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DNA from pre-erythrocytic stage malaria parasites is detectable by PCR in the faeces and blood of hosts ☆

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ABSTRACT

Following the bite of an infective mosquito, malaria parasites first invade the liver where they develop and replicate for a number of days before being released into the bloodstream where they invade red blood cells and cause disease. The biology of the liver stages of malaria parasites is relatively poorly understood due to the inaccessibility of the parasites to sampling during this phase of their life cycle. Here we report the detection in blood and faecal samples of malaria parasite DNA throughout their development in the livers of mice and before the parasites begin their growth in the blood circulation. It is shown that parasite DNA derived from pre-erythrocytic stage parasites reaches the faeces via the bile. We then show that different primate malaria species can be detected by PCR in blood and faecal samples from naturally infected captive macaque monkeys. These results demonstrate that pre-erythrocytic parasites can be detected and quantified in experimentally infected animals. Furthermore, these results have important implications for both molecular epidemiology and phylogenetics of malaria parasites. In the former case, individuals who are malaria parasite negative by microscopy, but PCR positive for parasite DNA in their blood, are considered to be "sub-microscopic" blood stage parasite carriers. We now propose that PCR positivity is not necessarily an indicator of the presence of blood stage parasites, as the DNA could derive from pre-erythrocytic parasites. Similarly, in the case of molecular phylogenetics based on DNA sequences alone, we argue that DNA amplified from blood or faeces does not necessarily come from a parasite species that infects the red blood cells of that particular host.

1. Introduction

The asymptomatic pre-erythrocytic development of the mammalian malaria parasite in the liver occurs immediately after sporozoites reach the organ following their deposition in the skin or microvasculature by an infected mosquito, and lasts for 5–27 days in the case of human malaria parasites, depending upon the parasite species (Coatney et al., 1971). In two human malaria parasite species, *Plasmodium vivax* and *Plasmodium ovale*, dormant liver stage parasites, hypnozoites, may persist for months or years following sporozoite inoculation (Coatney et al., 1971).

The molecular and biological characterisation of pre-erythrocytic parasite forms has, until now, necessitated invasive sampling techniques such as the removal of liver tissue from infected hosts. As a consequence, relatively little is known about the development of the parasite within hepatocytes compared with its biology during the erythrocytic cycle (Lindner et al., 2012).

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank database under accession number **KJ400029**.

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Molecular epidemiological studies on malaria often involve the PCR amplification of parasite DNA extracted from peripheral blood samples. It is nearly always assumed that this DNA derives from viable parasites infecting red blood cells (RBCs) at the time of sampling. If DNA from liver stage parasites is detectable in the peripheral blood, however, this assumption may not be true. This is of particular importance in situations in which individuals within a population may have sterile immunity to the blood stages of the parasite, or may be refractory to infection. In such cases, liver stage parasite DNA present in the peripheral blood may lead to the erroneous conclusion that otherwise uninfected hosts harbour submicroscopic blood infections. A case in point may be recent reports of the detection of *P. vivax* in the blood of Duffy negative individuals in West Africa (Mendes et al., 2011), where diagnosis was performed by PCR from blood samples without microscopy. Similarly, there are implications for studies in which the host specificities of malaria parasites are investigated using only DNA extracted from peripheral blood or faeces. It is conceivable that individuals may be infected with sporozoites of a species that are able to invade and develop within hepatocytes, but the resulting merozoites of which are unable to invade erythrocytes; as is the case, presumably, in the vast majority of *P. vivax* infections in Duffy negative humans (Culleton and Carter, 2012).

Here we show that DNA from the pre-erythrocytic stages of the rodent malaria parasite *Plasmodium yoelii yoelii* is detectable in both the peripheral blood and faeces of mice throughout the parasite's development in the liver, and that it enters the faeces through the bile. We also demonstrate the presence of DNA from different primate malaria parasite species in faecal and blood samples taken at one time point from a naturally infected macaque.

2. Materials and methods

2.1. Ethics statement

Laboratory animal experimentation was performed in strict accordance with the Japanese Humane Treatment and Management of Animals Law (Law No. 105 dated 19 October 1973 modified on 2 June 2006), and the Regulation on Animal Experimentation at Nagasaki University, Japan. The protocol was approved by the Institutional Animal Research Committee of Nagasaki University (permit: 1207261005-2). As Vietnam currently has no laws covering the use of animals in scientific procedures, the experiments performed in Khanh Phu were conducted in accordance with the Japanese law and were approved by the Director of the Provincial Health Department.

2.2. Mice, mosquitoes and parasites

Seven-week old female CBA mice, *Anopheles stephensi* mosquitoes and *P. y. yoelii* strains 17X1.1pp and CU parasites were used as previously described (Inoue et al., 2012; Ramiro et al., 2012).

2.3. Sporozoite challenge and sampling

Mice were infected with 25,000 sporozoites (see Supplementary Data S1) in a total volume of 100 μL of PBS via i.v. inoculation into the tail vein. Ten microlitres of blood was sampled from each mouse 10 min before inoculation, 10 min after inoculation, every 6 h for the following 60 h, every 12 h for the next 48 h, and then every 24 h for the next 5 days. Thin blood smears were prepared at the same time points. Faecal samples were collected at the same time points and processed as described in Supplementary Data S1. In experiments designed to determine how parasite DNA enters the faeces, mice were inoculated with 25,000, 15,000 or 1,500 sporozoites as above. Forty-two hours post-sporozoite inoculation, blood and faecal samples were taken, and liver and gall bladder tissue removed. Whole gall bladders, containing on average 3 μL of bile, and 200 mg sections of liver were removed for DNA extraction.

In order to determine the length of time taken for i.v. administered sporozoites to clear from blood circulation, mice were inoculated with 25,000 sporozoites and blood samples taken every 15 min for 180 min.

In experiments involving mixed strain infections, blood-stage infections of *P. y. yoelii* CU were initiated in mice through the i.v. administration of 1×10^6 infected RBCs, and 3 days later they were challenged with 25,000 sporozoites of *P. y. yoelii* 17X1.1pp. Control mice were inoculated with single strains via the same routes. Blood and faecal samples were taken every 12 h from 6 to 90 h p.i. DNA was extracted from samples and processed by real time quantitative PCR (qPCR) as described in the Supplementary Data S1.

2.4. Sampling of captured monkeys; Vietnam

In Khanh Phu, a commune in the mountains along the south-central coast of Vietnam, caged macaques were sampled monthly for blood and, less frequently, their faeces. The monkeys (named K1, K2 and K3) were microscopically free from malaria parasites for at least the first 7 months after their introduction to a cage that was situated in a forest where *Anopheles dirus* is abundant and has been found infected with *Plasmodium falciparum*, *P. vivax*, *Plasmodium malariae* and *Plasmodium knowlesi* (Marchand et al., 2011). DNA extraction, PCR amplification of the cytochrome B (*cytB*) gene and sequencing protocols are given in Supplementary Data S1.

2.5. Data analysis

Data were analysed using Microsoft Excel™ (Microsoft, USA) and GraphPad Prism™ 6 (GraphPad Software, Inc., USA).

3. Results

3.1. DNA from pre-erythrocytic stage parasites is detectable by PCR in the faeces and blood of mice

To investigate whether DNA from pre-erythrocytic stage parasites is present in the faeces and blood of infected hosts, sporozoites of *P. yoelii* were inoculated into groups of mice, with each mouse receiving 25,000 sporozoites. Thin blood smears were taken at the time points described in Section 2.3 and parasitaemia quantified by microscopy (Fig. 1A). Tail-vein blood samples (10 μL) and faeces (150–450 mg) were collected at the same time points, and analysed by qPCR for the presence of parasite DNA (Fig. 1B and C). Parasitaemia follows the course typical of the *P. yoelii* strain used in this experiment, with parasites appearing in the blood at 48 h post-sporozoite inoculation, reflecting the 48 h pre-erythrocytic development of the parasites in the liver (Killick-Kendrick and Peters, 1978) (Fig. 1A).

Parasite DNA was detectable in the peripheral blood throughout their development in the liver. At 10 min p.i., a relatively large amount of DNA was measured in the peripheral blood of mice, presumably due to the presence of sporozoites that had either not yet invaded hepatocytes, or were non-viable but had not yet been cleared from the circulation. At 6 h p.i., there was approximately 50 times less DNA measurable in the peripheral blood, presumably due to the successful invasion of sporozoites into hepatocytes, and the clearance of non-viable sporozoites. Thereafter we noted a gradual increase in the amount of parasite DNA in the peripheral blood concomitant with the development and replication of

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