Chapter 2
Plasmodium knowlesi: Emergent Human Malaria in Southeast Asia

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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 $100\times$ 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
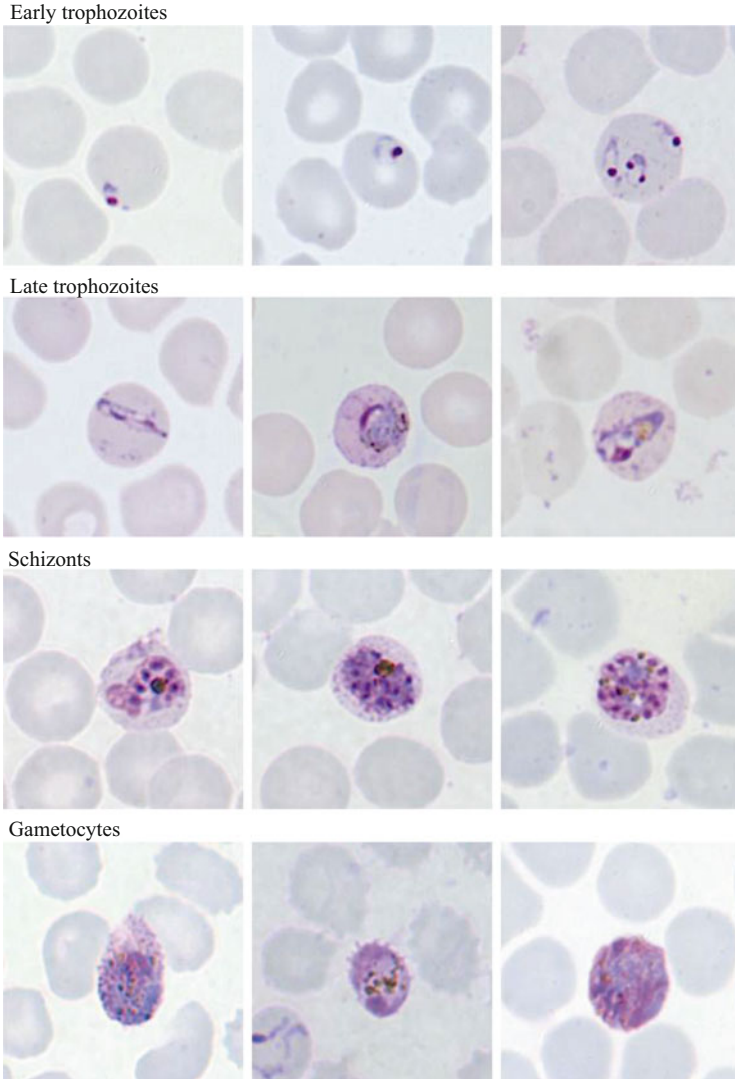
Early trophozoites

Late trophozoites

Schizonts

Gametocytes

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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