia and appears to be a zoonotic infection [73].

Schistosomiasis endemic areas are characterized by low socioeconomic conditions, poor sanitary facilities, bad habits of the people as regards urination and defecation in water canals and at the same time exposing themselves to this polluted

water by bathing, swimming for recreation, washing utensils and clothes, and walking barefoot during irrigation for agriculture, and in fishing [74]. Inadequate hygiene and play habits make children especially vulnerable to infection. The building of dams, irrigation systems, and reservoirs and migration to urban areas and refugee movements are introducing and spreading the disease to new areas. Increasing population size and the corresponding needs for power and water often result in development schemes and environmental modifications that also lead to increased transmission. With the rise in ecotourism, increasing numbers of tourists are contracting schistosomiasis.

All species are contracted in the same way, through direct contact with freshwater infested with the free-living form of the parasite known as cercariae. Eggs are excreted in human urine and feces and, in areas with poor sanitation, contaminate freshwater sources. The eggs break open to release a form of the parasite called miracidium. Freshwater snails become infested with the miracidium, which multiply inside the snail and mature into multiple cercariae that the snail ejects into the water. The cercariae quickly penetrate unbroken skin, the lining of the mouth, or the gastrointestinal tract. Once inside the human body, the worms penetrate the wall of the nearest vein and travel to the liver where they grow and sexually mature. Mature male and female worm pair and migrate either to the intestines or the bladder where egg production occurs. One female worm may lay an average of 200–2,000 eggs per day for up to 20 years. Most eggs leave the bloodstream and body through the intestines. Some of the eggs are not excreted and can lodge in the tissues.

Symptoms of schistosomiasis vary with the species of worm and the phase of infection. Heavy infestation may cause fever, chills, lymph node enlargement, and also liver and spleen enlargement. Initial invasion of the skin by cercariae may cause tingling sensation or light rash, commonly referred as “swimmer’s itch,” due to irritation at the point of entrance. Other symptoms can occur which include fever, aching, cough, diarrhea, or gland enlargement. Another primary condition is called “Katayama fever.” Its symptoms include fever, lethargy, the eruption of pale temporary bumps associated with severe itching (urticarial) rash, liver and spleen enlargement, and bronchospasm.

Intestinal symptoms include abdominal pain and diarrhea which may be bloody. When eggs become lodged in the intestinal wall, it causes an immune system reaction called granulomatous reaction that can lead to obstruction of the colon and blood loss. The infected individual may have what appears to be a potbelly. Eggs can also become lodged in the liver, leading to high blood pressure through the liver, enlarged spleen, the buildup of fluid in the abdomen (ascites), and potentially life-threatening dilations or swollen areas in the esophagus or gastrointestinal tract that can tear and bleed profusely (esophageal varices).

Urinary symptoms may include frequent urination, painful urination (dysuria), and blood in the urine (hematuria). The loss of blood can lead to iron deficiency anemia. A large percentage of persons, especially children, who are moderately to heavily infected experience urinary tract damage that can lead to blocking of the urinary tract and bladder cancer. Table 12.2 showed the prevalence data of schistosomiasis in Southeast Asian countries.

Table 12.2 Prevalence data of schistosomiasis in Southeast Asian countries

Country	Prevalence of schistosomiasis	Year reported	Reference
Cambodia and Lao PDR	77 % (1995), 1 % (2003)	2010	[75]
	26.8 % (Khong Island), 2 % (Sadao)	2007	[76]
Indonesia	0.49 % (2005 and 2006) (Lindu Valley)	2008	[77]
	0.79 % (2005), 1.08 % (2006) (Napu Valley)		
Malaysia	13.3 % (Police personnel)	1996	[78]
	6.8 % (Sarawak)	2001	[79]
Philippines	18.9 % (1975), 10 % (2008) (Maguindanao)	2008	[80]
	1.8 % (2012) (Maguindanao)	2012	[81]
Thailand	0.03 % (Ubon Ratchathani province)	1999	[82]

12.13 Cambodia and Lao PDR

Found in the Mekongi river that runs through Cambodia and parts of Lao PDR, *S. mekongi* has posed a persistent public health problem since its discovery in 1957 [72, 83]. In the 1990s, up to three quarters of the population in some areas of Cambodia were infected with schistosomiasis. Cambodia then launched an integrated control program to people residing in schistosomiasis endemic areas, and in the late 1994 and since the implementation of these control measures, the percentage of persons with schistosomiasis has dropped significantly from about 77 % in 1995 to less than 1 % in 2003 [75]. While this integrated control program has been extremely effective in lowering the prevalence of schistosomiasis, there have been many concerns regarding the sustainability of these intervention efforts. For example, the prevalence in Hat-Xai-Khoun village, Khong Island, although has decreased significantly, it only came down to 26.8 % (from 80 % in 1989), while at Sadao in Cambodia, it was detected to be around 2 % in 2005 when there was none in 2004 [76].

The role of reservoir hosts in the persistence of transmission has been demonstrated [84, 85]. In addition, it has been shown that prevalence of infection in the snails at Khong Island [86] and Sadao [87] has not declined as significantly as in the case of infection in the human population. In 2004, 11 new snail populations were recorded, occurring in six river systems of Lao PDR and Cambodia. As a result, the potential human population at risk has risen from 150,000 to over 1.5 million following the discovery of these new snail populations. Such observations suggest that control of this disease clearly is a task that is not easy to maintain [88].

12.14 Indonesia

Schistosomiasis in Indonesia is only endemic in the province of Sulawesi. It is only *S. japonicum* that has been reported in Indonesia [70, 71]. It was found limited to two very isolated areas, the Napu and Lindu Valleys [89]. The averaged prevalence was 0.49 % in seven villages in Lindu Valley during 2005 and 2006. In Napu Valley, the average prevalence among 17 villages was 0.79 % in 2005 and increased slightly to 1.08 % in 2006 [77].

12.15 Malaysia

Schistosomiasis in Malaysia is caused by *S. malayensis* which was first described in 1988 in Peninsular Malaysia [73]. Humans and rats are the only known natural hosts [90]. Several studies have indicated that humans are not an important host for this parasite [91–93] and its infection appears to be zoonotic in nature and was unlikely to become a significant public health problem.

Nonetheless a study done on Police Field Force personnel showed that 13.3 % of participants were positive or borderline for schistosomiasis via serological screening. Stool samples however were negative for schistosome eggs [78]. Another serological study, performed on indigenous interior tribes (Orang Ulu) in upper Rejang River Basin Sarawak Malaysia determined by ELISA method, demonstrated that 6.8 % of the individual surveyed were positive for malayensis schistosomiasis [79]. Unfortunately, stool examination was not done and therefore cannot be concluded that human was involved as agent for propagating the schistosome life cycle in Malaysia.

12.16 Philippines

Schistosoma japonicum is the only trematode affecting humans and has been documented as an important problem in the Philippines [94]. In 1975, it was indicated that five million people lived in schistosomiasis endemic areas and over 700,000 individuals were infected. Maguindanao, a province in the Autonomous Region of Muslim Mindanao, was ranked first in the list of schistosomiasis endemic provinces in the Philippines with a prevalence rate of 18.9 % and in 2005 reduced to 10 % [80].

When prevalence rate was compared between male and female, it was shown to be higher among males than that of females. In Luzon, for example, prevalence rate is almost four times higher in males than in females suggesting the occupational hazard of farming and fishing among the males [80].

In 2006, although it was estimated that the population in the endemic areas had grown to 12 million, the implementation of extensive chemotherapy programs has helped reducing the active infection to around 150,000 [95]. In the most recent survey, the prevalence rate recorded for Maguindanao was at 1.8 % [81].

12.17 Singapore

There is no report of schistosomiasis from Singapore in recent years. There was only one case reported long time ago of an 83-year-old female presented *S. japonicum* infection with bloody diarrhea [96].

12.18 Thailand

In Thailand, *S. japonicum* and *S. mekongi* which cause intestinal schistosomiasis can be seen especially in the northeastern region [97, 98]. Relatively high mekongi schistosomiasis infection rate has been recovered in humans and dogs along the country's borders with neighboring country of both Lao PDR and Cambodia. One of the endemic areas of mekongi schistosomiasis, the Kong Island [99], is in the immediate vicinity of Ubon Ratchathani province, the third most populated city in the northeastern part of Thailand. A study conducted in the province employing indirect- and dot-blot ELISA using a *Schistosoma* heterophile substance, keyhole limpet hemocyanin (KLH) as the antigen, indicated that very small percentage (0.03 %) of Ubon Ratchathani province inhabitants have been exposed to *S. mekongi* [82].

Few hospital cases of urinary schistosomiasis [100, 101] and S. *japonicum* causing eosinophilic appendicitis [102] have been reported in Thailand.

12.18.1 Food-Borne Trematodes

More than 40 million people are infected and more than 10 % of the world's population are at risk of food-borne trematode infections [103–106]. These infections, similar to other infections previously discussed, were also limited to populations living in low-income countries particularly in Southeast Asia and were very much associated with poverty.

In Southeast Asia the clinically important food-borne trematodes include *Opisthorchis viverrini*, *Clonorchis sinensis*, *Fasciola* spp., and *Paragonimus* [105].

The transmission of food-borne trematodes is restricted to areas where the first and second intermediate hosts coexist and where humans have the habit of eating

raw, pickled, or undercooked fish and other aquatic products. Parasite eggs from infected humans or animals reach freshwater bodies through contaminated fecal matter, e.g., through non-hygienic defecating habits of humans or the use of human feces for fertilizer (night soil). Food-borne trematodes have widespread zoonotic reservoirs. Cats, dogs, foxes, pigs, and rodents are definitive hosts for *C. sinensis*, and domestic ruminants serve as reservoirs for *F. hepatica* infections. Once eggs have reached a suitable body of freshwater, they develop and release a miracidium. It enters an aquatic snail, which acts as first intermediate host. Inside the snail, within several weeks, the miracidium transforms into cercariae. They are released into the freshwater environment and attach, penetrate, and encyst as metacercariae in susceptible second intermediate hosts. Infection with food-borne trematodes is accomplished through ingestion of metacercariae by eating raw or insufficiently cooked freshwater fish (*C. sinensis*, *Opisthorchis* spp.), freshwater crab or crayfish (*Paragonimus* spp.), aquatic plants, or by drinking contaminated water (*Fasciola* spp.).This determines the focal distribution of the food-borne trematode infections [103, 105], and endemic areas therefore can be identified as the area where the people eat raw, pickled, and semi-cooked freshwater species of crabs, shrimps, and crayfishes.

Symptoms of clonorchiasis include anorexia, indigestion, abdominal pain, weakness, weight loss, diarrhea, jaundice, portal hypertension, ascites, gastrointestinal bleeding, gallstone formation, inflammation, and hyperplasia of the biliary epithelium leading to deposition of fibrous tissue. Invasion of the pancreatic duct occurs in patients with heavy infections.

Many of the signs and symptoms of opisthorchiasis are similar to those described for clonorchiasis. With chronic heavy infections, patients present with enlarged gallbladder, cholecystitis, cholangitis, liver abscess, and gallstones.

Fasciola spp. (*F. gigantica* and *F. hepatica*) infection causes abdominal pain frequently localized to right hypochondrium, anorexia, weight loss, malaise, mild intermittent fever, mild hepatomegaly, jaundice, biliary abnormalities, traumatic and necrotic lesions in hepatic tissue, and fibrosis of biliary ducts.

Paragonimiasis is characterized by the following symptoms: chest pain, cough with rust-colored sputum, fatigue, fever, focal hemorrhagic pneumonia, migrating subcutaneous nodes granuloma formation, and fibrotic encapsulation in the lung parenchyma. Abdominal pain causes decreased appetite and diarrhea. Flukes tend to migrate to ectopic sites and can cause cerebral paragonimiasis which is fatal. Table 12.3 showed the prevalence data of food-borne trematodes in Southeast Asian countries.

(i) *Clonorchis sinensis*

In 2002 it was reported that more than 18 million people were infected with fish-borne trematodes worldwide [124]. In Southeast Asia *C. sinensis* is prevalent especially in Vietnam. During a study conducted on community that eats raw fish, *C. sinensis* was recovered from 51.5 % of the participants [107]. More recent study performed on fish-farming community in Nam Dinh showed that 32.2 % fish farm household members were infected with *C. sinensis* [108].

Table 12.3 Prevalence data of food-borne trematodes in Southeast Asian countries

Country	Prevalence of food-borne trematodes	Year reported	Reference
(i) *Clonorchis sinensis*			
Vietnam	51.5 %	2007	[107]
	32.2 %	2011	[108]
Thailand	23 % (Central)	2009	[109]
(ii) *Opisthorchis viverrini*			
Cambodia	4.6 %	2012	[110]
Lao PDR	50 % (Southern provinces)	2000	[111]
	23 % (Thakhek), 15 % (Vientiane)	2003	[112]
	85 % (Southern region)	2007	[48]
Thailand	9.6–19.3 %	2003	[113]
	2.1–70.8 % (Khon Kaen District)	2004	[114]
	7–13 % (Khukan District)	2004	[115]
	64 % (Central)	2009	[109]
Vietnam	21 %	2004	[116]
(iii) *Fasciola* spp.			
Vietnam	1,350 cases (2008), 3,000 cases (2009)	2001	[117]
Lao PDR	2.4 % (stool examination), 13.8 % (serology)	2008	[118]
(iv) *Paragonimus westermani*			
Lao PDR	51 % (villagers), 14.5 % (school children)	2008	[119]
Vietnam	12.7 % (Sinho district), 3.3 % (Luc Yen district)	2011	[120]
Thailand	10 % (samples of the 1980s), 4.9 % (samples of 2005)	2008	[121]
	15.8 % (samples of 1988)	1988	[122]
	0.51 % (samples of 2000)	2001	[123]

Clonorchis sinensis infection was also reported in central Thailand. Based on analysis of microscopy-positive PCR products, it was revealed that 23 % of individuals were infected with *C. sinensis* [109].

(ii) *Opisthorchis viverrini*

It was estimated that ten million people are infected with *O. viverrini* [125]. In Southeast Asia, *O. viverrini* is endemic in Cambodia, Lao PDR, Thailand, and Vietnam [105].

In Cambodia, the prevalence of this trematode has been reported as 4.6 % based on a study conducted on the residents and school children of Takeo Province. They were found positive for *O. viverrini* egg [110].

World Health Organization (WHO) estimated that over two million people are infected with *O. viverrini* in Lao PDR [126]. In the cities of Thakhek (Khammouane province) and Vientiane capital, infection rates were found to be at 23 % and 15 %, respectively [112]. Highest infection rate was seen in the southern provinces, where it exceeded 50 % in school children [111]. The

infection remains common in Lao PDR with an extensive distribution in the southern region with prevalence detected approaching 85 % [48].

It was reported that approximately six million people harboring *O. viverrini* in Thailand live in the northern and northeastern regions [127]. An epidemiologic survey showed that the average prevalence of opisthorchiasis in Thailand for 2001 was relatively high, ranging from 9.6 to 19.3 % [113]. Other studies conducted in 2004 showed that the average prevalence of *O. viverrini* infection was 24.5 % (ranging from 2.1 to 70.8 %) in various districts of Khon Kaen [114] while *O. viverrini* infection in Khukan District, Si Sa Ket Province ranging from 7 to 13.6 % [115]. *Opisthorchis viverrini* was also reported in central Thailand where 64 % of individuals were found infected based on analysis of the microscopy-positive PCR products [109].

A survey conducted in Vietnam from 1976 to 2002 on over 30,000 human stool samples from 15 provinces demonstrated average infection rate of *Clonorchis/Opisthorchis* in those provinces was 21 % [116].

(iii) *Fasciola* spp.

Fasciola hepatica and *F. gigantica* are the causative agents of liver fluke disease in domestic animals and humans. *F. hepatica* is common in sheep-raising countries like parts of Europe, the Middle East, and South America, while in Southeast Asia, *F. gigantica* is more commonly found. The two species can coexist in some countries.

In Southeast Asia, Vietnam has reported an increase of *Fasciola* infection. Here, human fascioliasis was distributed mainly in central region and the midland provinces. Based on hospital cases captured, The Vietnamese Ministry of Health reported an approximately 3,000 cases of fascioliasis in 2009 which was an approximate 45 % increase of the same infection reported in 2008. They also confirmed that almost all patients presented hepatic symptoms [117].

Fascioliasis is also found in Lao PDR where the prevalence of human fascioliasis was shown to be 2.4 % based on stool examination but increased to 13.8 % when systematic serology testing was employed [118].

(iv) *Paragonimus westermani*

There are about 15 species of *Paragonimus* known to infect humans. *Paragonimus westermani* is the most common worldwide. More than 20 million people are infected with *Paragonimus* spp. [128]. In Southeast Asia, human paragonimiasis is found in Lao PDR, Vietnam, and Thailand [105, 128].

A field investigation employing intradermal skin tests conducted in Lao PDR between 2003 and 2005 on 308 villagers and 633 school children showed that among the 308 villagers tested, almost 51 % presented positive reaction with male exhibiting a higher positive rate (66.2 %) than the female (37.3 %). As for the school children, the skin test positive reaction rate was 14.5 % [119].

In Vietnam a seroepidemiological surveys showed 12.7 % participants in Sinho district of Laichau province and 3.3 % of participants in Lucyen district

of Yenbai province were antibody positive against the *Paragonimus* antigen [120].

Using serodiagnostic method, IgG-ELISA for paragonimiasis on samples obtained in the 1980s and in 2005 from two villages near Chet Khot Waterfall, Kaeng Khoi District, in Saraburi province, Thailand, it was demonstrated that paragonimiasis occurred in 10 % of the total population of the 1980s and 4.9 % of those of 2005 [121]. Based on this finding, it can be concluded that the prevalence of paragonimiasis has decreased in Thailand (at least in the study area concerned). This conclusion is supported by another report of paragonimiasis in another paragonimiasis endemic area, Noen Maprang District, Phitsanulok Province, where its earlier prevalence of 15.8 % [122] was later dropped to 0.51 % during the follow-up study in 2000 [123].

12.19 Conclusion

Based on the data presented, the parasitic infections caused by cestodes and trematodes in Southeast Asia are clearly still a major burden on public health. With many countries having limited, incomplete, and outdated data, it is very possible to find that the status of these infections occurring currently in these countries differ from what was reviewed. This is because perpetuation and enhancement of these parasitic infections discussed are very much influenced by many factors such as geography, environment, socioeconomic, development, the population's religious and social beliefs, lifestyle, and customs which are not an easy proposition to tackle in order to achieve improvement for the existing situation.

Southeast Asia will remain endemic for these infections if progress pertaining to information and awareness about the extent and burden of the problem, suitable diagnostic and management capacity, and appropriate prevention and control strategies are not forthcoming. All these not only need to be well documented but must be applied in order to bring to the attention of the affected communities and the decision makers. Decision makers' understanding on burden of diseases, its impact on the health and agriculture, and overall development of the population effected is so crucial for any appropriate and relevant policies to be formulated and successfully implemented. Only then sustainability of any control and preventive strategies put in place is guaranteed and situation improved.

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Index

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