# *Plasmodium knowlesi*: A Malaria Parasite of Monkeys and Humans*

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## Keywords

human malaria, mosquitoes, *Anopheles leucosphyrus*, Southeast Asia, Borneo

## Abstract

*Plasmodium knowlesi* is a malaria parasite of monkeys of Southeast Asia that is transmitted by mosquitoes of the *Anopheles leucosphyrus* group. Humans are frequently infected with this parasite and misdiagnosed as being infected with *Plasmodium malariae*. The parasite was a major monkey animal model for developing antimalarial vaccines and investigations of the biology of parasite invasion. *P. knowlesi* is the first monkey malaria parasite genome to be sequenced and annotated.

## INTRODUCTION

*Plasmodium knowlesi* is found primarily in monkeys on peninsular Malaysia. It was probably first seen by Franchini (31) in the blood of *Macaca fascicularis* monkeys. Later, Napier & Campbell (58) investigated the tendency of the parasite to produce hemoglobinuria in *M. fascicularis* and *M. mulatta* monkeys. The original animal was given to Dr. Das Gupta, who maintained the parasite by subpassage (49). Knowles & Das Gupta (50) described the blood stages and transmitted the parasite to humans. Sinton & Mulligan (80) noted the distinctive stippling of the red cells, the presence of an accessory dot, and the 24-h schizogonic cycle, which convinced them that the parasite represented a new species. They named it *Plasmodium knowlesi* in honor of Dr. R. Knowles, who was the first to infect humans with the parasite. Until recently, *P. knowlesi* was known only as a parasite of monkeys and was extensively studied, primarily in macaques, for basic immunological, chemotherapeutic, and biological relationships between malaria parasites and their primate hosts. This parasite was used because of its high rate of infectivity and high mortality in rhesus monkeys (*Macaca mulatta*). In drug and vaccine studies, efficacy could be readily measured by the high rate of death of the host.

The recent finding that many humans were infected with this organism in Sabah and Sarawak, Malaysia, and subsequently elsewhere throughout southeastern Asia has led people to consider *P. knowlesi* to be the fifth human malaria parasite (90). The resemblance of the late blood-stage forms of *P. knowlesi* to those of *P. malariae* made it difficult to differentiate these two species by blood film examination. Separation was accurately made only by the recent application of PCR technology. Distribution of the parasite in the natural monkey hosts and transmission to humans appear to be restricted to mosquito vectors of the *Anopheles leucosphyrus* group confined to Southeast Asia. Because it is a human pathogen, *P. knowlesi* was the first monkey malaria parasite genome to be sequenced (61).

## PARASITE

### Life History

The developmental cycle in the erythrocyte lasts approximately 24 h (**Figure 1**); this is the only malaria parasite of primates with this quotidian erythrocytic cycle. All other species have cycles of approximately 48 or 72 h (19). Following ingestion of the microgametocytes and

## CLASSIFICATION

*Plasmodium knowlesi:*
Domain: Eukaryota
Kingdom: Chromalveolata
Superphylum: Alveolata
Phylum: Apicomplexa
Class: Aconoidasida
Order: Haemosporida
Family: Plasmodiidae
Genus: *Plasmodium*
Species: *knowlesi* Sinton and Mulligan 1932
The clade with the most closely related species consists of *P. coatneyi* and *P. knowlesi*.

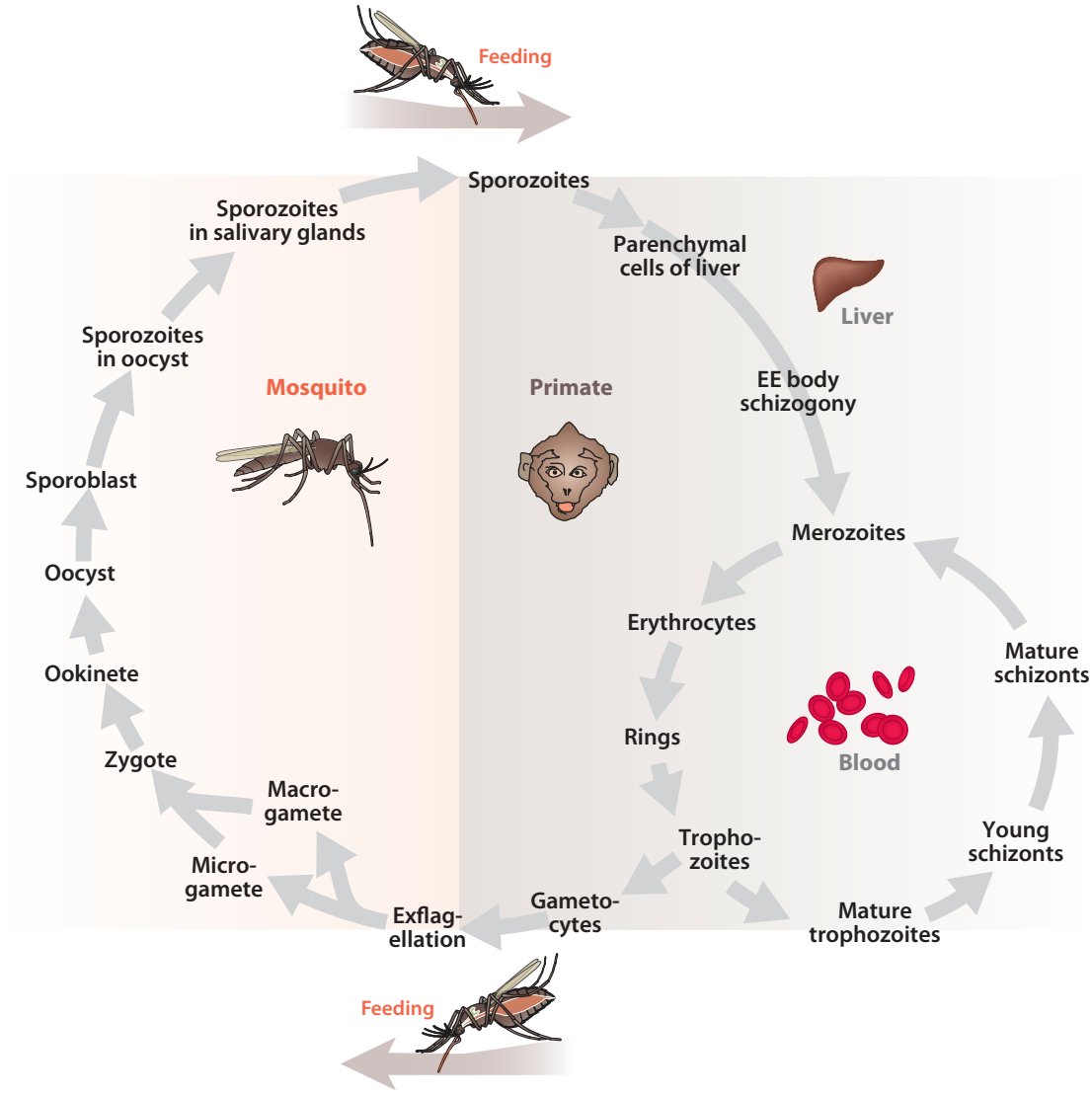


**Figure 1**

Life cycle of the malaria parasite. Sporozoites are injected into the primate by a mosquito bite. This is followed by the development of the exoerythrocytic (EE) body, the production of merozoites that invade the erythrocytes, the production of gametocytes that are taken into the gut of the mosquito during feeding, and the sporogonic cycle in the mosquito that results in the production of sporozoites that are now injected into the primate to complete the cycle.

macrogametocytes by the mosquito during feeding, the fusion of the gametes takes place in the gut of the vector to produce the ookinete that migrates to the wall of the mosquito's gut, where it develops into the oocyst. This sporogonic development on the gut of the mosquito requires approximately 9 to 10 days at 25°C (19). At this time, thousands of sporozoites are released from each oocyst and are then pumped by the heart to the salivary glands. Sporozoites are concentrated in the acinar cells of the salivary glands. During feeding, up to 100 sporozoites are injected into the primate host. The sporozoites are transported by the circulatory system to the liver,

where they bind tightly to the sinusoidal cell layer, cross the Kupffer cells, and finally invade liver parenchymal cells. Within these cells, the exoerythrocytic (EE) stages develop. This stage requires approximately 5 days to reach maturity. Thousands of merozoites are released from each EE stage and invade the erythrocytes of the primate host. Gametocytes are subsequently produced to complete the life cycle of the parasite.

## Erythrocytic Stage

The parasites, as they appear in the erythrocytes, are illustrated in drawings prepared by G.H. Nicholson (**Figure 2**, panels 1–25) from Reference 19. Photomicrographs of the blood stages as seen in Giemsa-stained blood films are shown in **Figure 3**. The young rings are similar to those of *P. falciparum*. The nucleus is spherical and many times seen lying inside the ring. When full grown, the nonamoeboid ring may occupy half or more of the host erythrocyte. Band forms (panel 11) appear similar to those of *P. malariae*. With the loss of the vacuole, the parasite shrinks and becomes compact, and pigment appears in the form of dark grains. The nucleus increases in size. With Giemsa stain, the cytoplasm stains a deep blue and the nucleus a deep red. The erythrocyte shows a faint stippling (panels 13–19). With the advent of schizogony, the nucleus divides, producing as many as 16 merozoites (10 on average). These merozoites then fill the host cell. The pigment then collects into one or more yellowish-black masses and eventually into a single mass in the mature schizont. The asexual cycle in the blood lasts only 24 hours and is known as a quotidian cycle.

The sexual forms grow more slowly, taking approximately 48 h to complete their development. The mature macrogametocyte (panel 24) is generally spherical and fills the host cell. The cytoplasm stains a distinctive blue and the nucleus takes a deep pink stain. Black pigment granules are scattered throughout the cytoplasm. The microgametocyte (panel 25) is sometimes smaller. The cytoplasm stains a medium pink, and the nucleus is a darker shade of pink. Pigment is jet black and is also scattered in the cytoplasm.

## Tissue Stage

Sporozoites injected into the bloodstream of the host enter the liver either by the hepatic artery or the portal vein. They bind to the sinusoidal cell layer, presumably mediated by proteoglycans protruding from the space of Disse (or perisinusoidal space). The sporozoites position themselves and pass through a Kupffer cell and the space of Disse to enter the liver parenchyma. Sporozoites eventually settle into individual parenchymal cells for development into the EE stage (EE body). The tissue forms of *P. knowlesi* were first described by Garnham et al. (33). The earliest forms seen were those taken at biopsy 92 h postinfection. In a subsequent study, Held et al. (40) injected infected salivary glands from *Anopheles dirus* mosquitoes directly into the liver of rhesus monkeys. Beginning 48 h postinjection, liver biopsies were taken at 8-h intervals through 120 h. At that time, young ring forms were present in the circulating blood of the animals. At the same time, nearly mature exoerythrocytic forms (EE bodies) were demonstrable in the liver sections, indicating a delay in the maturation of some of the EE forms. The greatest rate of growth of these EE bodies appeared to take place between 72 and 96 h after sporozoite injection (24).

Subsequently, tissue stages were demonstrated in different species of New World monkeys. **Figure 4** shows maturing and fully mature EE bodies developing in the parenchymal cells of squirrel monkeys.

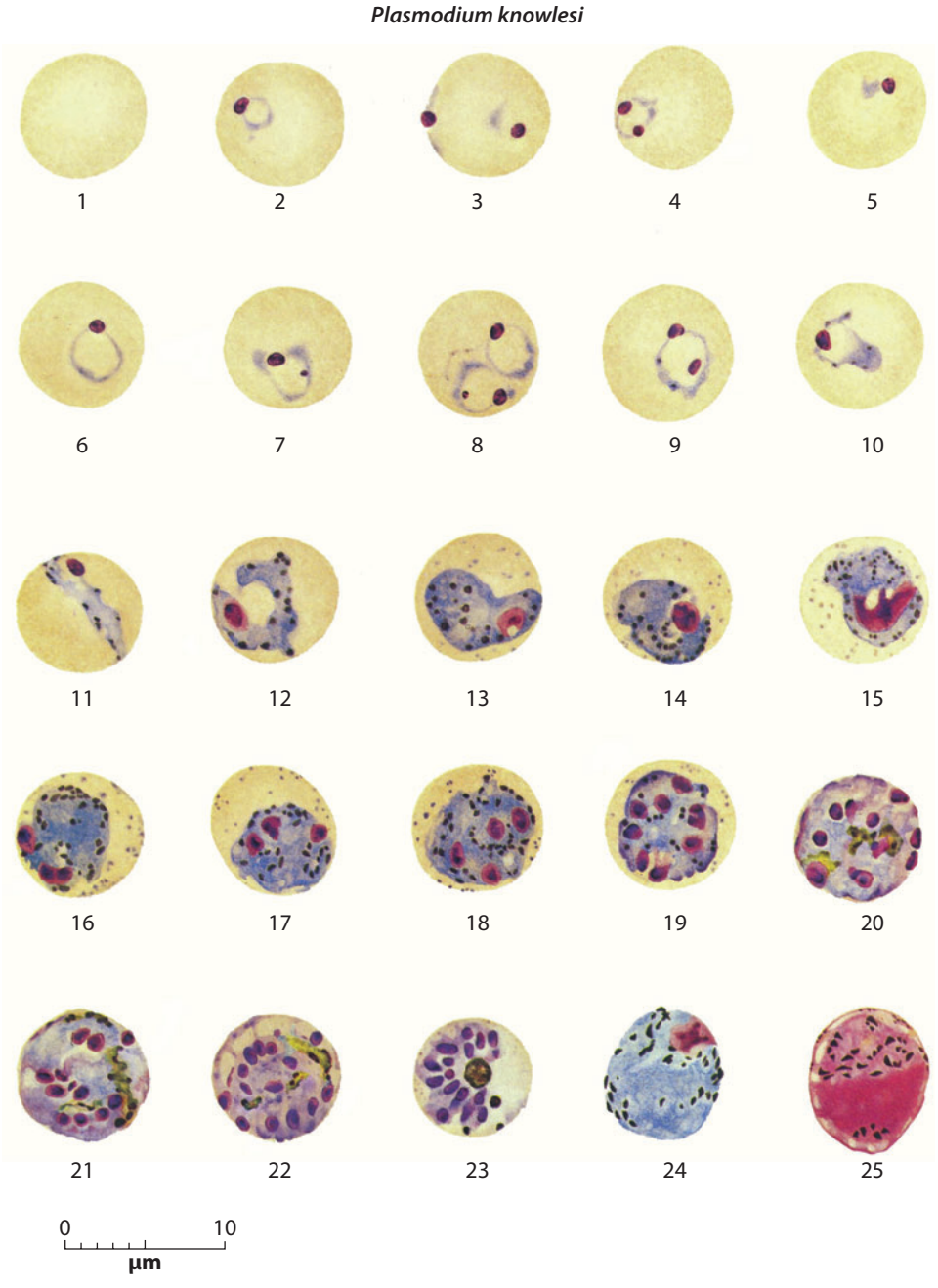


**Figure 2**

Erythrocytic stages of *Plasmodium knowlesi*. Drawings prepared by G.H. Nicholson.

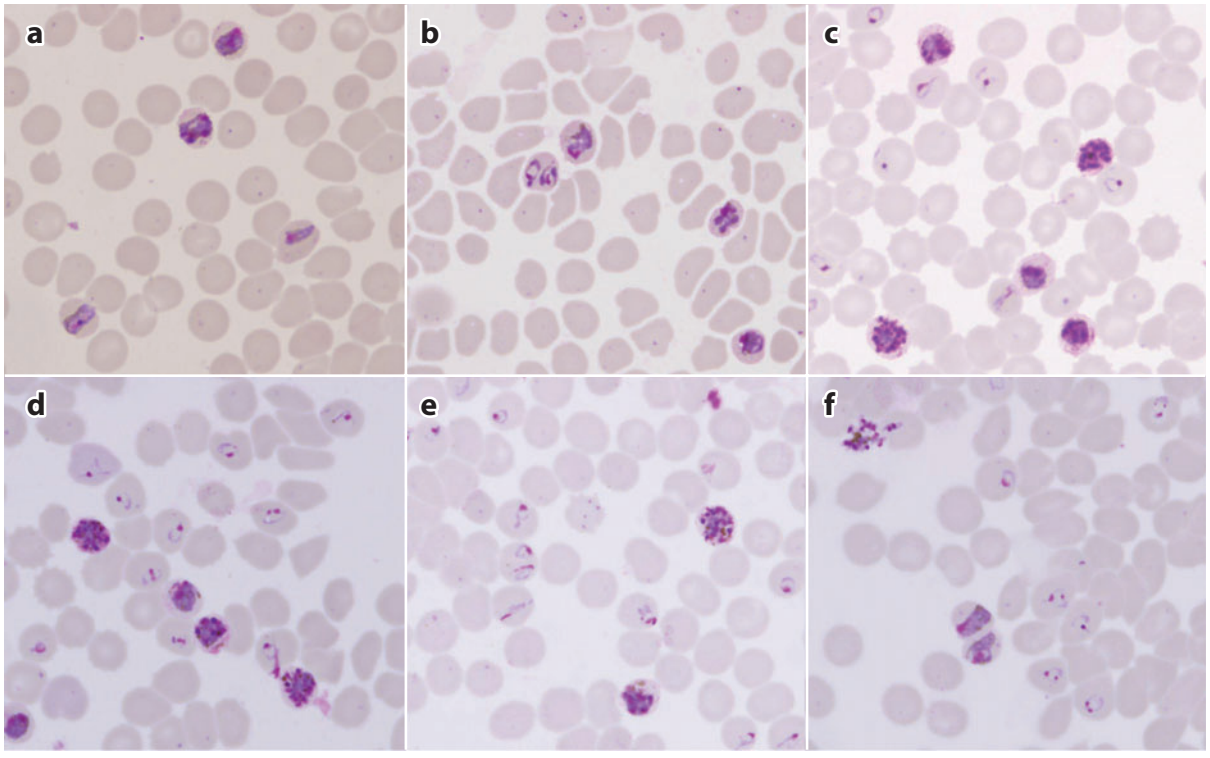


**Figure 3**

Photomicrographs of erythrocytes of a rhesus monkey infected with *Plasmodium knowlesi*. Panels *a*, *b*, and *f* show band forms similar to some trophozoite forms of *Plasmodium malariae*. Panels *d–f* show young ring forms typical of *P. malariae* and *P. falciparum*. Panels *c–e* show mature schizonts. The number of merozoites in the mature forms are usually greater than that observed in *P. malariae*.



## INFECTION OF HUMANS

### Experimental Infections

The first experimental infection of humans was that performed by Knowles & Das Gupta (50). This was followed by numerous reports of humans treated for neurosyphilis by infection with *P. knowlesi* (15, 16, 18, 43, 44, 54, 55, 60, 85). In these studies, most authors characterized the disease in humans as mild with a tendency toward spontaneous self-recovery. However, Ciuca et al. (17) reported in 1955 that after 170 transfers, the infection became virulent and had to be terminated with drugs. Following the natural infection of a person in Malaysia in 1965, 20 volunteers were infected with the H strain of *P. knowlesi*, 8 via the bites of *A. dirus* mosquitoes, and 12 by blood passage (13, 19). Parasite counts as high as 1,200 per microliter were encountered as late as the twenty-eighth day of patent parasitemia. Most patients cleared their detectable parasitemia by day 16. Infections were transmitted from human to human and from human to rhesus monkey by mosquito bite.

### Natural Infections

The first reported natural transmission of *P. knowlesi* to humans was the case of a 37-year-old American who had spent 5 days in the bush in Malaysia in 1965 (12). However, the close

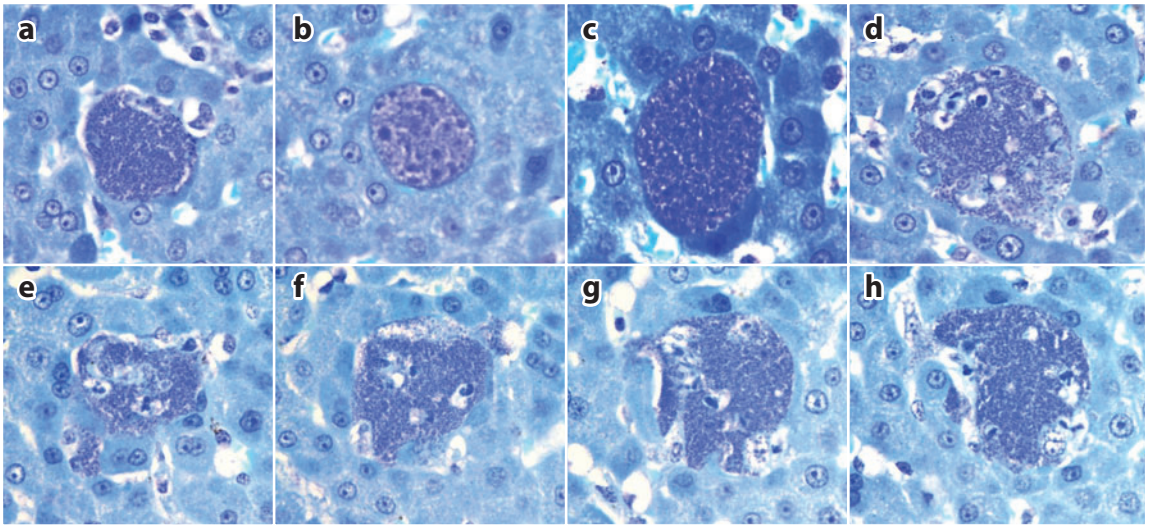


**Figure 4**

Exoerythrocytic (EE) stages of *Plasmodium knowlesi* in 5-μm sections of the liver of *Saimiri boliviensis* monkeys. Panels *a* and *b*: 96-h EE bodies; panels *c* and *d*: 120-h EE bodies; and panels *e–h*: serial sections of an individual 120-h EE body.



resemblance of the blood stages of this parasite to that of *P. malariae* suggested that detection of other human cases would be readily missed or misdiagnosed. Singh et al. (78) clarified the situation through the use of PCR techniques. In a retroactive study in Malaysian Borneo, 58% of blood samples from people that had been diagnosed with malaria actually contained the monkey malaria parasite *P. knowlesi*. Microscopic examination had indicated the presence of *P. malariae*, yet this parasite was not detected by PCR. There had been four fatal human infections due to *P. knowlesi* (27). *M. fascicularis* monkeys infected with *P. knowlesi* apparently had served as the continued source of infection, and *Anopheles latens* (a member of the *Leucosphyrus* group of mosquitoes) served as the vector (87).

Since the initial indirect studies in Borneo, human infections have been reported from additional areas of Southeast Asia including peninsular Malaysia (86), Singapore (59), the Philippines (52), and Thailand (45, 69). The distribution of this parasite is apparently confined to Southeast Asia because mosquitoes of the *Anopheles* (*Cellia*) *leucosphyrus* Donitz group are the only mosquitoes shown to be capable of transmitting this parasite.

## NONHUMAN INFECTIONS

The natural hosts for *P. knowlesi* in Malaysia are the crab-eating macaque (*M. fascicularis*), the pig-tailed macaque (*M. nemestrina*), and the langur (*Presbytis melalophos*). Experimentally, almost all primates, including humans, are susceptible to infection. Both Old and New World monkeys are used in biological, chemotherapeutic, and immunological studies with *P. knowlesi* (21). Removal of the spleen greatly increases the parasite counts in animals that could normally tolerate infections. The most commonly used experimental host monkey has been the rhesus monkey (*M. mulatta*). Infection is usually fatal if the monkeys are inoculated with parasitized erythrocytes. Following infection via sporozoites, approximately 70% of the animals die with overwhelming parasitemia (22, 25). The remaining animals control their infections and infect susceptible mosquitoes, often over a period of many days. The endpoint for studies for early vaccine trials in this host was often

death; therefore, interpretation of the effectiveness of such studies was straightforward. Those who performed immunization trials with rhesus monkeys (6, 7, 23, 29, 36, 46, 70, 73, 77) might have been better served had they used the natural host, *M. fascicularis*. The disadvantage of using *M. fascicularis* is that many of the wild-caught animals that are imported have already been naturally infected. Nonetheless, much information has been obtained from these studies on *P. knowlesi* in rhesus monkeys.

## IMMUNOLOGIC AND ANTIGENIC STUDIES

*P. knowlesi* has been one of the major models in the search for a vaccine for malaria because (*a*) it can be grown in culture, (*b*) antigens similar to those of human malaria parasites are produced by *P. knowlesi*, and (*c*) in trials, the animals can be challenged via parasitized erythrocytes or via sporozoites with predictable outcomes for the controls.

### In Vitro Culture

Soon after the in vitro culture of *P. falciparum* was developed by Trager & Jensen (84), the culture of *P. knowlesi* was established (34, 62–64, 91). This made the parasite available to those investigators lacking the facilities to work with primates. In addition, both the erythrocyte and sporozoite stage parasites could be maintained in the frozen state and then used to initiate infections in animals after many years of storage. Normally, parasites are preserved using Glycerolyte® (20, 26); successful preservation with DMSO (dimethyl sulfoxide) has also been reported (5).

### Antigen Isolation and Characterization

Many of the major antigens studied for the development of human vaccines have also been isolated from *P. knowlesi* for study in monkeys or culture. Aikawa et al. (1) localized the protective 143-/140-kDa antigens by using antibodies and ultramicrotomy. Barnwell et al. (3, 4) studied the expression of the variant antigen on the erythrocyte membrane of cloned *P. knowlesi*. Chitnis & Miller (14) identified the erythrocyte-binding protein domains involved in erythrocyte invasion. The Duffy gene, which is involved with cellular receptors, was also a center of investigation. Chaudhuri et al. (11) purified and characterized the Duffy blood group antigens. Fried et al. (32) identified two cysteine-rich, lipophilic proteins on the surface of ookinetes (Pks20 and Pks24). Howard & Barnwell (41) analyzed the surface membrane antigens on erythrocytes infected with *P. knowlesi*. Hudson et al. (42) examined a merozoite surface protein of the parasite with a molecular weight of 14,000. Klotz et al. (48) examined vaccination-induced variation in the 140-kDa merozoite surface antigen. The *P. knowlesi* schizont-infected cell agglutination (SICA) antigen gene family and its variation, compared to *P. falciparum*, were described by al-Khedery et al. (2). Korir & Galinski (51) demonstrated the relationship between *P. knowlesi* SICA variant antigens and *P. falciparum* EMP1. Schmidt-Ullrich et al. (74, 75) studied the 65,000-Mr glycoprotein at the surface of infected erythrocytes and the protective 74,000-Mr antigen in membranes of schizont-infected rhesus erythrocytes. Ellis et al. (30) cloned and expressed the malarial sporozoite surface antigen in *E. coli*. Ruiz et al. (72) reported the organization and expression of the *P. knowlesi* circumsporozoite antigen, and Sharma et al. (76) reported the immunogenicity of the nonrepetitive regions of the circumsporozoite protein.

### Vaccine Trials

Rhesus monkeys have been the primary host for trials using candidate experimental *P. knowlesi* vaccines and Freund's adjuvant. Brown & Hills (6) and Brown & Tanaka (7) immunized rhesus

monkeys using formalin-inactivated whole parasites and Freund's adjuvant. Butcher et al. (8) determined the antibody-mediated mechanisms that were induced by vaccination with *P. knowlesi* merozoites. Cabrera et al. (9, 10) immunized rhesus monkeys with a parasite preparation that had been put through a French pressure cell press. Animals were protected 4 years after immunization against a heterologous strain of the parasite. Collins et al. (23) immunized rhesus monkeys by using heat-stable, serum-soluble antigens. David et al. (28) immunized monkeys with a 140-kDa merozoite surface protein and Deans et al. (29) conducted vaccine trials in rhesus monkeys with a minor, invariant 66-kDa merozoite antigen. Gwadz & Green (36) and Gwadz et al. (35) reported the vaccination of rhesus monkeys with irradiated sporozoites of *P. knowlesi.* Gwadz & Koontz (37) also reported the development of transmission-blocking immunity in monkeys immunized with gamete antigens. Kaushik et al. (46) protected rhesus monkeys with a merozoite vaccine, and Khanna et al. (47) vaccinated rhesus monkeys using whole antigen and an aqueous suspension of muramyl dipeptide as an adjuvant. Mitchell et al. (56, 57) also used a merozoite vaccine to effectively immunize rhesus monkeys. Rieckmann et al. (70) immunized rhesus monkeys with blood-stage antigens of *P. knowlesi*. Schenkel et al. (73) vaccinated rhesus monkeys by the use of nonviable antigen and Simpson et al. (77) vaccinated rhesus against malaria by use of sucrose density gradient fractions of blood-stage antigens. Targett & Fulton (83) were among the first researchers to successfully use rhesus monkeys and *P. knowlesi* for the study of malarial vaccines.



## VECTORS

### Natural Vectors

The first mosquito species to be incriminated as a vector was *Anopheles hackeri*, collected in peninsular Malaysia (89). Sporozoites were dissected from this mosquito and then injected into a rhesus monkey that subsequently developed *P. knowlesi* malaria. *A. latens* is the vector in Sarawak (82, 87). Using nested PCR, Vythilingam et al. (86) detected *P. knowlesi* in *A. cracens* mosquitoes collected in peninsular Malaysia. *P. knowlesi* has not been found in monkeys outside the range of mosquitoes of the *A. leucosphyrus* group. The parasite is so lethal to rhesus monkeys that the geographic range of this primate is limited by the range of the vector mosquitoes for *P. knowlesi*. According to Manguin et al. (53), the *Leucosphyrus* group consists of the *Dirus* Complex, which includes seven species, and the *Leucosphyrus* Complex, which includes four species. These are forest mosquitoes but are occasionally present in the open areas on the forest fringes.

### Experimental Vectors

Sporozoite-positive salivary glands have been reported in *A. stephensi* (33, 38, 39, 79), *A. annularis* (79, 80), *A. aztecus* (33), *A. atroparvus* (39, 88), *A. freeborni* (22), and *A. dirus* (22, 25). In only the last species were sporozoites found in abundance. *A. stephensi* was first used to transmit the parasite from monkey to monkey (38, 39). However, the sporozoite counts in the salivary glands were low. Chin et al. (13) experimentally transmitted *P. knowlesi* to human volunteers and monkeys using laboratory-reared *A. dirus* mosquitoes. Collins et al. (22, 25) repeatedly transmitted the H strain of the parasite to rhesus monkeys using *A. dirus*; Sullivan et al. (81) transmitted three different strains of *P. knowlesi* to New World monkeys using the same vector. Rosenberg (71) determined that *A. freeborni* mosquitoes could not transmit the infection because sporozoites are unable to invade the salivary glands. Sporozoites develop within the oocysts normally. Other mosquitoes that support normal development of the oocysts only are *A. maculatus*, *A. quadrimaculatus*, and *A. atroparvus* (19).

## TREATMENT

Treatment with schizonticidal drugs is sufficient to prevent or control infections. Because *P. knowlesi* does not relapse from residual forms in the liver, treatment with primaquine is not needed. Chloroquine is most commonly used to treat monkeys infected with this parasite (300 mg for an adult rhesus or *M. fascicularis* given over 3 days) (W.E. Collins, unpublished observations). There are no reports of resistant strains. The standard treatment with chloroquine for humans would be 1500 mg for a 60-kg adult. Prasad et al. (65–68) studied the kinetic effects of chloroquine as well as its effect on complement levels, cellular immune responses, and the phagocytic function of monocytes by using *P. knowlesi*–infected rhesus monkeys. In addition, infections in monkeys have been treated successfully with quinine, sulfonamides, mefloquine, and the newer artemisinin drugs (W.E. Collins, unpublished observations).

## DIAGNOSIS

Currently, diagnosis is made by nested PCR using different primers (45, 78). On blood films from monkeys, infected erythrocytes are not enlarged and any stippling is faint and restricted to more mature trophozoites. The additional presence of trophozoite band forms would indicate *P. knowlesi* in monkeys or either *P. malariae* or *P. knowlesi* in humans as a tentative diagnosis. The definitive diagnosis is the 24-h developmental cycle of the erythrocytic stage. Mosquitoes are infected by subpassage to rhesus monkeys (89), and sporozoites and oocysts in captured mosquitoes are identified by using nested PCR (82, 86).

Future studies should determine the different members of the *Leucosphyrus* group of mosquitoes that are transmitting *P. knowlesi* in Southeast Asia. Moreover, humans with malaria acquired in the vicinity of monkey habitat should be tested by PCR for infection with *P. knowlesi* in addition to the other human parasites.

### SUMMARY POINTS

1. *Plasmodium knowlesi* is the primary parasite of monkeys of Southeast Asia and it occasionally infects humans.
2. The parasite morphologically resembles *P. malariae*; specific diagnosis of human infection is determined by nested PCR.
3. Infection may occur anyplace within the distribution range of members of the *A. leucosphyrus* complex of mosquitoes if infected monkeys, such as *M. fascicularis*, *M. nemestrina*, and *Presbytis melalophos*, are present.
4. In most instances, the vectors of this parasite are forest-dwelling, canopy-feeding mosquitoes such as *A. latens* and *A. cracens*.
5. Workers or travelers who enter this environment are at risk of being fed upon by infected mosquitoes and of developing infection and disease.
6. This parasite has been widely used in the development of different types of experimental antimalarial vaccines.
7. The primary hosts for immunologic studies are *M. mulatta* monkeys, although other primates such as New World *Aotus* spp. and *Saimiri* spp. have been infected.
8. The parasite has been grown in culture and successfully stored in the frozen state.

## FUTURE ISSUES

1. How extensive is the infection of humans with *P. knowlesi* in Southeast Asia?
2. Are other species of *Plasmodium* (*P. inui*, *P. cynomolgi*) being transmitted to humans?
3. Are infections of *P. knowlesi* transmitted from human to human?
4. Are vectors other than *A. latens* and *A. cracens* responsible for the transmission of *P. knowlesi* to monkeys and humans?

## DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.



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