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Human Infections and Detection of *Plasmodium knowlesi*

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SUMMARY

Plasmodium knowlesi is a malaria parasite that is found in nature in long-tailed and pig-tailed macaques. Naturally acquired human infections were thought to be extremely rare until a large focus of human infections was reported in 2004 in Sarawak, Malaysian Borneo. Human infections have since been described throughout Southeast Asia, and *P. knowlesi* is now recognized as the fifth species of *Plasmodium* causing malaria in humans. The molecular, entomological, and epidemiological data indicate that human infections with *P. knowlesi* are not newly emergent and that knowlesi malaria is primarily a zoonosis. Human infections were undiagnosed until molecular detection methods that could distinguish *P. knowlesi* from the morphologically similar human malaria parasite *P. malariae* became available. *P. knowlesi* infections cause a spectrum of disease and are potentially fatal, but if detected early enough, infections in humans are readily treatable. In this review on knowlesi malaria, we describe the early studies on *P. knowlesi* and focus on the epidemiology, diagnosis, clinical aspects, and treatment of knowlesi malaria. We also discuss the gaps in our knowledge and the challenges that lie ahead in studying the epidemiology and pathogenesis of knowlesi malaria and in the prevention and control of this zoonotic infection.

INTRODUCTION

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium*. Over 150 species have been described to date, infecting mammals, birds, and reptiles (1). Despite having

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such a large number of hosts, in general, malaria parasites tend to be host specific. For example, humans are the natural hosts for 4 species, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, while long-tailed macaques (*Macaca fascicularis*) are hosts for 5, *P. knowlesi*, *P. fieldi*, *P. coatneyi*, *P. cynomolgi*, and *P. inui* (2). Zoonotic malaria was considered to be extremely rare until a large focus of *P. knowlesi* infections in the Kapit Division of Sarawak, Malaysian Borneo, was described in 2004 (3). Since then, human cases have been described in virtually all Southeast Asian countries, and *P. knowlesi* is now considered the fifth species of *Plasmodium* causing malaria in humans (4, 5). In this review on knowlesi malaria, we describe the early studies on *P. knowlesi* and focus on the epidemiology, diagnosis, clinical features, and treatment of knowlesi malaria. We also discuss the gaps in our knowledge and the challenges that lie ahead in studying the epidemiology and pathogenesis of knowlesi malaria and in the prevention and control of this zoonotic infection.

Life Cycle of *Plasmodium* in Humans

The life cycle of malaria parasites in humans and other primates begins when a female anopheline mosquito injects sporozoites into the host while taking a blood meal (1). These parasites are taken by the bloodstream to the liver, where they invade hepatocytes, undergo asexual multiplication, and develop into schizonts. The hepatic schizonts that rupture release thousands of merozoites that invade erythrocytes (RBCs) to continue their development. Within the erythrocyte, the merozoite develops into a ring or early trophozoite form, which in turn develops into a mature trophozoite that undergoes asexual multiplication to form a schizont containing numerous merozoites. The erythrocytic schizont ruptures, releasing merozoites that invade erythrocytes, thereby completing the erythrocytic cycle. Some of the merozoites also develop within the erythrocytes into male and female gametocytes, which are taken up during a blood meal by female anopheline mosquitoes, in which they continue their development.

There are no clinical signs and symptoms when the malaria parasites are developing in the liver. These are associated with the cycle of the parasites in the erythrocytes. The duration of the erythrocytic cycle depends on the species of *Plasmodium*: *P. knowlesi* has the shortest cycle, approximately 24 h, while for *P. falciparum*, *P. vivax*, and *P. ovale*, it is approximately 48 h, and for *P. malariae*, it is 72 h (1). Therefore, if untreated, the parasite counts or parasitemia will continue to increase approximately every 24, 48, or 72 h, depending on the species of *Plasmodium*. In synchronous infections of single clones, particularly for *P. knowlesi*, *P. vivax*, *P. ovale*, and *P. malariae*, there is a fever peak which occurs following the release of merozoites by rupturing schizonts, resulting in quotidian, tertian, or quartan fever patterns. However, the fever patterns may be daily and may not be at these regular intervals for all species of *Plasmodium* early in infection and also in cases where mixed species or more than one brood of parasites is present (6, 7).

Discovery and Early Studies

P. knowlesi was first isolated and studied in detail at the Kolkata School of Tropical Medicine in India in the early 1930s, after it was noticed by Campbell in a blood film from a long-tailed macaque that had been imported from Singapore (8–10). Campbell and Napier, who were working on leishmaniasis, inoculated *P. knowlesi*-infected blood into two long-tailed macaques and a rhesus macaque (*Macaca mulatta*) and reported that it produced a fulminating infection in the rhesus macaque and a mild infection in the long-tailed macaques. Knowing that malaria was the research focus of Knowles and Das Gupta, they handed the macaque to them for a series of experiments that confirmed that blood passage of *P. knowlesi* among its natural hosts, *Macaca fascicularis*, resulted in low parasitemia (8). In contrast, blood passage into rhesus macaques, which are indigenous to India and are not the natural hosts of *P. knowlesi*, resulted in extremely high parasitemia and fatal infections, unless the macaques were treated with antimalarials. Knowlesi and Das Gupta then successfully infected three human volunteers with knowlesi malaria following macaque blood passage in two volunteers and human blood passage in the third volunteer (8). Those researchers observed that all three human recipients of *P. knowlesi*-infected blood developed malaria and commented that although the fever pattern was daily, further work was required to confirm the periodicity of the fever. They also observed that the morphology of the parasites resembled that of *P. malariae*. The quotidian nature of the fever pattern, indicating that *P. knowlesi* had a 24-h erythrocytic cycle, was confirmed by Sinton and Mulligan (11, 12). They studied the morphology of the parasite in detail in nonhuman primate hosts and named this new malaria parasite in honor of Robert Knowles.

From the 1920s until the mid-1950s, prior to the discovery of penicillin, patients with tertiary neurosyphilis were successfully treated by inducing fever with *P. vivax*, a treatment that was pioneered by Wagner-Juaregg (13) and often referred to as malariotherapy (14). Since *P. knowlesi* had a shorter erythrocytic cycle than *P. vivax*, it was used for malariotherapy by physicians in a number of countries (15–18), particularly by Ciuca and coworkers in Romania (19–21). Those researchers stopped using the *P. knowlesi* strain when they noticed that it had become more pathogenic following 170 serial passages in patients with neurosyphilis (21).

It was known that humans could acquire knowlesi malaria by blood passage soon after *P. knowlesi* was isolated in 1931. However, the first case of a natural infection in a human was only reported 34 years later, when a U.S. Army surveyor acquired the infection while working in the forest in the state of Pahang in Peninsular Malaysia (22). This finding was the result of an extraordinary sequence of events. The surveyor had returned to the United States, where his doctor observed malaria parasites that resembled the ring forms of *P. falciparum*. His doctor sent him to the National Institutes of Health Clinical Center in Bethesda, MD, where they saw "band forms" of malaria parasites resembling *P. malariae*. They obtained a blood sample before treating him and sent the sample to Atlanta, GA, where chemotherapy trials on *P. malariae* were being conducted on volunteers at the U.S. Penitentiary in Atlanta. Inoculation of blood into the first volunteer and six other human volunteers produced quotidian or daily fever patterns, and subsequent inoculation of infected human blood into three rhesus macaques produced fatal infections, thereby confirming that the surveyor was naturally infected with *P. knowlesi* (23). This single case was followed by a presumptive human case of knowlesi malaria that was acquired in Johore, Peninsular Malaysia, in 1971, where detection was based on parasite morphology and serological methods, since no pretreatment blood was available for inoculation into rhesus macaques (24).

The accidental infection of humans by mosquito bites in the

early 1960s in two different laboratories in the United States with *P. cynomolgi* (25, 26), another malaria parasite of long-tailed macaques (1), resulted in the initiation of studies to investigate whether malaria was a zoonosis, since zoonotic malaria would have hampered the Malaria Eradication Program launched by the WHO. These investigations by a team from the U.S. National Institutes of Health working in close collaboration with colleagues at the Institute for Medical Research in Kuala Lumpur, Peninsular Malaysia, were intensified with the report of the naturally acquired human infection with *P. knowlesi* (22). A total of 1,117 blood samples were collected from villagers living in the vicinity where the American army surveyor had been working in the forest in Pahang State, Peninsular Malaysia (27). These samples, comprising only 28 that were malaria positive by microscopy, were pooled and injected into rhesus macaques, but none of the macaques acquired malaria. However, *P. knowlesi* was identified in 2 of 4 long-tailed macaques from the study area. The researchers therefore concluded that natural infections of humans with *P. knowlesi* and other simian malaria parasites within the study population were extremely rare and that there probably existed two cycles of transmission of *P. knowlesi*: one among the macaques at the canopy level and another, less common, one from macaques to humans. Subsequently, malariologists downplayed the significance of the zoonotic potential of malaria.

The generally held view that zoonotic malaria was an extremely rare event changed following the discovery of a large focus of human infections with *P. knowlesi* in the Kapit Division of Sarawak, Malaysian Borneo (3). Singh and colleagues were prompted to study microscopy-confirmed cases of *P. malariae* since there appeared to be foci of these cases in Sarawak. One of these foci was in the Kapit Division, where microscopy-confirmed *P. malariae* cases in 1999 accounted for 40% of the 270 microscopy-confirmed cases for the state of Sarawak. Furthermore, in contrast to *P. malariae* infections, which normally result in asymptomatic infections with low parasitemia (<5,000 parasites per μl blood) and affects people of all age groups (1, 6), most of the cases in Kapit were reported in adults seeking treatment at hospitals, and parasitemia was higher than that expected for *P. malariae* malaria. Initial investigation of 4 such "*P. malariae*" samples from Kapit Hospital by nested PCR assays indicated that *Plasmodium* DNA was present but that the patients were not infected with *P. malariae* or with the other 3 species of *Plasmodium* tested. DNA sequencing of two nuclear genes, the small-subunit (SSU) rRNA genes and the circumsporozoite gene, followed by phylogenetic analyses indicated that 8 "*P. malariae*" patients were infected with *P. knowlesi* (3). PCR primers that were specific for *P. knowlesi* were developed, and a prospective study was undertaken with 208 blood samples collected from patients admitted to Kapit Hospital with malaria, 141 of whom had been diagnosed with *P. malariae* by microscopy (3). PCR assays revealed that knowlesi malaria accounted for 58% of 208 admissions for malaria at Kapit Hospital: 112 were single *P. knowlesi* infections, 8 were *P. knowlesi* coinfections with other *Plasmodium* species, and none were *P. malariae*.

EPIDEMIOLOGY

Distribution

Following the description of the large focus of human knowlesi malaria cases in the Kapit Division of Malaysian Borneo in 2004 (3), there have been reports of infections acquired in Kapit and other locations in Malaysian Borneo (28–43) and in Peninsular Malaysia (28, 44–47). In some hospitals in Malaysian Borneo, knowlesi malaria accounts for the majority of malaria cases admitted to hospitals (29, 38, 43). Human cases are not restricted to Malaysia, with reports of infections acquired in Thailand (48–53), the Philippines (54–56), Myanmar (57, 58), Singapore (59–61), Vietnam (62, 63), Indonesia (64, 65), Brunei (66), and Cambodia (67) (Fig. 1). Transmission of knowlesi malaria to humans has therefore been reported in all the countries in Southeast Asia except Laos. Most of the human knowlesi malaria cases have been detected in Sarawak and Sabah, Malaysian Borneo, mainly because extensive studies utilizing molecular detection methods have been undertaken in these two states. With regard to Sarawak, since molecular methods for malaria detection were employed at the Malaria Research Centre, Universiti Malaysia Sarawak, over 881 local *P. knowlesi* cases and only 6 *P. malariae* cases have been identified from 2000 to 2011 (3, 5, 37, 38). All 6 were logging camp workers who had acquired their infections overseas, thereby indicating that there are no indigenous cases of *P. malariae* in Sarawak. The situation appears to be similar in Sabah, where the number of *P. knowlesi* infections detected by PCR at the Public Health Laboratory and at Universiti Malaysia Sabah greatly outnumbers those of *P. malariae* (30, 40, 41). The reports to date indicate that adults are more commonly infected than children. The actual incidence of knowlesi malaria in each of the countries in Southeast Asia, including Malaysia, is unknown, mainly because only a limited number of studies utilizing molecular detection methods have been undertaken, and it is not possible to accurately identify *P. knowlesi* by microscopy due to its morphological similarities with *P. malariae* and *P. falciparum* (68). Additional extensive studies utilizing molecular detection methods would probably uncover more human knowlesi malaria cases in Southeast Asia.

Reservoir Hosts

The natural hosts of *P. knowlesi* that were initially identified were long-tailed (*M. fascicularis*) and pig-tailed (*Macaca nemestrina*) macaques from Singapore (8) and Peninsular Malaysia (69, 70), and there has also been a report of a single *P. knowlesi* isolation from a leaf monkey (*Presbytis melalophos*) from Peninsular Malaysia (71). Subsequently, *P. knowlesi* infections were detected in macaques from Cebu (72) and Palawan Island (73), Philippines. More recent reports, using molecular detection methods and sequencing, have identified *P. knowlesi* infections in wild long-tailed macaques in Sarawak, Malaysian Borneo (74); Peninsular Malaysia (44); Singapore (61); and Southern Thailand (49) and in wild pig-tailed macaques in Southern Thailand (49) and Sarawak (74). These two macaque species are distributed throughout Southeast Asia (75, 76) (Fig. 1) and are the most common nonhuman primates in this region. The observation that peridomestic long-tailed macaques in Singapore (61) and macaques living close to a temple in Thailand did not harbor *P. knowlesi* or other simian malaria parasites is a reflection of the absence of a competent mosquito vector for parasite transmission. The highest prevalence of *P. knowlesi* has been observed in wild macaques of the Kapit Division in Sarawak, Malaysian Borneo, where 87% of 83 long tailed-macaques and 50% of 26 pig-tailed macaques were *P. knowlesi* positive. The very high prevalence of *P. knowlesi* and other malaria parasites in these macaques (94% were malaria positive) from 17 locations indicates that malaria transmission is in-

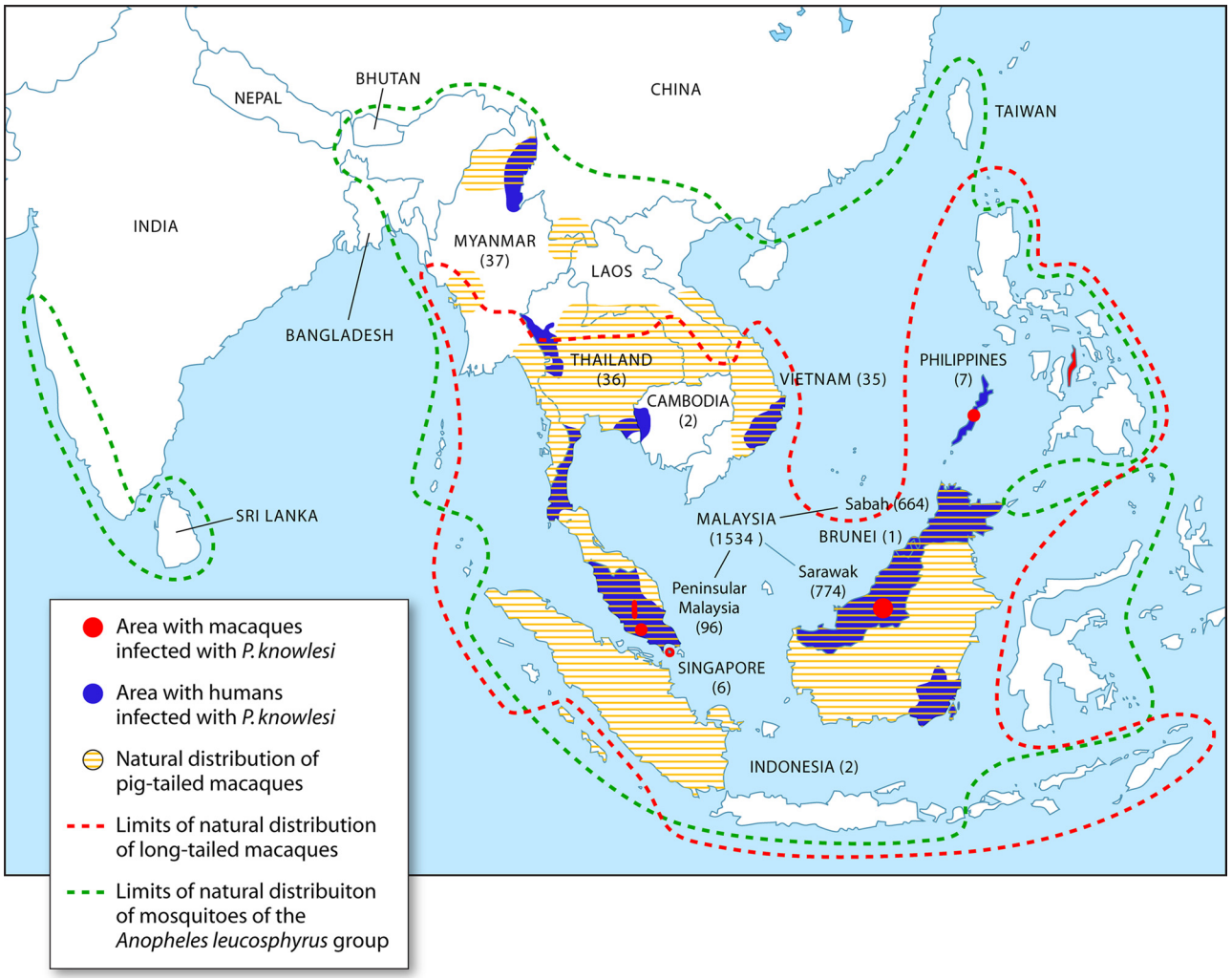

FIG 1 *Plasmodium knowlesi* infections reported in humans and macaques and limits of natural distribution of mosquito vectors and of macaques. The numbers in parentheses represent numbers of *P. knowlesi* cases reported for each Southeast Asian country or region in Malaysia. (Adapted from reference 5 with permission from Elsevier.)

tense among the wild-macaque population in the Kapit Division of Malaysian Borneo.

Vectors

The vectors of knowlesi malaria are forest-dwelling mosquitoes that belong to the *Anopheles leucosphyrus* group, and their distribution in Southeast Asia largely overlaps that of long-tailed and pig-tailed macaques (77, 78) (Fig. 1). The first vector found in nature to be infected with *P. knowlesi* was *A. hackeri*, a predominantly zoophilic mosquito, in Selangor State, Peninsular Malaysia, in 1961 (79). Experimental studies in the United States undertaken in the 1960s with the H strain of *P. knowlesi* (isolated from the American surveyor) showed that among the anophelines tested for the presence of sporozoites in the salivary glands and their capability of transmitting *P. knowlesi* to rhesus macaques, *A. balabacensis* was the most competent vector, followed by *A. stephensi*, *A. maculatus*, and *A. freeborni* (80, 81). Human-to-human, monkey-to-human, and human-to-monkey transmission of *P. knowlesi* was demonstrated by using *A. balabacensis*, with incubation periods in the vector of 12 to 13 days. In Kapit, Malaysian Borneo, where most of the human knowlesi malaria cases have been described, *A. latens* has been incriminated as the vector (82, 83). This mosquito species feeds mainly between 7 and 10 p.m. in the forest, is attracted to both long-tailed macaques and humans, and prefers to feed on macaques at higher elevations. *A. cracens* has recently been incriminated as a vector for knowlesi malaria in Pahang, Peninsular Malaysia, with peak biting times between 8 and 9 p.m. (44, 84). This species is highly zoophilic and was previously found to feed on macaques at the canopy level and on humans at the ground level. None of these vectors of *P. knowlesi* were found to be infected with the human malaria parasites *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* during recent entomological surveys in Sarawak (82, 83) and Peninsular Malaysia (44). Interestingly, at 4 collection sites in the forest and forest fringe areas near a village in south central Vietnam, *A. dirus* mosquitoes were found to harbor sporozoites of *P. knowlesi* alone and mixed with *P. vivax* or *P. falciparum* and also mixed with both *P. falciparum* and *P. vivax* (62, 85). Since *A. dirus* is the only known vector for human malaria in this area (62), this raises the possibil-

ity that human-to-human transmission of *P. knowlesi* could occur and may actually be occurring in this region in Vietnam.

Populations at Risk

For any vector-borne disease, transmission is highly dependent on the bionomics and distribution of the vectors. Since the vectors of *P. knowlesi* are restricted to members of the *A. leucosphyrus* group, which are found in the forest and forest fringe, populations that are particularly at risk of acquiring knowlesi malaria are people who live there or those that venture into the ecological habitat of macaques and the anopheline vectors of *P. knowlesi* for either work or leisure. For example, in Sarawak, Malaysian Borneo, where most cases of knowlesi malaria have been reported, the majority of knowlesi malaria patients are adults who are subsistence farmers, hunters, and logging camp workers (3, 5, 28). Those acquiring their infections in Vietnam appear to be very similar to those in Sarawak in that they are people in the forest fringe who enter the forests to collect bamboo and rattan and work on their farms on mountain slopes (62), and there have also been cases of children living in forest communities with knowlesi malaria (63). In Singapore, servicemen acquired knowlesi malaria while training in a forested area (61). Visitors to Southeast Asia have not been spared, with reports of adult travelers from Sweden (31), Finland (47), France (52), Spain (53), the Netherlands (42), Taiwan (56), the United States (55), and the United Kingdom (66) returning home with *P. knowlesi* infections following visits to Sarawak (31, 42), the Philippines (55, 56), Peninsular Malaysia (47), Brunei (66), and Thailand (52). Furthermore, a helicopter pilot returned to New Zealand with knowlesi malaria following a working stint in the interior of Sarawak, Malaysian Borneo (36), and an Australian acquired it while working in South Kalimantan Province, Indonesian Borneo (64).

Molecular Epidemiology and Evolutionary and Demographic History

In order to expand our understanding of the epidemiology of knowlesi malaria, *P. knowlesi* isolates derived from humans and macaques have been characterized and compared. The studies from the Kapit Division of Sarawak revealed that some of the *P. knowlesi* isolates derived from wild macaques shared identical mitochondrial DNA (mtDNA) and circumsporozoite protein gene (*csp*) sequences with those derived from humans (74). Furthermore, wild macaques had higher numbers of haplotypes of mtDNA and alleles of the *csp* gene per infection than humans, probably reflecting the high level of malaria transmission among wild macaques in Kapit. Limited molecular studies in Peninsular Malaysia (44) and Singapore (61) also demonstrated shared alleles of *csp* among humans and macaques, while in Thailand, alleles of the merozoite surface protein 1 gene (*msp-1*) were found to be similarly diverse in both hosts (49). In the Sarawak studies, the mtDNA haplotypes and the mtDNA lineage were not associated exclusively with either vertebrate host, and the cumulative molecular evidence together with the epidemiological and entomological data support the view that knowlesi malaria is primarily a zoonosis and that wild macaques are the reservoir hosts.

By comparing DNA sequence data of nuclear genes of *P. knowlesi* derived from both macaque and human hosts, it is not possible to ascertain whether knowlesi malaria is a newly emergent zoonosis. However, through the analysis of *P. knowlesi* mtDNA sequences, it is possible to extend our understanding of the evolutionary and demographic history of *P. knowlesi*. Through such analyses, the estimated time to the most recent common ancestor (TMRCA) of *P. knowlesi* was 98,000 to 478,000 years ago (74), which indicates that *P. knowlesi* is derived from an ancestral parasite population. It is as old as or older than the human malaria parasites *P. falciparum* and *P. vivax*, for which the TMRCAs have been estimated to be 50,000 to 330,000 years ago (86, 87) and 53,000 to 265,000 years ago (88, 89), respectively. The emergence of *P. knowlesi* from an ancestral parasite predates the arrival and settlement of humans in Southeast Asia approximately 70,000 years ago (90) but not that of the genus *Macaca*. Macaques migrated to Asia from Africa approximately 5.5 million years ago, and the *M. fascicularis* group emerged about 3.7 million to 4.0 million years ago (91–93). It is highly likely that *P. knowlesi* migrated with the natural macaque hosts to Borneo during the Pleistocene era, when sea levels were lower than they are now and the island of Borneo was connected to mainland Southeast Asia (94). Analysis of *P. knowlesi* mtDNA sequence data also showed that *P. knowlesi* parasites in Sarawak underwent rapid population growth between 30,000 and 40,000 years ago, concordant with a time of human population growth in Southeast Asia (95). Similar analyses of mtDNA of *M. fascicularis* and *M. nemestrina* have not been undertaken, so it is not possible to ascertain whether the population expansion of *P. knowlesi* was due to that of the human or the macaque hosts or even that of the mosquito vectors. Nevertheless, molecular evidence indicates that *P. knowlesi* is an ancient parasite and strongly suggests that it is not newly emergent in the human population. Precisely when humans first became infected with *P. knowlesi* is not known. In Sarawak, the first malaria survey using microscopy was undertaken in 1952, where out of 421 malaria cases detected during community surveys in 6 areas, 142 (33.7%) were *P. malariae* cases (96). In two areas, *P. malariae* accounted for 68.8% and 76.3% of malaria cases detected. Analysis by PCR of DNA extracted from archival "*P. malariae*" slides from 1996 in Sarawak (37) indicates that these were *P. knowlesi* infections, and recent studies showed that parasites identified by microscopy as *P. malariae* were *P. knowlesi*. Slides from the 1952 surveys are not available for analysis by PCR assays, but it is highly likely that these microscopy-confirmed *P. malariae* infections were actually *P. knowlesi* infections. In conclusion, the molecular data indicate that *P. knowlesi* is an ancient parasite and that it has most probably been infecting humans in Southeast Asia ever since it first emerged in macaques in this region.

LABORATORY DIAGNOSIS

Microscopy

The most widely used method for detection of malaria in rural settings is microscopy, since it is a relatively cheap, rapid, quantitative, and sensitive technique. Microscopists in Southeast Asia are largely trained to identify the three main species of *Plasmodium* that cause malaria in humans in the region, namely, *P. falciparum*, *P. vivax*, and *P. malariae*. Each of these species has morphological characteristics that should enable a well-trained microscopist to identify them fairly accurately (97). For example, for both *P. falciparum* and *P. malariae*, there is no enlargement of malaria-infected RBCs. However, due to sequestration of late trophozoites and schizonts of *P. falciparum*-infected erythrocytes in blood capillaries, only ring forms or early trophozoite and crescent-shaped gametocytes are observed in peripheral blood films prepared from patient samples (Fig. 2), unless parasitemia is very

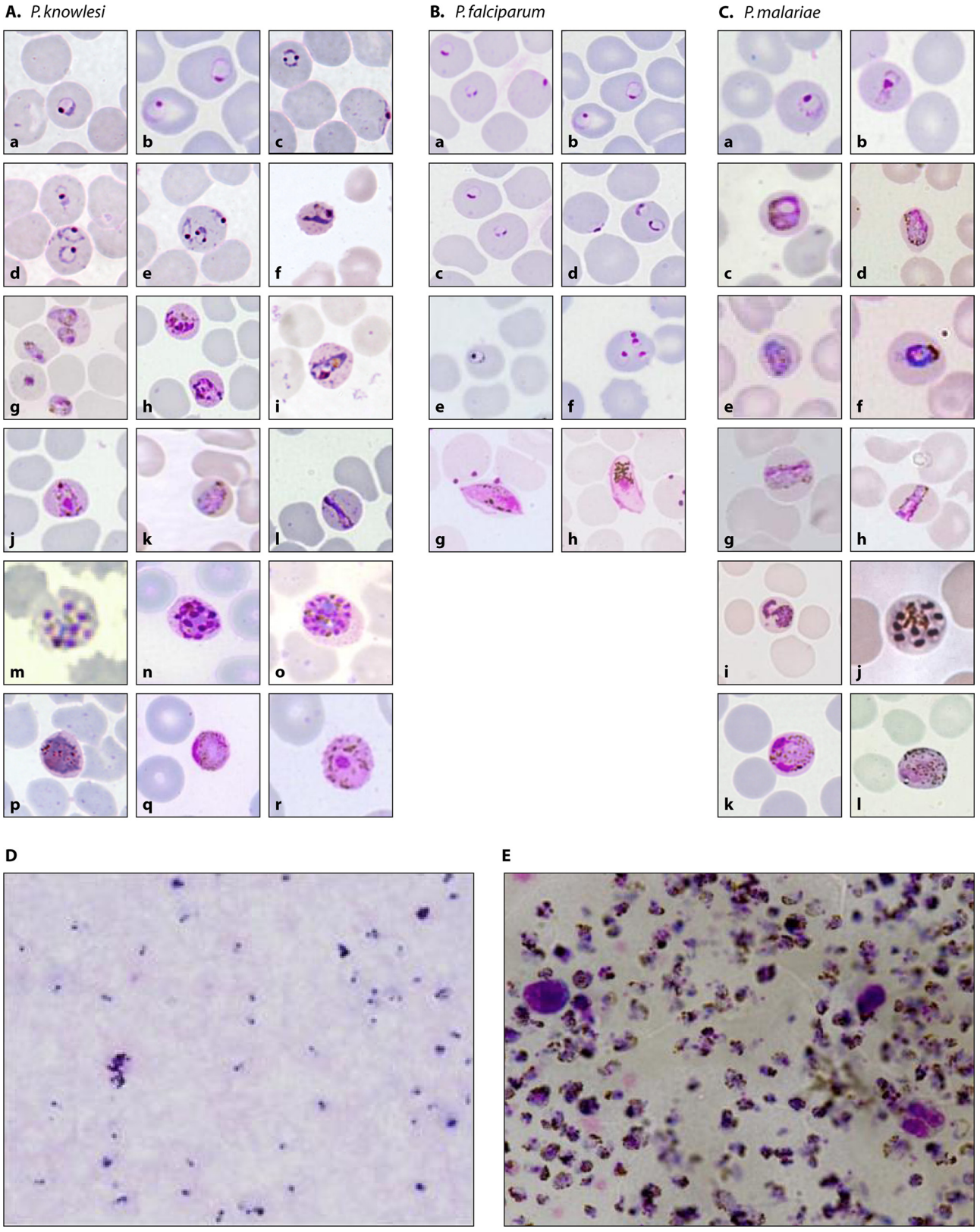

FIG 2 Erythrocytic stages of *P. knowlesi*, *P. malariae*, and *P. falciparum* observed in Giemsa-stained peripheral blood films. (A to C) Thin blood films with early trophozoites of *P. knowlesi* (Aa to e), *P. falciparum* (Ba to f), and *P. malariae* (Ca); late trophozoites, including band forms, of *P. knowlesi* (Af to l) and *P. malariae* (Cb to i); schizonts of *P. knowlesi* (Am to o) and *P. malariae* (Cj); and gametocytes of *P. knowlesi* (Ap to r), *P. falciparum* (Bg and h), and *P. malariae* (Ck and l). (D and E) Thick blood films showing early trophozoites of *P. knowlesi* resembling those of *P. falciparum* (D) and heavy parasitemia from a fatal *P. knowlesi* infection (E). (Photographs in panels Aa to e, m, and o and Cc, j, and l have been reproduced from reference 5 with permission from Elsevier, and photographs in panels A and C have been reproduced from reference 68.)

high (98). For *P. malariae*, all erythrocytic stages are seen in blood films, and some trophozoites stretch across the erythrocyte and appear as "band forms." However, even well-trained microscopists not uncommonly have difficulty distinguishing early trophozoites of *P. vivax* from those of *P. falciparum*, particularly when parasitemia is low. Furthermore, although these species of *Plasmodium* should be correctly identified by microscopy, misidentification does occur, as exemplified by a study by Cox-Singh et al., where 43/440 (10%) patients with PCR-confirmed *P. vivax* infection in Sarawak were misdiagnosed as having *P. malariae/P. knowlesi* infection (28).

It is not possible to accurately identify *P. knowlesi* by microscopy, since the morphological features of the early trophozoites of *P. knowlesi* are identical to those of *P. falciparum*, with double-chromatin dots, multiple infections per erythrocyte, and no enlargement of infected erythrocytes. The rest of the blood stages of *P. knowlesi* resemble those of *P. malariae*, including band-form trophozoites (Fig. 2). The morphological similarities between *P. knowlesi* and *P. malariae* were first noted by Knowles and Das Gupta when they induced knowlesi malaria by blood passage in three human subjects in 1932 (8). They wrote that in humans, the parasites show little or no amoeboid activity; the red corpuscles are not enlarged; with Leishman's or Giemsa's stain, no stippling is seen; and the general morphology rather recalls that of *P. malariae* of humans.

Careful examination of well-stained slides shows minor differences in morphology between *P. knowlesi* and *P. malariae*, such as a certain proportion of early trophozoites of *P. knowlesi* with double-chromatin dots and schizonts of *P. knowlesi* having up to 16 merozoites, compared to 6 to 12 for *P. malariae* (68). However, early trophozoites and mature schizonts would not be observed in all *P. knowlesi* infections, but more importantly, these minor differences between the two species would be missed in a busy routine diagnostic laboratory in a developing country, where only thick blood films are normally examined when parasitemia is low and where microscopists have limited time to screen a large number of samples. Although parasitemia in knowlesi malaria infections can be extremely high (Fig. 2), low parasitemia is relatively common in knowlesi malaria patients. In a prospective malaria study in Kapit, 30% of 107 knowlesi malaria patients presented with parasitemia of <500 parasites/μl blood (33). Most *P. knowlesi* infections have been identified as *P. malariae* infections in routine diagnostic laboratories, as exemplified by the observation that out of 349 samples identified as single *P. knowlesi* infections by PCR, and before the microscopists at 12 hospitals in Sarawak were informed about *P. knowlesi*, 317 (91%) were identified as *P. malariae*, 18 (5%) were identified as *P. vivax*, and 14 (4%) were identified as *P. falciparum* by microscopy (28). Accurate identification of *P. knowlesi* by microscopy is a diagnostic challenge, particularly when parasitemia is low. Perhaps the best observation and prediction were made by Garnham, who wrote in his book entitled *Malaria Parasites and Other Haemosporidia* that "a *P. knowlesi* infection in a human being could easily pass unrecognized as such in routine laboratories, where it would probably be diagnosed as *P. malariae*, or if rings only were present, as *P. falciparum*" (1). Due to the difficulties in distinguishing *P. knowlesi* from *P. malariae* by microscopy, and given that *P. knowlesi* infections can lead to death (28, 32, 34, 43), at a recent WHO consultation meeting on *P. knowlesi*, it was recommended that in areas where *P. knowlesi* has been described, microscopists should report all *P. malariae*-positive results as *P. malariae/P. knowlesi* (99). Similar reporting should also be made by microscopists in areas where the disease is not endemic for slides from travelers who have visited the forest or forest fringe areas in Southeast Asian countries where human *P. knowlesi* cases have been described.

Molecular Detection Methods

Molecular detection methods have been developed for the accurate identification of malaria parasites, and these methods have consistently proven to be more sensitive and specific than microscopy (100–104). The molecular detection assays that have been described for *P. knowlesi*, the gene targets for the PCR primers in each assay, and the number of *P. knowlesi* infections detected in field isolates at each laboratory are summarized in Table 1 (3, 28, 30–34, 37–41, 44–46, 51, 54, 58–60, 62, 63, 67, 102, 105–110). The first PCR assay developed for the detection of *P. knowlesi* was a nested PCR assay with primers Pmk8 and Pmkr9, based on the small-subunit rRNA genes (3). This gene was selected because a widely used nested PCR assay for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* was being utilized for molecular epidemiological studies of malaria in Sarawak, Malaysian Borneo (101, 111). Genus-specific primers (primer rPLU6 with either primer rPLU1 or rPLU5) are used in the first round of PCR amplification, followed by species-specific primers in a separate second round of PCR amplification. The sensitivity of this method of detection is between 1 and 6 parasites/μl blood, when a DNA template is prepared with a simple boiling procedure with a chelating agent from blood spots collected onto filter paper (112). The initial knowlesi-specific primers that were developed in Sarawak, Pmk8 and Pmkr9, were found in certain laboratories to nonspecifically amplify a proportion of *P. vivax* isolates, resulting in *P. vivax* infections being identified as mixed *P. vivax* and *P. knowlesi* infections (63, 65, 113). In epidemiological studies undertaken in Sarawak, 512 single *P. vivax* infections and 28 *P. vivax* infections mixed with *P. knowlesi* infections have been detected (3, 28, 33). If these mixed infections were all due to nonspecific amplification of *P. vivax*, this would represent 5.2% false-positive *P. knowlesi* results for the *P. vivax* samples. In contrast, a laboratory in Thailand reported that 20% (6 of 30) of samples from *P. vivax* patients resulted in PCR amplification by *P. knowlesi* primers Pmk8 and Pmkr9 (113). With any PCR assay, optimization of amplification parameters is crucial, and further optimization of the assay for knowlesi malaria may have reduced the percentage of false-positive results observed. Nevertheless, new *P. knowlesi*-specific primers based on the SSU rRNA have now been developed by the laboratory in Thailand (primers PkF1140 and PkR1550) (113) and by the laboratory in Sarawak (primers Kn1f and Kn3r) (74). Recently, a single-step PCR assay for the detection of *P. knowlesi* was described, with a sensitivity of detection of 10 parasites/μl blood, using a DNA template prepared with a commercial DNA extraction kit, but this assay has yet to be validated for specificity and sensitivity with clinical and other samples from the field (108).

A number of real-time PCR assays targeting the SSU rRNA genes for detection of *P. knowlesi* have also been described (Table 2). However, with the exception of the assay described by Divis et al. (105), none of the assays have been validated against a significant number of clinical samples. Real-time PCR assays have an advantage over nested PCR assays and single-step PCR assays in that they are more rapid at providing identification and can give

TABLE 1 Molecular detection assays for *P. knowlesi*[a]

Type of assay	Gene target	Primers or probe(s)	Sensitivity	No. of human *P. knowlesi* infections identified (location of laboratory)	Reference(s)
Nested PCR	SSU rRNA (S type)	Pmk8 + Pmkr9	1–6 parasites/μl blood	852 (Sarawak)	3, 28, 31–33, 38, 54, 67
				405 (Sabah)	40, 41
				65 (Sabah)	30
				77 (Peninsular Malaysia)	44
				7 (Peninsular Malaysia)	45
				2 (Peninsular Malaysia)	46
				1 (Singapore)	59
				1 (Singapore)	60
				32 (Vietnam)	62
				3 (Belgium)	63
				1 (Thailand)	51
				36 (China)	58
				1 (Netherlands)	39
		PkF1060 + PkR1550	1–10 parasite genomes/sample	1 (Sarawak)	67
				130 (Australia)	43
		Kn1f + Kn3r	1–6 parasites/μl blood	25 (Sarawak)	74
		PK18SF + PK18SRc	NR	35 (Thailand)	48–50
				1 (Cambodia)	67
	Dihydrofolate reductase-thymidylate synthase	PK-Lin-F + PK-Lin-R		0 (Thailand)	102
LAMP	Apical membrane antigen 1		10 plasmid copies/sample	13 (Peninsular Malaysia)	106
	β-Tubulin	F3, B3, FIP, BIP, FLP, BLP	100 plasmid copies/sample	0 (Obihiro, Japan)	107
Single-step PCR	Unidentified genes		1 parasite/μl blood	0 (Atlanta, GA)	108
Real-time PCR	SSU rRNA	PK1 + PK2	10 copies/μl	0 (Rochester, NY)	109
		NVPK-P	5 copies/reaction	1 (Netherlands)	42
		PKe′F, PKg′R	100 copies/μl	2 (France)	110
		Pk	10 copies/μl	40 (Sarawak)	105

[a] LAMP, loop-mediated isothermal amplification; NR, not reported.

quantitative data, but they are expensive to run and require a substantial initial financial investment. These assays are more likely to be available in diagnostic laboratories in developed countries and referral laboratories in developing countries. Due to their relatively high costs, real-time PCR assays and other molecular detection assays, such as loop-mediated isothermal amplification (LAMP) assays (Table 2), will not replace microscopy in routine diagnostic laboratories in rural areas in the tropics where malaria is prevalent.

Rapid Diagnostic Tests

Immunochromatographic rapid diagnostic tests (RDTs) have been developed for detection of malaria and are useful for investigations of outbreaks in rural settings where electricity is unavailable and in laboratories in developed countries where laboratory technologists are unfamiliar with detecting malaria by microscopy (114). The RDTs contain antibodies that are specific for histidine-rich protein 2 (HRP-2) of *P. falciparum* or are specific for lactate dehydrogenase (LDH) of either *P. falciparum* or *P. vivax*. They commonly also include "pan-malarial antibodies" directed at aldolase or LDH of *Plasmodium*. All the current commercially available RDTs were developed before it was discovered that *P. knowlesi* is a significant cause of human malaria, and consequently, these RDTs were not evaluated against *P. knowlesi*.

Four different RDTs have been used on travelers with knowlesi malaria, and one has been used on macaques experimentally infected with *P. knowlesi*, producing mixed results (Table 2) (31, 39, 42, 52, 53, 64, 115). For the BinaxNOW RDT, with the exception of one report where there was a positive *P. falciparum* and pan-malaria result, all other reports indicated a positive pan-malaria result and a negative *P. falciparum* result. Furthermore, negative pan-malaria results were observed for 5 out of 9 samples examined, with parasitemia ranging from 185 to 1,587 parasites per μl blood and from 0.0005 to 0.1%, highlighting the low sensitivity of detection of knowlesi malaria with BinaxNOW. The OptiMal test and the OptiMal-IT tests, which use antibodies to LDH of *P. falciparum* that have been found to cross-react with *P. knowlesi* (116), indicated the presence of *P. falciparum* or *P. falciparum* mixed with either *P. vivax*, *P. ovale*, or *P. malariae* in 4 samples from humans and 3 from macaques. From this limited number of reports so far, it appears that the *Plasmodium* species identified in a sample from a knowlesi malaria patient depends on the RDT being utilized, with OptiMal identifying the infection as a single or mixed *P. falciparum* infection, BinaxNOW identifying it as a non-*P. falciparum* malaria infection, and the other two RDTs identifying it as a *P. vivax* infection. More importantly, *P. knowlesi* infection in patients with low parasitemia would not be detected

TABLE 2 Rapid diagnostic tests that have been used to detect *P. knowlesi*

RDT[a]	Result(s)	Parasitemia[b]	Place where test was conducted	Reference
Tests on human samples				
BinaxNOW Malaria	Negative (*P. falciparum*), positive (pan-malaria)	84,000 parasites/μl blood	Rotterdam, Netherlands	39
	Negative (*P. falciparum* and pan-malaria)	1,587 parasites/μl blood		
	Negative (*P. falciparum* and pan-malaria)	138 parasites/μl blood		
	Negative (*P. falciparum*), positive (pan-malaria)	0.8%	Toulouse, France	52
	Positive (*P. falciparum* and pan-malaria)	7,700 parasites/μl blood (0.2%)	Singapore	60
	Negative (*P. falciparum* and pan-malaria)	0.1%	Stockholm, Sweden	31
	Negative (*P. falciparum* and pan-malaria)	250 parasites/μl blood	Madrid, Spain	53
	Negative (*P. falciparum* and pan-malaria)	185 parasites/μl blood	Enoggera, Australia	64
	Negative (*P. falciparum* and pan-malaria)	0.0005%	Amsterdam, Netherlands	42
	Negative (*P. falciparum* and pan-malaria)	NR	Auckland, New Zealand	36
OptiMAL	Positive (*P. falciparum* and pan-malaria)	84,000 parasites/μl blood	Rotterdam, Netherlands	39
	Positive (*P. falciparum* and pan-malaria)	1,587 parasites/μl blood		
	Negative (*P. falciparum* and pan-malaria)	138 parasites/μl blood		
	Positive (*P. falciparum*), negative (pan-malaria)	7,700 parasites/μl blood (0.2%)	Singapore	60
Core Malaria Pan/Pv/Pf	Positive (*P. vivax* and pan-malaria)	0.8%	Toulouse, France	52
Tests on macaque samples				
OptiMal-IT	Positive (*P. falciparum* and pan-malaria)	28.3%	Japan	115
	Positive (*P. falciparum* and pan-malaria)	11.2%		
	Negative	0.04%		
Entebe Malaria Cassette	Positive (*P. vivax*), negative (*P. falciparum*)	28.3%	Japan	115
	Positive (*P. vivax*), negative (*P. falciparum*)	11.2%		
	Negative (*P. vivax* and *P. falciparum*)	0.04%		

[a] BinaxNOW detects HRP-2 of *P. falciparum* and aldolase of *Plasmodium*; OptiMal detects LDH of *P. falciparum* and *Plasmodium*; Core Malaria Pan/Pv/Pf detects HRP-2 of *P. falciparum*, LDH of *P. vivax*, and LDH of *Plasmodium*; and Entebe Malaria Cassette detects HRP-2 of *P. falciparum*, LDH of *P. vivax*, and LDH of *Plasmodium*.
[b] NR, not reported.

by these RDTs. Since *P. knowlesi* has a short 24-h erythrocytic cycle and has the potential to be fatal (28), it is important that cases with low parasitemia are correctly diagnosed. For travelers from areas where the disease is not endemic, it is recommended that microscopists examine blood films for malaria parasites when RDTs are negative. However, there is a danger that for patients with a negative RDT where blood films are not examined, the attending physician would attribute the signs and symptoms to nonmalaria infections, and appropriate antimalarial treatment would not be provided. There is a need to evaluate the currently available commercial RDT kits against clinical isolates of *P. knowlesi* and also to develop new sensitive and rapid detection tests suitable for use in remote rural areas.

CLINICAL COURSE

Historical Data

Only recently have we begun to understand the complete clinical spectrum of naturally acquired *P. knowlesi* infections in humans. However, the first reports of the clinical impact can be found among the initially reported experimental infections of humans by Knowles and Das Gupta (8) and subsequent studies on therapeutic *P. knowlesi* infections in patients with neurosyphilis (16, 18–21). From these reports, the impact of infection by blood passage had a variable effect on patients. Nicol believed that the disease induced was mild and brief, prompting him to be skeptical over the application of *P. knowlesi* for use in malariotherapy (18). On the other hand, Ciuca and coworkers in Romania noted that the parasite became too virulent for use following 170 human-to-human blood passages, noting an increase in the need to terminate infections with antimalarials and parasitemia frequently reaching 500,000 parasites per μl of blood (21). Detailed descriptions by van Rooyen and Pile (15) indicated that patients could become unwell with shock, while Milam and Kusch (16) noted that the infections could suddenly change "from moderate severity to one of rather serious proportions." Different *P. knowlesi* parasite strains and the effect of multiple subpassages between humans may have accounted for the variable clinical outcomes observed by these early workers. Two studies in the United States also provided evidence that infections in African Americans were milder than those in Caucasians and that some African Americans were refractory to infection by *P. knowlesi* through blood passage (16, 117).

Although it is difficult to extrapolate these findings to knowlesi malaria infections in previously healthy individuals, it is clear that a full spectrum of illness may be present, including severe disease. In the first reported naturally acquired case of knowlesi malaria in the U.S. Army surveyor, symptoms of anorexia, mild fatigue, and some nausea were followed by a sore throat, shaking chills, high fever, and profuse sweating (22), typically nonspecific infectious symptoms commonly seen in malaria. The *P. knowlesi* strain isolated from this patient, the H strain, was used in a series of experiments where malaria was induced by blood passage and mosquito

bites in 20 volunteers at the U.S. Penitentiary in Atlanta, GA, in the 1960s (22, 23, 97). In contrast to what had been observed previously (16, 117), these experiments showed that African Americans were infected easily, and there were no differences observed in infections between African Americans and Caucasians (97). Among the 12 blood-induced infections, the clinical manifestations were reported as "moderate to severe with attacks terminating spontaneously after 2 weeks." The clinical course for the 8 sporozoite-induced infections was observed to be not much different from that of blood-induced infections (97). However, after 8 to 11 days of parasitemia and fever, three of the sporozoite-induced cases required chloroquine to terminate the infections, since they were deemed to have severe infections (23). Although clinical details were not provided in this small series of experiments, they indicated that infection with a single strain of *P. knowlesi* could lead to a spectrum of disease.

Recent Studies

The first report of clinical details for a large number of naturally acquired knowlesi malaria cases appeared in 2004, when retrospective clinical data for 106 patients in Sarawak, Malaysian Borneo, were described (3). In the same year, a case of naturally acquired knowlesi malaria from southern Thailand was reported (48). Between 2004 and 2008, clinical details of knowlesi malaria were reported in a further eight cases, including cases in Sarawak, the Philippines, Singapore, and Peninsular Malaysia (28, 54, 56, 59). The most notable of these reports was that by Cox-Singh et al. (28), where four cases of fatal *Plasmodium knowlesi* from Sarawak were described. Our understanding now of clinical symptoms seen in naturally acquired human infections with *P. knowlesi* is derived from single case reports (31, 32, 36, 39, 46–48, 52, 53, 55–57, 59, 64); a small case series of 7 patients in the Klang Valley, Peninsular Malaysia (45); a retrospective study of 94 patients in Kapit, Sarawak (3); three prospective studies from Kapit, Sarikei, and Sabah, with 107, 110, and 130 patients, respectively (33, 43, 118); and three retrospective studies from Sabah (29, 34, 35). Although the literature covers patients from a wide geographical zone across Southeast Asia and of many ethnicities and various background levels of malaria immunity, most of the data are weighted to Malaysia.

Demographics

The majority of cases of knowlesi malaria have been reported for adults, with fewer reports and smaller case series for children. This is likely to reflect generally low transmission rates combined with environmental interactions that predispose adults to come into contact with infected vectors. Similarly, males are more represented in the literature, especially in case reports (31, 36, 39, 47, 52, 53), case series (35), and two recent prospective studies in Sarawak and Sabah (35, 118), while studies in the Kapit region of Sarawak have found a more equal distribution (3, 33). Reports from selected areas of Sabah indicated that 10% of patients were under 15 years of age, and males accounted for 74% of cases (41). The percentage of females was found to be higher for patients with severe disease in initial studies (33, 35), but Barber et al. reported that there were only 8 (21%) females out of 38 severe cases (43). There is a wide age range among patients, including older patients, where comorbidities may be present. These factors combined with low endemicity and limited malaria exposure may explain the observation of an association between age, sex, and severity of disease (33, 35), although a recent study found this association to be confounded by parasitemia in a multivariate analysis (43).

Symptoms

The symptoms of acute knowlesi infection are of a nonspecific infectious illness similar to those seen in falciparum and vivax malaria (33, 43). Fevers, chills, and rigors are the most dominant features reported, while headaches, myalgia/arthralgia, malaise, and poor appetite are also commonly present. Cough (48 and 56%), abdominal pain (31 and 52%), and diarrhea (18 and 29%) were additional symptoms noted in prospective studies of 107 and 130 patients, respectively, presenting with acute knowlesi malaria (33, 43). Gastrointestinal symptoms were also dominant features in four fatal cases described by Cox-Singh et al. (32), and parasitemia in knowlesi malaria has been associated with abdominal pain (43). In Vietnam, where coinfection with other malaria species was present, the clinical features appeared to be less dominant, with only 6 of 32 (19%) patients reporting fever (62). The median duration of illness prior to presentation to a health care facility for knowlesi malaria has been reported to be between 4 and 5 days (3, 33, 35, 43). In some cases, however, the duration of illness was several weeks (33, 64).

Clinical Examination Findings

The most common examination findings reported for 107 prospectively studied knowlesi malaria patients were tachypnea, fever, and tachycardia (33). Palpable liver and spleen were reported in 24 to 40% and 15 to 26% of cases, respectively, in two prospective studies of 107 and 130 patients (33, 43). Clinical signs of severe disease including low oxygen saturations, tachypnea, chest crackles (indicating acute respiratory distress or coexisting pneumonia), hypotension, and jaundice have been documented (28, 32–35, 43, 45). In one fatal case with a history of poorly controlled hypertension, focal neurology was present, but it is not clear whether this was a coexistent cerebrovascular event, since brain imaging was unavailable. A cerebral malaria-like syndrome has not been reported, but consciousness may be impaired secondary to the severity of illness in the context of multiorgan failure or hypoglycemia (32, 33).

Laboratory Findings

Thrombocytopenia. Thrombocytopenia is the most frequently reported blood abnormality and appears to be almost universal in knowlesi malaria infections (28, 31–36, 39, 42, 43, 45, 47, 48, 52, 53, 55, 59, 64, 118). In the prospective study undertaken at Kapit Hospital, 98% of 107 patients presented with thrombocytopenia, and within 24 h of admission, the remaining 2% became thrombocytopenic (33). Despite the extremely high proportion of patients with thrombocytopenia, and a third of these patients being severely thrombocytopenic ($<$50,000 platelets per μl blood), bleeding complications were rarely seen. In limited comparative studies, the severity and frequency of thrombocytopenia were higher with knowlesi malaria than with falciparum and vivax malaria (33), while an inverse association between platelet counts and parasitemia has been observed (43), a feature also seen with both falciparum and vivax malaria infections (33, 119, 120). Willmann et al. recently described laboratory markers of severity in knowlesi malaria infections and found thrombocytopenia ($\leq$45,000 platelets per μl blood) to be associated with complicated disease, with a sensitivity of 71% but a positive predictive value of 22% (118).

Throughout Southeast Asia, dengue fever is a differential cause of a febrile illness associated with thrombocytopenia (121). For the first case of a naturally acquired *P. knowlesi* infection in Singapore, the initial diagnosis was dengue fever, and a blood film was examined only on the third day following admission to the hospital (59). We observed that for 104 patients with acute thrombocytopenia during a 4-month period, 4 patients (10% of knowlesi malaria cases) at Kapit Hospital were given an initial working diagnosis of dengue fever (C. Daneshvar, unpublished data). For knowlesi malaria, where thrombocytopenia can occur with very low parasitemia, careful examination of well-prepared blood films is needed and should be repeated daily if results are initially negative. Delays in diagnosis of knowlesi malaria could be critical to the outcome, and thrombocytopenia seems an appropriate warning flag to intensify clinical suspicion of this rapidly dividing parasite in patients with a recent history of travel to the forest and forest fringes of Southeast Asia.

Anemia. Unlike falciparum malaria (122), severe anemia is not a commonly reported feature at the time of presentation for adults with knowlesi malaria. Mild anemia may be observed, with a frequency of <5% in 107 patients prospectively studied (33). The mean red blood cell volume (MCV) was preserved, (median, 85.6 fl), with 8 (7.5%) patients having mild microcytosis (<80 fl) (Daneshvar, unpublished). During the course of admission, there was an initial drop in hemoglobin levels, compatible with data reported for falciparum infections (123), which recovered in all 87 of the patients studied by day 28 (33). In a recent study of 130 patients with acute knowlesi malaria, two patients met the criteria for severe anemia (hemoglobin concentration of <7 g/dl) on day 1 and day 8 of admission (43).

Other hematological findings. For knowlesi malaria, the prothrombin and thromboplastin times are usually preserved (33). However, in a retrospective case series including severe disease, 7 patients had significant derangement, but no associated bleeding complications were noted (35). Other hematological abnormalities may be seen, including lymphopenia (lymphocyte counts of <800 per μl), which was observed in 6.5% of 107 patients in a prospective study at Kapit Hospital (33).

Renal function. Renal function may be significantly deranged; of 107 patients with acute knowlesi malaria infections, 3 (2.8%) had established renal failure (creatinine level of >265 μmol/liter after fluid resuscitation for >24 h) (33). Two recent prospective studies of 130 and 110 cases reported frequencies of renal failure of 6.9% and 14.5%, respectively (43, 118). Careful fluid resuscitation and antimalarial treatment are usually sufficient, and renal impairment is reversible. A recent case series from the Klang Valley of Peninsular Malaysia reported that 2 out of 7 knowlesi malaria patients had acute renal failure (45). It is, however, an ominous sign associated with fatal cases (28, 34). Electrolyte abnormalities, including hyponatremia (Na concentration of <136 mmol/liter), are frequently seen (24% of 107 patients) and are self-correcting with treatment of malaria (33). Sodium concentrations have been reported to be negatively associated with parasitemia (43).

Liver function. Liver function may be abnormal, and a mild elevation of levels of aminotransferase enzyme is frequently seen in cases of knowlesi malaria. Generally, synthetic functions (clotting factors) appear to be preserved; however, albumin concentrations are lower in patients with severe disease at the time of admission (33).

Hematological and biochemical parameters respond rapidly following treatment, with the exception of hemoglobin levels, serum albumin concentrations, and liver enzyme levels, which typically return to normal limits by day 28 (33).

Clinical Aspects in Children

There are few reports on knowlesi malaria in children. Barber et al. described retrospective findings of *P. knowlesi* infections in 16 children from the district of Kudat in Sabah, Malaysian Borneo (29). No children were found to have severe disease upon admission, and they all successfully responded to antimalarial treatment. Thrombocytopenia was observed for 94% of children with single knowlesi infections, and anemia (<11 g/dl) was present at admission in 56% children and developed in all children during hospital admission. Compared with 14 children with falciparum malaria, significant differences were found in hemoglobin concentrations (higher in knowlesi than in falciparum malaria infection) and platelet counts (lower in knowlesi than in falciparum malaria infection). Of 188 patients at Kapit Hospital reviewed consecutively, 8 out of 121 (6.6%) cases of single knowlesi infections occurred in children (Daneshvar, unpublished). The median age of the children was 11 years (range, 9 to 12 years), and the median parasite count was 940 parasites per μl blood (range, 440 to 26,270 parasites per μl blood). All children were thrombocytopenic, two were mildly anemic, two had increased alanine aminotransferase concentrations, and one had jaundice (bilirubin level of 38 μmol/liter). In one patient with a parasite count of 26,270 parasites per μl blood, a petechial rash over the shins and retinal hemorrhage were found upon examination. All eight children with knowlesi malaria responded to treatment.

Complicated Knowlesi Malaria

Severe disease. The first reported cases of fatal *P. knowlesi* infections were of four patients aged between 39 and 69 years (28). They presented with a 3- to 7-day history of a fever associated with nonspecific features that included shortness of breath, abdominal pain, and vomiting. All four cases had high parasitemia (75,000, 112,000 and 764,720 parasites per μl, scored as "++++"), severe thrombocytopenia, renal failure, hypotension, jaundice, and deranged liver enzymes. In the first patient, malaria was not considered for 3 days due to the dominance of abdominal symptoms, leading clinicians to a diagnosis of presumed bacterial gastroenteritis, while the second patient had a perforated gastric ulcer with associated pneumoperitoneum. The third case died within 4 h of admission to the hospital, while the fourth case developed acute respiratory distress after 4 days of admission, required a prolonged period of care in the intensive care unit, and then died of complications from a tracheostomy hemorrhage.

Features of severe malaria defined by laboratory findings reflect extensive studies of *P. falciparum* infections (122). Such prognostic markers include a white cell peripheral leucocytosis count of >12,000 cells/μl, a serum creatinine concentration of >265 μmol/liter, a urea concentration of >21.5 mmol/liter, a hemoglobin concentration of <7.1 g/dl, and a blood glucose level of <2.2 mmol/liter (124–126). A 3-fold increase in aminotransferase enzyme levels and increased serum lactate and low bicarbonate concentrations are also associated with a poor outcome in falciparum malaria (122). The relevance of these thresholds and application to knowlesi malaria require further evaluation.

Although most cases of knowlesi malaria respond to treatment and resolve without complications, complicated and fatal cases

TABLE 3 Summary of published reports on severe and fatal knowlesi malaria cases

Study type (reference)	Study location	No. of cases	Avg age of patients (yr)	No. (%) of patients with clinical sign[h]							Outcome
				Hyperparasitemia[a]	Hypotension[b]	Acute kidney injury[c]	Jaundice[d]	Hypoglycemia[e]	Lactic acidosis[f]	ARDS/ pulmonary edema[g]	
Retrospective fatal case series (28)	Sarawak	4	53	3 (75)	3 (75)	4 (100)	4 (100)	1 (100)[j]	1 out of 1	3 (75)	All died
Prospective (33)	Sarawak	10	63.5	3 (30)	2 (20)	3 (30)	4 (100)	1 (10)	1 (10)	6 (60)	2 died (20%)
Fatal case report (32)	Sabah	1	40	1 (100)	1 (100)	1 (100)	NA	NA	NA	1 (100)	Died
Retrospective (45)	Peninsular Malaysia	3	55	3 (100)	2 (67)	2 (67)	2 (67)	1 out of 1	1 out of 1	2 (67)	All survived
Retrospective case series (35)	Sabah	22	56.5	NA	11 (50)	12 (55)	9 (41)	3	6	13 (59)	6 died (27%)
Prospective (127)	Sarawak	2	32.5	1 (50)	0	1 (50)	1	NA	NA	NA	All survived
Prospective (43)	Sabah	38	55	18 (47)	13 (34)	9 (24)	20 (53)[i]	0 (0)	4 (11)	14 (37)	All survived
Prospective (118)	Sarawak	17	49.6	8 (47)	1 (5.9)	16 (94.1)	6 (35.3)[i]	NA	NA	3 (17.6)	4 died (23.5%)

[a] Parasite counts of >100,000 parasites/μl.
[b] Systolic blood pressure of <80 mm Hg or started on ionotropes.
[c] Creatinine level of >265 μmol/liter (3 mg/dl) or requiring dialysis.
[d] Total bilirubin level of ≥43 μmol/liter (3 mg/dl).
[e] Glucose level of <2.2 mmol/liter (40 mg/dl).
[f] Lactate level of >6 mmol/liter.
[g] Respiratory rate of >30 breaths per minute, presence of pulmonary infiltrates, or requiring ventilation.
[h] NA, data not available.
[i] Total bilirubin level of ≥43 μmol/liter plus parasitemia of >20,000 parasites/μl or creatinine level of >132 μmol/liter (1.49 mg/dl).
[j] Data available for one patient.

are being increasingly reported (Table 3) (28, 32–35, 43, 45, 127). In a prospective study that excluded patients with significant comorbidities with apparent end-organ disease, the application of the World Health Organization criteria for severe falciparum malaria (122) indicated that 7 of 107 (6.5%) patients had signs and laboratory features of severe disease at the time of presentation, and a further 3 developed severe disease during admission (33). Two patients in this study died, with a case fatality rate of 1.8% (confidence intervals [CI], 0.6% to 6.6%). Recently, further studies have contributed to our understanding. A retrospective study of admissions to a tertiary referral hospital in Sabah, Malaysian Borneo, reported complications occurring in 22 of 56 (39%) cases (35). It should be noted that 17 of the 22 severe cases had been referred from district hospitals, and this number is therefore likely to be an overestimation of the true prevalence of complicated disease. A more recent comparative prospective study from the same site reported complications in 38 (29%) of 130 patients, where the referral criteria included having moderate parasitemia from referring district hospitals (43). That study also found that having knowlesi malaria was associated with an increased odds ratio of developing severe malaria over falciparum malaria (odds ratio, 2.96; 95% CI, 1.19 to 7.38). More extensive studies are needed to determine the case fatality rate, but these studies demonstrate the breadth of complications and severity of disease that may occur in knowlesi malaria infections.

Acute respiratory distress syndrome. Patients may present with tachypnea, hypoxemia, and pulmonary infiltrates on chest radiograph, consistent with acute respiratory distress syndrome (ARDS). The two largest case series reported frequencies of 5.6% and 10.7% for 107 and 130 knowlesi malaria cases studied, respectively (33, 43). In severe cases of knowlesi malaria reported in the literature with sufficient detail, ARDS was present in 43 out of 83 (52%) cases, with a crude mortality rate of 37% (Fig. 3). Frequently associated complications were reported to be present (median = 3), and in four patients, ARDS developed following admission, a pattern similar to that seen with falciparum malaria (128–130). Logistic regressions in limited case series indicated positive and independent associations with parasitemia and neutrophilia and an inverse association with hemoglobin concentrations at admission (33, 43).

Acute renal failure. Overall, acute renal failure was reported in 3 of 107 (3%) prospectively studied patients with acute knowlesi infections (33). Willmann et al. observed acute kidney injury in 16 (94.1%) of 17 cases with severe knowlesi malaria (118). In pooled data for complicated disease, acute renal failure was present in 36 out of 86 (42%) reported cases, with an associated mortality rate of 42% (Fig. 3). Acute tubular necrosis was observed postmortem in a fatal case of knowlesi malaria (32). Full renal support may be required, although supportive treatment may be sufficient with normalization of renal function (33, 35, 45). Renal failure in association with a "Blackwater fever" clinical phenotype has been reported (35). Parasitemia, neutrophilia, and age are independently associated with serum creatinine, although this complication may occur in apparently young healthy patients (33, 35, 43). Interleukin-1ra (IL-1ra) levels were found to positively correlate with the serum creatinine (131).

Other features. Hypotension after fluid resuscitation requiring ionotropic support has been reported for knowlesi malaria (33, 35, 45). This occurred in 35 of 86 (41%) reported complicated cases and was associated with a mortality rate of 31%. Hypoglycemia is not a dominant feature of knowlesi malaria infections; however, when present, it is associated with other multiple complications (median, 5.5) and has a very high mortality rate (4 of 6 cases). Acidosis is also a feature of severe knowlesi malaria infections associated with a high mortality rate (Fig. 3). Cerebral malaria-like syndromes have not been reported for knowlesi malaria.

Parasitemia appears to be a strong predictor of complications in knowlesi malaria infection, with an area under the receiver operating characteristic (ROC) curve of 0.9 (95% confidence interval, 0.82 to 0.98; $P < 0.001$) (33). The specificity at a threshold

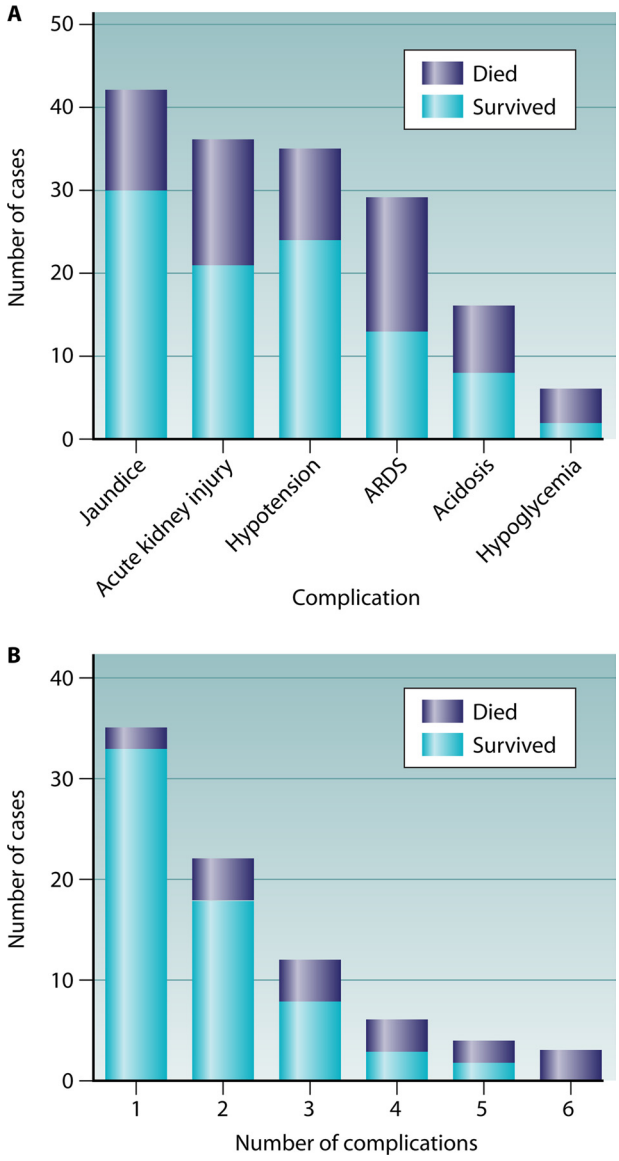

FIG 3 Complications and outcomes (A) and the number of complications and outcomes (B) for 86 cases of severe knowlesi malaria. ARDS, hypoxia with a respiratory rate of >30, presence of pulmonary infiltrates, or requiring ventilation; acute kidney injury, creatinine levels of >265 μmol/liter (3 mg/dl) or requiring dialysis; hypotension, systolic blood pressure of <80 mm Hg or started on ionotropes; jaundice, total bilirubin level of ≥43 μmol/liter (3 mg/dl); acidosis, lactate level of >6 mmol/liter; hypoglycemia, glucose level of <2.2 mmol/liter (40 mg/dl). (Data obtained from references 28, 32–35, 43, 45, and 127.)

of 100,000 parasites per μl was 100%, while the sensitivity of 30% indicates that this threshold is probably too high and highlights that severe cases can occur at relatively low parasitemia. Further studies have confirmed this, with thresholds of >20,000 or >35,000 parasites per μl having an 11- or 10-fold increase in the odds of having severe disease, respectively (43, 118).

Complications in knowlesi malaria may occur as single-organ dysfunction (35 of 86 [41%] cases) but more commonly occur with multiorgan involvement (Fig. 3). From published reports, three or more complications were present in 25 of 86 cases (30%). As one would expect, the more complications present, the higher the mortality rate (Fig. 3). Further work involving a larger number of cases is needed to ascertain whether such complications are present upon admission or develop over the course of admission as well as their role in predicting outcome in knowlesi malaria.

Pathogenesis. The pathogenesis of severe knowlesi disease is not fully understood. A recent study reported postmortem findings for a patient in Sabah, Malaysian Borneo, who died within 2 h of admission, having presented in shock and found to have multiorgan failure (32). This case showed accumulation of infected erythrocytes, indicating possible sequestration of malaria parasites and hemorrhagic complications in vital organs, but a lack of chronic inflammatory infiltrate. This suggests that there are some histological similarities with falciparum malaria but that a distinct pathophysiology may occur in severe knowlesi malaria.

A study of pretreatment cytokine concentrations at admission showed that knowlesi malaria patients with complicated disease had higher levels of cytokines including tumor necrosis factor alpha (TNF-α), IL-6, IL-8, IL-1ra, and IL-10 than patients with uncomplicated disease (131). The anti-inflammatory cytokines IL-1ra and IL-10 were associated with parasitemia in knowlesi malaria. For patients with uncomplicated knowlesi malaria, the overall levels of proinflammatory, anti-inflammatory, and macrophage-derived cytokines were lower than those for patients with uncomplicated falciparum malaria. Patients with severe knowlesi malaria differed from those with severe falciparum malaria, with higher IL-6, TNF-α, and macrophage inflammatory protein 1β (MIP-1β) (CCL4) levels but not higher IL-10 levels. While further studies are clearly needed to understand the immunopathology of knowlesi malaria, this study indicates that the pathogenesis of severe disease may be different from that seen in falciparum malaria.

A small study of *ex vivo* cytoadherence demonstrated that late-trophozoite- and schizont-infected erythrocytes from patients with knowlesi malaria have the capacity to bind to the human endothelial cell receptors intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM) but not to CD36 (127). The role of this in the pathogenesis of knowlesi malaria is not yet understood; however, the potential for these endothelial cell receptors to be expressed in knowlesi malaria patients gives rise to the possibility of sequestration of *P. knowlesi*-infected erythrocytes in blood capillaries of different organs. Whether total sequestration or sequestration of a subset of *P. knowlesi*-infected erythrocytes truly occurs during the natural life cycle or occurs only in a proportion of patients during an intense inflammatory response and whether this has a role in pathogenesis need to be determined. Further studies examining the dynamic changes in cytokine levels and gene expression and the role of host and parasite genetics will complement our understanding of pathogenesis while also revealing insights into other malaria species.

TREATMENT

Although patients with knowlesi malaria have been successfully treated with a wide range of antimalarial drugs, the optimal treatment for either uncomplicated or complicated disease is unknown. From the neurosyphilis era in the 1930s, quinacrine (Atebrin), pamaquine (Plasmochin), proguanil (Paludrine), and quinine were used to terminate fevers, often in combination (15, 16, 132). van Rooyen and Pile described "the almost dramatic destruction of parasites" following administration of quinine intramuscularly

TABLE 4 Antimalarials for treatment of naturally acquired human *P. knowlesi* infections

Treatment	No. of cases	No. of deaths	Parasite clearance time(s)[a]	Reference(s)
Chloroquine	247	3	PCT_{50}, 3 h[b]; PCT, 1 day ($n = 97$); PCT, 2.5 days ($n = 15$); PCT, 2 days ($n = 13$)	3, 29, 33–35, 39, 43, 45, 48, 52, 56, 59
Artesunate followed by artemether-lumefantrine[c]	58	0	PCT, 2 days	43
Quinine	48	9	PCT, 2 days ($n = 3$); PCT, 2.5 days ($n = 11$); PCT, 4 days ($n = 16$)	3, 28, 29, 33, 35, 45, 47
Artemether-lumefantrine	36	0	PCT, 1 day ($n = 8$); PCT, 2 days ($n = 28$)	35, 43
Artemether-mefloquine[d]	10	0	NR	43
Dihydroartemisinin-piperaquine	7	0	PCT, 1 day	43
Artesunate	7	2	PCT, 2 days	34, 35
Atovaquone-proguanil	3	0	PCT, 2 days ($n = 1$)	42, 55, 64
Mefloquine	1	0	NR	31
Atovaquone-proguanil-artemether-lumefantrine	1	0	NR	36
Chloroquine-doxycycline	1	0	NR	45
Chloroquine-sulfadoxine-pyrimethamine	2	2	NR	28
Chloroquine-sulfadoxine-pyrimethamine-primaquine-quinine	1	1	NR	28
Mefloquine-quinine-artemether-lumefantrine	1	0	Early treatment failure with mefloquine	46
Quinine-artemether-lumefantrine-doxycycline	1	0	NR	45
Sulfadoxine-pyrimethamine	1	1	NR	34
Quinine-chloroquine-doxycycline-primaquine	1	1	NR	34

[a] PCT_{50}, time to 50% reduction in admission parasitemia; PCT, time to complete parasite clearance. NR, not reported.
[b] For gametocidal activity, the PCT_{50} was 10.4 h (95% CI, 9.0 to 12.2 h), and the PCT_{90} was 34.4 h (95% CI, 29.9 to 40.4 h).
[c] Patients received at least one dose of intravenous artesunate.
[d] Three patients also received at least one dose of intravenous artesunate.

(15). One may assume that there is little antimalarial drug resistance, as knowlesi malaria is primarily a zoonosis, so *P. knowlesi* parasites have not been subjected to any significant antimalarial drug pressure. Indeed, in nonhuman primate studies, tetracycline, clindamycin, trimethoprim, erythromycin, and artemisinins have all been shown to have an antiparasitic effect in *P. knowlesi* infections (133–138). Despite this, quinacrine was not effective in controlling acute parasitemia in humans (15), and it was noted that resistance was easily induced with recurrent exposure to mefloquine, proguanil, and pyrimethamine in rhesus macaques (139, 140). Interestingly, quinacrine is closely related to mefloquine. In fact, a recent case report indicated that despite treatment with mefloquine, parasitemia continued to increase (46).

In the earliest reports of naturally acquired knowlesi malaria infection, chloroquine was provided as a clinical cure (3, 22, 48). In Malaysia, the combination of chloroquine and primaquine (primarily as a gametocidal drug) is recommended for the treatment of *P. malariae* and was effective for 82 patients at Kapit Hospital subsequently identified as having single knowlesi malaria infections (3). Also, in this retrospective study, 2 patients received quinine, and 10 patients received a combination of chloroquine, primaquine, and sulfadoxine-pyrimethamine (Fansidar). There were no reported deaths or treatment failures in any of these patients, and the overall median parasite clearance time was 2.4 days.

Case reports indicated that chloroquine alone and atovaquone with proguanil, mefloquine, artemisinins, quinine, and doxycycline can be successfully used to treat knowlesi malaria (Table 4). An observational prospective study supported successful treatment with chloroquine (141). In that study, 73 patients with uncomplicated knowlesi malaria who had not been exposed to antimalarial drugs in the preceding 14 days were treated with a total dose of 25 mg/kg of body weight of chloroquine, administered as 10 mg/kg, followed by 5 mg/kg at 6, 24, and 48 h. In line with the Malaysian Ministry of Health treatment guidelines for *P. malariae* infections, 2 doses of primaquine (15 mg) were administered at 24 and 48 h, mainly as a gametocidal drug. That study showed that patients subjectively felt better within 24 h and that the median fever clearance time was 26 h. Parasite clearance was rapid, with a time to 50% reduction in admission parasitemia (PCT_{50}) and a PCT_{90} of 3.1 and 10.3 h, respectively, significantly faster than for vivax infections. For most patients, parasite clearance occurred within 48 h. No resistance, recrudescence, or reinfection was observed during 28 days of follow-up for 60 of these patients. Chloroquine appeared to have gametocidal activity, although the parasite clearance time (PCT) was less rapid than for asexual erythrocytic stages of *P. knowlesi* (PCT_{50}, 10.4 h [95% CI, 9.0 to 12.2 h]; PCT_{90}, 34.4 h [95% CI, 29.9 to 40.4 h]). In view of this, primaquine is unlikely to have a role in the treatment of knowlesi malaria, since there is not known to be a latent liver (hypnozoite) stage for *P. knowlesi* (1). Limited data on the PCTs of quinine and artemether-lumefantrine are also available (35). This retrospective study reported PCTs of 2.5 days and 1 day for quinine ($n = 10$) and artemether-lumefantrine ($n = 8$), respectively, for patients with uncomplicated infection.

Given the 24-h replication cycle of *P. knowlesi*, early aggressive treatment is warranted for patients with relatively high parasitemia, to prevent sudden increases in parasitemia and the development of complications. In prospectively studied patients with uncomplicated infection, one-third of knowlesi malaria patients experienced an increase in parasitemia during the first 6 h of treatment with chloroquine, twice that seen for vivax malaria (141). In this setting, patients with knowlesi malaria were identified and immediately treated. Unfortunately, this is not always the case. Delayed diagnosis and confusion over the morphological appearances, leading to a diagnosis of *P. malariae* still being issued by diagnostic laboratories in rural hospitals, complicate the situation

further. This was recently illustrated in a report reviewing retrospective data on 6 knowlesi malaria fatalities in Sabah, Malaysian Borneo. Despite convincing demographic evidence being available on the existence of knowlesi malaria and the paucity of *P. malariae* infections, 5 patients with high parasitemia and severe malaria were diagnosed as having *P. malariae* infection, and only two received immediate parenteral treatment (34). At the recent WHO consultation meeting on *P. knowlesi*, it was recommended that in the absence of assays for molecular confirmation of *P. knowlesi* infection, hospital laboratories in areas where *P. knowlesi* has been described should report all microscopy-diagnosed *P. malariae* cases as *P. malariae*/*P. knowlesi* so that clinicians can treat and manage patients accordingly (99).

There are currently no reported randomized control trials of treatment for severe and uncomplicated knowlesi malaria. William et al. reported retrospective data on 22 patients with severe disease in Sabah, Malaysian Borneo, where intravenous quinine and artesunate were used in 16 and 6 patients, respectively (35). Those treated with intravenous quinine had a PCT of 4 days, with a case fatality rate of 31%, while patients in the artesunate group had a parasite clearance time of 2 days, with a case fatality rate of 17%. Barber et al. reported the use of artemisinins (as combined oral therapies and intravenously as a single agent) in severe and nonsevere knowlesi malaria ($n = 119$) and reported a median fever clearance time of 1 day and a PCT of 2 days (43). In that study, there were no fatal cases, which those authors attributed to the early use of intravenous artesunate. These findings support the recommendations of the WHO informal consultation meeting on *P. knowlesi*, where a pragmatic approach with artemisinin combination therapies was advocated together with managing complicated disease according to guidelines for management of severe falciparum malaria (99).

FUTURE DIRECTIONS AND CHALLENGES

Our current knowledge on the clinical course, treatment, pathogenesis, and epidemiology of knowlesi malaria has been derived from a limited number of case reports, small retrospective and prospective studies, and relatively small field studies. The data acquired indicate that knowlesi malaria is primarily a zoonotic disease with long-tailed and pig-tailed macaques as the major reservoir hosts. Furthermore, the studies showed that although *P. knowlesi* infections cause a spectrum of disease and can be potentially fatal, knowlesi malaria can be successfully treated with a number of antimalarials. However, there is still much more that needs to be known about knowlesi malaria. There remains a paucity of data from large prospective clinical studies to enable physicians to appreciate the true spectrum of knowlesi malaria. As yet, the largest prospective study including all patients with acute knowlesi malaria infections attending a regional health care facility has studied only 107 patients (33), and a more recent one based at a referral hospital included only 130 knowlesi malaria patients (43). Larger prospective studies on well-defined cohorts with the full spectrum of disease will enable us to define criteria for what constitutes severe disease in knowlesi malaria. Further studies will be able to provide additional information on regional variations in clinical outcomes.

Treatment studies are urgently needed to inform the optimum management of patients with knowlesi malaria infections. At a practical level, with the widespread use of artemisinin combination therapies, such studies may not initially be attractive; however, defining baseline parasite clearance and stage specificity and extended *in vivo* resistance studies will serve as markers for detecting resistance in the future. It may be that the rapid clearance of parasites will need specific methods to be devised to accurately define some of these parameters, and a role for quantified molecular techniques will be explored. The pharmacodynamics of antimalarials, both orally and parentally, in patients with severe and uncomplicated disease need to be established.

The unusually frequent presence of complications of knowlesi malaria reported in the literature makes it essential to undertake studies of the optimal treatment for clearance of parasites and of how best to manage the associated syndromes (28, 34, 43). There have been no detailed studies exploring management strategies for acute respiratory distress syndrome, renal failure, and fluid status; the use of adjunctive therapies such as dialysis, blood transfusions, or ionotropes; and the role for antibiotics. Such studies will be possible only with multicentered approaches, collaboration, and endorsement by the Ministries of Health in the countries affected. Crucial to such studies will also be the ability to rapidly detect knowlesi malaria while excluding the presence of falciparum malaria. In theory, such studies may seem simple; however, those working in these environments are well aware of the logistical problems encountered.

Detailed prospective clinical studies can generate the information and material required to explore the pathogenesis of knowlesi malaria in depth and will undoubtedly give insights into the pathogenesis of other malaria species. In fact, in some respects, the presence of knowlesi malaria in a population in a region of low endemicity may help to simplify studies on pathogenesis, while the existence of a natural animal model could provide further insights. Although it is clear that research on severe knowlesi malaria needs to be undertaken, accurate diagnostic difficulties, the relatively low incidence of knowlesi malaria and the low prevalence of severe cases, low parasite burdens, and difficulty in accurately quantifying serial changes suggest that there are significant challenges ahead.

The data contributed by hospital-based studies may be only the tip of the iceberg of *P. knowlesi* infections, and there is a need for longitudinal studies of communities that live in the forest or forest fringe to ascertain whether there is a significant burden of infection in these exposed populations that either resolves without treatment or never becomes symptomatic. Furthermore, there is a need to conduct large-scale malaria surveys with molecular detection methods in areas other than Sarawak and Sabah in Southeast Asia, to determine the actual prevalence of knowlesi malaria. The utilization of molecular tools will also assist in determining whether human-to-human transmission is currently occurring. Central to these studies is the need to develop inexpensive and sensitive rapid diagnostic tests that are easy to use in rural settings. In addition, *P. knowlesi*-specific serological assays need to be developed, since they will be able to provide valuable information with regard to previous exposure to *P. knowlesi* in communities living in the forest fringe and forests. Such denominators will provide crucial information to guide us toward a better understanding of the knowlesi malaria landscape.

It is unknown whether the recent increase in the number of reported knowlesi malaria cases in Southeast Asia is a genuine increase or whether it is the result of heightened awareness and enhanced efforts for detection with recently developed sensitive and specific molecular detection methods coupled with a decline

in the number of human malaria cases. Detailed genetic analyses of sequences of *P. knowlesi* isolates obtained from both human and macaque infections from different parts of Southeast Asia, similar to the study undertaken in Kapit, Sarawak, would assist in determining whether there is any regional variation in the emergence of knowlesi malaria.

The studies on the vectors of knowlesi malaria in nature that have been undertaken to date have all had very restricted geographic and temporal samplings. For example, in the study in Kapit, sampling was undertaken at a single longhouse, a farm, and only one location in the forest in the Kapit District, one of three districts within the Kapit Division, which has an area of 38,934 km^2. In a large state such as Sarawak (124,000 km^2), the vectors for knowlesi and other malarias may vary at different locations. Therefore, there is a need for more extensive entomological studies to be undertaken to identify the vectors of knowlesi malaria and to study their bionomics in different ecological habitats in Malaysia and other regions in Southeast Asia where human knowlesi malaria cases have been reported. A detailed understanding of the vector bionomics and transmission of knowlesi malaria is crucial to the successful implementation of malaria control programs.

Now that malaria elimination is back on the agenda (142), one of the main challenges is the prevention and control of zoonotic knowlesi malaria in rural areas where there is a large pool of parasites in the macaque reservoir hosts. A reduction in the number of these macaque reservoir hosts would be one option, but implementing such a measure would be impractical and difficult. Currently used methods for malaria control, which include provision of insecticide-treated bed nets and residual spraying of houses with insecticides, would not be efficient against vectors that are predominantly outdoor feeders, like the ones identified in the Kapit Division of Sarawak, Malaysian Borneo (82, 83). Prevention of humans from being bitten by vectors would be the main method of prevention and control of knowlesi malaria in these settings. This would involve the use of insect repellents, but these are prohibitively expensive for subsistence farmers, hunters, logging camp workers, and other rural people whose daily activities take them to the forest and forest fringe. The use of insecticide-impregnated hammocks has been successful for controlling forest malaria in Vietnam (143), but hammocks are not traditionally used in Malaysian Borneo, where most *P. knowlesi* cases occur, and so such a control measure would be difficult to implement successfully.

In conclusion, although much is known about the epidemiology and clinical manifestations of knowlesi malaria, these data are based on limited studies, and there remain many unanswered questions. What is the actual burden of knowlesi malaria in Southeast Asia? What are the criteria and the optimum management strategies for severe knowlesi malaria? Is the pathophysiology of knowlesi malaria different from that of falciparum malaria? Is human-to-human transmission by mosquitoes currently occurring? Will deforestation or ecological changes to the forests by logging lead to changes in vector behavior and composition and eventually cause *P. knowlesi* to switch hosts from macaques to humans? With renewed interest in knowlesi malaria and the availability of appropriate molecular tools, some of these questions may be answered in the near future, while for others, it will require patience, persistence, and continued commitment from clinicians, laboratory-based scientists, entomologists, and epidemiologists. In the meantime, clinicians and health care workers should be made aware of the potential for fatal outcomes in *P. knowlesi* infections, and there should be continued surveillance of knowlesi malaria.

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