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Infection of mosquitoes from in vitro cultivated *Plasmodium knowlesi* H strain

Jennifer S. Armistead [a,b,1], Roberto R. Moraes Barros [a,1], Tyler J. Gibson [a], Whitney A. Kite [a], J. Patrick Mershon [a], Lynn E. Lambert [c], Sachy E. Orr-Gonzalez [c], Juliana M. Sá [a], John H. Adams [b], Thomas E. Wellems [a,*]

[a] Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA
[b] Department of Global Health, College of Public Health, University of South Florida, Tampa, FL 33620, USA
[c] Laboratory of Malaria Immunology and Vaccinology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

In vitro studies of sexual blood stages of the most fatal malaria species, *Plasmodium falciparum*, have revealed key processes by which gametocytes develop and transmit infection from humans to anopheline mosquitoes. However, most malaria cases outside sub-Saharan Africa are caused by other *Plasmodium* spp., frequently *Plasmodium vivax* and *Plasmodium knowlesi*, a zoonotic parasite of macaque monkeys. Gametocytes of *P. vivax* and *P. knowlesi* exhibit distinct morphology, faster development, and a shorter life span compared with gametocytes of *P. falciparum*, reflecting the evolutionary separation and biological differences of these species. Unlike *P. falciparum*, *P. vivax* cannot be cultivated in vitro, necessitating access to infected primates for laboratory studies. In contrast, *P. knowlesi* asexual stages have been successfully adapted to cultures in macaque and human red blood cells, but these stages have not been reported to produce gametocytes infective to mosquitoes. Here, we show that gametocyte production and sporadic, low-level mosquito infectivity of a *P. knowlesi* strain was not improved by application of a "crash" method commonly used to induce gametocytes in *P. falciparum* cultures. However, Percoll-gradient purified schizonts from this strain yielded highly synchronised populations that, in three of six experiments, produced infections at an average rate of 0.97–9.1 oocysts in *Anopheles dirus* mosquitoes. Oocyst counts were most abundant in mosquitoes that were fed from the synchronised cultures 36 h after schizont purification. Gametocytes in these cultures occurred at low prevalence and were difficult to observe. Transcription from orthologs of *P. falciparum* gametocyte-specific markers did not correlate with infectivity of the *P. knowlesi* parasites to mosquitoes. The ability to infect mosquitoes from in vitro-cultivated *P. knowlesi* will support research on the unique features of this emerging pathogen and facilitate comparative studies of transmission by the different human malarias.

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1. Introduction

Transmission of *Plasmodium* parasites that cause malaria requires maturation of infective gametocytes within host red blood cells (RBCs). When ingested in a bloodmeal by anopheline mosquitoes, gametocytes yield gametes in the insect midgut that initiate sexual reproduction required for the parasite life cycle. Continuous in vitro culture of the asexual blood stages of *Plasmodium falciparum* (Haynes et al., 1976; Trager and Jensen, 1976), the parasite species responsible for the deadliest human malaria, was a landmark in malaria research. Extension of these culture methods to generate sexual-stage gametocytes (Ifediba and Vanderberg, 1981) together with membrane-feeding techniques to infect mosquitoes (Graves, 1980; Ponnudurai et al., 1982), have facilitated discoveries of sexual commitment mechanisms (Mantel et al., 2013; Regev-Rudzki et al., 2013; Brancucci et al., 2014; Coleman et al., 2014; Kafsack et al., 2014), characterisation of gametocyte-specific genes and processes (Meibalan and Marti, 2017), genetic crosses (Walliker et al., 1987; Wellems et al., 1990; Hayton et al., 2008), as well as evaluations of gametocytocidal drugs (Lucantoni et al., 2017) and transmission-blocking vaccine candidates (Goncalves and Hunziker, 2016).

* Corresponding author at: 12735 Twinbrook Parkway, Room 3E-10, Rockville, MD 20852, USA.
E-mail address: twellems@niaid.nih.gov (T.E. Wellems).
[1] These authors contributed equally.

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Of the other human malaria parasites, *Plasmodium vivax* and *Plasmodium knowlesi* are also responsible for large public health burdens of illness, predominantly outside Africa. Despite the closer phylogenetic relationship of these two species than of either to *P. falciparum* (Loy et al., 2017), only *P. knowlesi* has been adapted to continuous culture in vitro, in rhesus macaque (Kocken et al., 2002) and in human RBCs (Lim et al., 2013; Moon et al., 2013). Cultivability of these parasites helps to support research on *P. knowlesi* malaria and control of the morbidity and mortality it causes in southeastern Asia (Yusof et al., 2014; Barber et al., 2017). Use of these parasites may also serve for biological and genetic models of *P. vivax* and the other species that are refractory to continuous cultivation. However, studies of *P. knowlesi* in culture have been limited to the asexual cycle in RBCs, as (unlike for *P. falciparum*) no production of sexual stage gametocytes has been reported from a cultivated line (Moon et al., 2013; Zeeman et al., 2013).

Plasmodium falciparum gametocytes typically require a development time of 10–12 days (Jeffery and Eyles, 1955) to their mature falciform shape (from which the species takes its name); they then remain infective for several days (Smalley and Sinden, 1977). In contrast, *P. knowlesi* gametocytes are thought to mature in approximately 1.5 days to large, round forms that fill and enlarge the host RBCs; these forms are then infective for only a matter of hours (Hawking et al., 1968). *Plasmodium knowlesi* in vivo infections have a characteristic synchronous 24 h cycle, that includes noontime rupture of schizonts and merozoite invasion of RBCs (Hawking et al., 1968). Gametocyte infectivity may thus be periodic, a feature that would maximise transmission efficiency to coincide with the blood-feeding of mosquitoes at night (Hawking et al., 1968), although this has been debated (Mideo et al., 2013). Other human and non-human primate *Plasmodium* spp., such as *P. vivax* and *Plasmodium cynomolgi*, demonstrate similar rapid, periodic production of mature, infective gametocytes (Hawking et al., 1966; Yang et al., 1984; Yang, 1996; Gautret and Motard, 1999), distinct from *P. falciparum* and closely-related plasmodia of the *Laverania* subgenus (Gautret and Motard, 1999).

In our studies, standard "crash" methods that induce gametocytes from *P. falciparum* cultures have failed to increase gamete production from a culture-adapted *P. knowlesi* line derived from the H strain. Here, we show that highly synchronised in vitro cultures of these parasites produced mosquito infections despite low or even sub-microscopic densities of gametocytes. Synchronised *P. knowlesi* yielded highest infectivity to *Anopheles dirus* mosquitoes 31–41 h after purified schizonts ruptured and infected rhesus macaque RBCs in freshly established cultures. However, the results varied and, in some cases, yielded few or no mosquito infections. Gametocytes occurred at low prevalence and were difficult to observe when mosquito infections developed; transcription from known orthologs of *P. falciparum* gametocyte-specific markers showed no correlation with infectivity of the *P. knowlesi* cultures to mosquitoes.

2. Materials and methods

2.1. Ethical statement

All non-human primates were obtained from US National Institutes of Health (NIH) approved sources. All care and use were in accordance with the NIH Animal Research Advisory Committee (NIH ARAC) Guidelines, under protocols approved by the US National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee and in compliance with the Animal Welfare Act and the Guide for the care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

2.2. Rhesus macaque infections

Four rhesus macaques (*Macaca mulatta*) bred in the US from Indian stock were used in these studies: DB5KA, male, splenectomised, 12 years old, malaria naïve, 11.92 kg; FZ8, female, 10 years old, malaria naïve, 7.18 kg; MCF, female, 4 years old, malaria naïve, 8.26 kg; DB2L, male, splenectomised, 11 years old, malaria naïve, 11.44 kg. The macaques were anesthetised with ketamine (10 mg/kg body weight) via i.m. injection prior to inoculations or venipuncture. Inoculations were performed with thawed parasitised RBCs (pRBCs) or sporozoites from cryopreservates of previous *P. knowlesi* H strain infections in rhesus macaques (cryopreservates kindly provided by Dr. Patrick Duffy, Laboratory of Malaria Immunology and Vaccinology, NIAID Division of Intramural Research, USA). The cryopreserved pRBCs were thawed and washed in sterile incomplete RPMI (iRPMI, which is RPMI-1640 medium supplemented with 25 mM Hepes, 10 mg/L of hypoxanthine, and 25 mM $NaHCO_3$ (KD Medical, Columbia, MD, USA)) and delivered i.v. to one animal (DB5KA). Cryopreserved sporozoites were thawed at 37 °C for 30 s and resuspended in PBS (1×: 10 mM PO_4^{3-}, 137 mM NaCl, 2.7 mM KCl, pH 7.4) supplemented with 0.6% human serum (Interstate Bloodbank, Memphis, TN, USA). Approximately 16,500 sporozoites were administered to each of three rhesus macaques (DB2L, FZ8, and MCF) via i.v. injection. Parasitemias of infected animals were monitored daily by microscopy using 20% Giemsa-stained thin blood films, and up to three times daily after reaching 0.1%. Total parasitemias were obtained after counting an estimated 10,000 RBC. Rhesus macaque infection studies where concluded according to animal protocol when parasitemias of ~5% were detected.

2.3. Plasmodium knowlesi cultivation in vitro

Uninfected rhesus macaque blood was collected by venipuncture into sodium heparin vacutainers (BD, Franklin Lakes, NJ, USA) and centrifuged at 800*g* for 3 min. Plasma was removed and RBCs were washed once, resuspended in iRPMI at 50% hematocrit and stored at 4 °C for up to 2 weeks. *Plasmodium knowlesi* H strain-infected pRBCs were collected from infected rhesus macaques into sodium heparin vacutainers (BD). After plasma removal by centrifugation, the pRBCs were combined with uninfected rhesus macaque RBCs in cultures with complete RPMI (cRPMI, which is iRPMI supplemented with 10 mg/L of gentamicin, and 1% Albumax II (Life Technologies, Carlsbad, CA, USA)) at 5% hematocrit and 37 °C under a 90% N_2, 5% CO_2, and 5% O_2 gas mixture, with media changes once daily, or twice daily if parasitemia was ≥4%. Cultures were monitored by microscopy using Giemsa-stained thin blood films, and maintained at 0.5–10% parasitemia. Total parasitemias and parasite stage distributions were recorded from counts of 5,000 RBCs. Cultures were transferred to iRPMI supplemented with 10% (v/v) pooled sera from 2 to 6 animals, for a minimum of three cycles prior to membrane-feeding assays. The three different pools of sera used for this purpose are listed in Supplementary Table S1.

2.4. Crash method for induction of gametocytes

Attempts to induce *P. knowlesi* gametocytogenesis by culture crash were based on a protocol established for *P. falciparum* (Saliba and Jacobs-Lorena, 2013). Mixed stage PkH/FZ8 cultures were diluted to 0.2% hematocrit with rhesus macaque RBCs in iRPMI supplemented with 10% (v/v) rhesus macaque pooled sera. A 75% volume of the overlying medium was removed and replaced every 24 h, but with no addition of RBCs, resulting in a small culture volume increase (and corresponding hematocrit reduction) of 1.5-fold after two cycles. Parasitemia was monitored by micro-

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