**Experimental transmission of *Plasmodium malariae* to *Anopheles gambiae***

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**Brief summary**

Our study shows an experimental transmission of *P. malariae* in a colony of *An. gambiae* s.s. mosquitoes using fresh parasite isolates obtained from asymptomatic individuals with microscopic and molecular evidence of *P. malariae*, either as mono-infections or mixed infections.

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**Abstract**

Our current knowledge of the clinical burden, biology, and transmission of *Plasmodium malariae* is extremely scarce. To start addressing some of those questions, we experimentally infected *Anopheles gambiae* mosquitoes with fresh *P. malariae* isolates obtained from asymptomatic individuals in Lambaréné, Gabon. The proportion of mosquitoes infected via direct membrane feeding assay with either *P. malariae* mono-infections (16%; 19/121) or co-infections (28%; 31/112) was higher after serum replacement compared to parallel groups without serum replacement (4%, 4/102; and 4%, 2/45; p<0.01 respectively). Our results show that isolates from asymptomatic carriers can be used for experimental studies of *P. malariae* transmission.

**Keywords.** *Plasmodium malariae*, *Anopheles gambiae*, experimental transmission, Gabon.

*Plasmodium malariae* is one of the five species that cause human malaria. Infections with this species are characterised by a slow, 72-hour intra-erythrocytic replication cycle, a unique ability to persist in the host for decades and high fever peaks despite self-limiting asexual blood stage density in non-immune patients [1]. *P. malariae* prevalence can reach up to 30% in some endemic regions [2, 3]. Similar infection rates are observed in field collected mosquitoes [3, 4]. *P. malariae* appears to be more prevalent than *P. ovale* spp. and is usually found occurring as co-infections with *P. falciparum* or *P. vivax*, with co-infection rates varying between 15% and 45% in Gabon [5]. An analysis of the landmark Garki project, however, revealed a striking temporal offset between *P. malariae* and *P. falciparum* prevalence and conversion rates, implying yet undefined interactions of potential importance for malaria control strategies [6].

*P. malariae* infections in endemic areas are typically asymptomatic and sub-microscopic [1], and thus, are frequently undetected, especially in mixed infections. This will underestimate the number of true cases [7]. More sensitive PCR protocols can dramatically improve the detectability of *P. malariae* infections [8]. Studies using molecular methods have found regional prevalence rates of *P. malariae* up to 40% [2]. It is important to note that *P. malariae* infections can also reach high prevalence in non-African endemic settings characterised by low *P. falciparum* prevalence rates [2]. The undetected persistence at low parasitaemia of *P. malariae* infections and potentially, the existence of a monkey reservoir cause intermittent outbreaks of *P. malariae* malaria in South America [9]. Moreover, there is evidence for possibly natural refractoriness to standard antimalarial treatment regimens [7].

In stark contrast to *P. falciparum* and *P. vivax*, our knowledge of the experimental transmission of *P. malariae* is very limited, including due to a lack of a continuous *in vitro* culture protocol for *P. malariae*. Previous reports have shown that mosquito species such as *Anopheles stephensi*, *An. freeborni, An. gambiae* and *An. dirus* transmit *P. malariae*. However, most of these studies were carried out using monkeys infected with the Uganda I/CDC strain [10]. Only one study reported the infectivity of *Anopheles* species from human samples that were previously inoculated with a *P. malariae* clinical isolate [11]. Therefore, this study aimed to establish an experimental transmission of *P. malariae* in a colony of *An. gambiae* s.s. (Kisumu strain) mosquitoes using fresh parasite isolates obtained from asymptomatic individuals with microscopic and molecular evidence of *P. malariae*, either as mono-infections or mixed infections. Because *P. malariae* oocysts are morphologically indistinguishable from other human malaria parasites, we used qPCR to verify infections in both infecting isolates and infected mosquitoes.

**METHODS**

All procedures involving human subjects used in this study were approved by the national ethics committee of Gabon (Nr: 040/2018/SG/CNE), the institutional review board of CERMEL (CEI-014/2018) and the ethics committee of the medical faculty and the university clinics of the University of Tübingen. The study was carried out from April to October 2019 as a household-based survey in rural communities of the Moyen-Ogooué province of Gabon. Finger prick blood samples were collected from volunteers aged >3 years after signed informed consent (Supplementary methods).Individuals with microscopic evidence of *P. malariae* infections (with or without *P. falciparum* or *P. ovale*), a haemoglobin concentration of ≥6 g/dL and no anti-malarial treatment for the last three months were invited to provide 5 mL of blood taken by venepuncture into a heparinised tube for direct membrane feeding assays (DMFA).All malaria slide-positive participants were treated with artemether-lumefantrine (Coartem®). A 500 μl aliquot was stored at -20°C in DNA/RNA Shield® (Zymo Research, Irvine, USA) for total RNA extraction using the quick-DNA/RNA™ Microprep Plus Kit (Zymo Research, Irvine, USA) according to manufacturer’s instructions. The detection of *P. malariae* infections was performed using qPCR (Supplementary methods and Supplementary Table S1).

Three to six days old female adult *Anopheles gambiae* s.s. (Kisumu strain) mosquitoes were starved for 12h before blood feeding. DMFAs were performed in the negative air pressure, BSL-3 area of the insectary to prevent accidental release of infected mosquitoes. The DMFA procedure was performed immediately after blood collection, 50 female mosquitoes per paper cup were fed via an artificial membrane (Parafilm®). Two experimental groups were tested; one group was allowed to feed on 1ml of whole blood and the second group was fed after serum replacement with 60% naïve European AB- human serum to exclude transmission blocking activities in semi-immune sera. Mosquitoes were fed for 30 minutes in the dark. Afterwards, unfed mosquitoes were removed and killed. Blood engorged mosquitoes were provided with a 10% sugar solution and kept at standard insectary conditions (Supplementary methods).

Mosquito infections were determined 10-12 days after blood feeding. At least randomly selected 20 mosquito midguts per cup were dissected and stained with mercurochrome (0.2% in PBS). Oocysts counted at 100x magnification under a light microscope. Additionally, 10 randomly selected fed mosquitoes per cup were stored in 500μl of RNAlater® at -20°C for molecular confirmation. After examination by microscopy, stained midguts were stored at -20ºC. Additionally, sporozoites were isolated from salivary glands on day 21 from mosquitoes dissected in the serum replacement. Confirmation of *Plasmodium* infections in *An. gambiae* mosquitoes fed with mono- or co-infected fresh isolates was carried out using a TaqMan assay (Supplementary Table 1). The data analysis is described in the Supplementary methods.

**RESULTS**

Out of 647 participants screened in 110 household surveys, 15 *P. malariae* infections were identified by microscopy (2.3%). Subsequent analysis of this subset of 15 infections by qPCR revealed that eight participants were mono-infected (8/15), six were co-infected with *P. falciparum* (6/15) and one was co-infected with *P. falciparum* and *P. ovale* (1/15).

At the time of membrane feeding, we detected a median of 663 asexual parasites/µl (range, 181-1,203) for *P. malariae* mono-infected isolates and a median of 447 asexual parasites/µl (range, 251-820) for co-infection isolates. The respective median *P. malariae* gametocyte counts were 71 gametocytes/µl (range, 17-236) and 103 gametocytes/µl, (range, 0-601) (Table 1).

We performed membrane feeding experiments with 13 isolates. A total of 847 female mosquitoes were successfully fed in 2 groups (whole blood, N=319 and serum replacement, N=528). Of the mosquitoes that survived until day 12 post-feeding (survivorship, 68%; 578/847) a minimum of 20 mosquitoes were examined per isolate and per experimental group. The majority of *P. malariae* positive isolates were infective, i.e. at least one mosquito was found to harbour oocysts (15%, 65/441), (Figure 1, Table 1 and Supplementary information). Two isolates failed to yield mosquito infections (Table 1). The presence of *P. malariae* oocysts in midguts of a subset of mosquitoes 10-12 days after feeding was confirmed in 4 out of 4 separate experiments using qPCR. None of these mosquitoes were positive for *P. falciparum* after feeding with co-infected *P. malariae*-*P. falciparum* samples.

Infection rates were lower after feeding with whole blood isolates compared to serum replacement isolates (4%, 4/102 *vs* 16%, 19/121; p=0.004). A similarly pronounced difference was also seen with co-infected isolates (4%, 2/45 *vs* 28%, 31/112, respectively; p=0.001) (Figure 1A). Serum replacement also increased the intensity of mosquito infections with *P. malariae* mono-infected isolates (geometric mean, 2.9; 7 independent experiments; representing a total n of 121 mosquitoes) compared to whole blood isolates (geometric mean 1.6; 6 independent experiments: representing a total n of 102 mosquitoes, p=0.004) (Figure 1B). Likewise, the number of oocysts per mosquito fed with co-infected isolates were higher in the serum replacement group (geometric mean, 5.1; 5 independent experiments; representing a total n of 112 mosquitoes) compared to the whole blood group (geometric mean, 1.3; 4 independent experiments; representing a total n of 45 mosquitoes, p<0.001) (Figure 1B).

Salivary gland dissections were performed on four DMFA-fed mosquito batches on day 21 after feeding with 30 mosquitoes dissected in the serum replacement. We estimated sporozoite numbers per mosquito at 53,700 and 1,350 for mosquitoes fed on two *P. malariae* mono-infected isolates and 2,500 and 9,580 for mosquitoes infected with two co-infected isolates.

*P. malariae* gametocytes were detected in 92% of isolates (12/13). Gametocytaemia was correlated both with the prevalence of mosquito infection (Pearson r=0.66 p=0.02) (Figure 1C) and the intensity of the infection (Pearson r=0.85 p<0.001) (Figure 1D) in mosquitoes fed with serum replacement isolates but not with whole blood isolates (r=-0.32; p=0.4 andr=-0.9; p=0.8, respectively).

**DISCUSSION**

The recently published genome of *P. malariae* should prove to be a valuable resource [7] and stimulate research of this neglected parasite. As it stands, however, even basic characteristics, such as clinical burden, immunity and transmission, remain ill-defined. To start addressing some of those questions, we set out to establish a protocol for the experimental transmission of *P. malariae* in a colony of *An. gambiae* s.s. (Kisumu strain) mosquitoes. Our data demonstrate moderate transmission rates using fresh parasite isolates collected from asymptomatic carriers in an endemic area around Lambaréné, Gabon.

Previous reports have shown double-digit proportions of all *Plasmodium*-infected *Anopheles* mosquitoes to carry *P. malariae* in endemic areas in South America [4] and Africa [3], indicating that *P. malariae* is frequently and efficiently transmitted. However, experimental transmission of *P. malariae* isolates to *Anopheles* mosquitoes has only rarely been described. Studies have shown that mosquitoes could be infected by feeding on *P. malariae* Uganda strains from monkeys of the new world [10]. More recently, Woodford et al. [11] demonstrated the infection of only one mosquito (infection rate: 2.9%) using the direct skin feeding assay (DFA) from a volunteer inoculated with a *P. malariae* blood stage isolate. Our results show a robust infectivity of *P. malariae* isolates to *An. gambiae* s.s.. The presence of *P. malariae* was confirmed by qPCR in whole mosquitoes as well as stained midguts in a subset of mosquitoes. The moderate infectivity rates in our study are most likely related to the low asexual parasite and gametocyte densities of the infecting isolates obtained from asymptomatic carriers. The observed transmission rates, however, are consistent with other *Plasmodium* species at low parasite densities and more importantly, at low gametocyte numbers. Infection rates in our study were similar to those described in *An. gambiae* infected by *P. falciparum* isolates from asymptomatic individuals in high-endemic settings in Burkina Faso, Ethiopia [12], and with *P. vivax* in Ethiopia and Thailand [13]. However, this low transmission rates could also be due to the fact that the Kisumu strain which was used is from a different geographical area. Molina-Cruz *et al*. found that infections carried out with *Anopheles* and *P. falciparum* species from the same geographical areas resulted in higher infection rates. This should be addressed in future studies on the transmission of *P. malariae* [14]. Our findings, although based on a small number of *P. malariae* infected participants, are therefore in line with other findings from African settings. Mosquito infections can arise from asymptomatic infections, including asymptomatic qPCR-detected infections [12].

In agreement with previous studies with *P. falciparum*, the replacement of serum of malaria-infected individuals by AB serum from non-immune donors prior to feeding resulted in higher mosquito infection rates compared to no-replacement controls. This is thought to be related to transmission-blocking activities in semi-immune sera, such as antibodies against antigens expressed by gametocytes [15]**.**

To conclude, our study demonstrated the successful use of a standard DMFA protocol for the experimental transmission of *P. malariae* to *Anopheles gambiae* s.s.. The present study is a step forward for studying parasite-vector interactions that determine the transmission of *P. malariae* in Africa.

**Supplementary Data**

Supplementary methods, supplementary Table 1 and supplementary information are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author**.**

**Notes**

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***Potential conflicts of interest.*** The authors declare that they have no conflict of interest.

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**Figure caption**

**Fig 1. Infectivity of *P. malariae* isolates to *An. gambiae* mosquitoes.** Mosquitoes were fed with unmodified isolates (whole blood, WB) or isolates after serum replacement (SR). **(A)** Bar chart showing the proportion of infected mosquitoes per treatment group(7 *P. malariae* mono-infection, 5 *P. malariae*-*P. falciparum* co-infections and 1 *P. malariae*-*P. falciparum*-*P. ovale* co-infection). Error bars indicate upper 95% confidence intervals. **(B)** Dot plot of infection intensity defined as number of oocysts per midgut per experimental group. **(C)** Scatter plot of gametocyte density *vs* proportion of infected mosquitoes. Each dot represents an independent experiment. **(D)** Scatter plot of gametocyte density *vs* infection intensity. Asterisks indicate significant (p< 0.05) differences between whole blood and serum replacement groups.

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