Ubiquitous *Hepatocystis* infections, but no evidence of *Plasmodium falciparum*-like malarial parasites in wild greater spot-nosed monkeys (*Cercopithecus nictitans*)☆

Ahidjo Ayouba[a], **Fatima Mouacha**[a], **Gerald H. Learn**[b], **Eitel Mpoudi-Ngole**[c], **Julian C. Rayner**[d], **Paul M. Sharp**[e], **Beatrice H. Hahn**[b], **Eric Delaporte**[a], and **Martine Peeters**[a,*]

[a]Institut de Recherche pour le Développement and University of Montpellier, 34394 Montpellier, France

[b]Departments of Medicine and Microbiology, University of Pennsylvania, Philadelphia, PA 19104, USA

[c]IMPM/CREMER, Yaoundé, Cameroon

[d]Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, United Kingdom

[e]Institute of Evolutionary Biology and Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh EH9 3JT, United Kingdom

Abstract

Western gorillas (*Gorilla gorilla*) have been identified as the natural reservoir of the parasites that were the immediate precursor of *Plasmodium falciparum* infecting humans. Recently, a *P. falciparum*-like sequence was reported in a sample from a captive greater spot-nosed monkey (*Cercopithecus nictitans*), and was taken to indicate that this species may also be a natural reservoir for *P. falciparum*-related parasites. To test this hypothesis we screened blood samples from 292 wild *C. nictitans* monkeys that had been hunted for bushmeat in Cameroon. We detected

*Corresponding author. Tel.: + 33-4 67 41 62 97; fax: + 33-4 67 41 61 41. martine.peeters@ird.fr.

Hepatocystis spp. in 49% of the samples, as well as one sequence from a clade of *Plasmodium* spp. previously found in birds, lizards and bats. However, none of the 292 wild *C. nictitans* harbored *P. falciparum*-like parasites.

Keywords

Malaria; *Plasmodium falciparum*; *Hepatocystis*; Non-human primates; *Cercopithecus nictitans*; Greater spot-nosed monkey

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Malaria is one of the most devastating infectious diseases, with 300-500 million new cases and more than one million deaths occurring annually. Five species of *Plasmodium*, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*, are known to cause malaria in humans. Among these, *P. falciparum* is particularly notable as it is the most malignant, being responsible for the overwhelming majority of human malaria mortality (Greenwood et al., 2005), and it is sufficiently divergent from most other species of *Plasmodium* that it has been placed in a separate subgenus, *Laverania*. Thus, the evolutionary origin of *P. falciparum* is of considerable interest. Until recently, the only other parasite known to fall within *Laverania* has been *P. reichenowi,* which infects chimpanzees (*Pan troglodytes*). It had been widely assumed that this reflects long-term host-parasite co-evolution, i.e., that the common ancestor of *P. falciparum* and *P. reichenowi* infected the common ancestor of humans and chimpanzees (Escalante et al., 1994). This view changed dramatically when several groups reported the detection, in samples from chimpanzees, bonobos and gorillas, of additional *Plasmodium* DNA sequences that fell within the *Laverania* clade (Ollomo et al., 2009; Rich et al., 2009; Duval et al., 2010; Krief et al., 2010; Prugnolle et al., 2010). However, it remains unclear how many different species of *Laverania* parasitise apes, which ape species are infected in the wild, and which of these represent the true source of human *P. falciparum*. These issues were seemingly resolved by an extensive molecular epidemiological study of wild chimpanzees and gorillas (Liu et al., 2010). Analysis of more than 2,000 ape faecal samples from throughout central Africa identified six distinct *Laverania* spp., three exclusively found in chimpanzees and three others found only in western gorillas (*Gorilla gorilla*). More importantly, in detailed phylogenetic analyses, all known human *P. falciparum* strains emerged from within the radiation of just one of the species infecting gorillas. Thus, these data identified western gorillas as the natural reservoir of the parasites that were the immediate precursor of human *P. falciparum* (Liu et al., 2010; Rayner et al., 2011).

However, this conclusion that human *P. falciparum* is of gorilla origin has recently been called into question (Prugnolle et al., 2011a). Screening blood samples from 338 captive and semi-free ranging monkeys representing 10 different primate species in Gabon, Prugnolle and colleagues (2011a) found *P. falciparum*-like sequences in one sample from a pet greater spot-nosed monkey (*Cercopithecus nictitans*). Although there was only a single positive sample, among 29 from this species, it was argued that *P. falciparum* could be present in wild monkeys, and that these monkeys, rather than gorillas, could have served as the original source of the parasites found in humans (Prugnolle et al., 2011a,b). If true, this would have profound implications for our understanding of past, and potential future, zoonotic transmissions of malaria parasites. The increasing human population density in African forests inhabited by apes and monkeys, together with changes in mosquito vector abundance due to deforestation and climate change, may alter the transmission dynamics of *Plasmodium* spp. from primates and other mammals (Keesing et al., 2010). In addition, malaria eradication strategies may create new ecological niches by reducing the burden of human malaria parasites. Thus, it is important to monitor the *Plasmodium* infections of non-human primates. To determine whether greater spot-nosed monkeys are indeed widely

infected with *P. falciparum*-like parasites, we screened blood samples from nearly 300 wild *C. nictitans*, the first comprehensive survey of this species as a possible reservoir for human malaria.

We selected blood samples from existing specimen banks that were originally collected in Cameroon to assess the risk of zoonotic transmission of simian retroviruses (Aghokeng et al., 2010). The samples were collected at bushmeat markets and around logging concessions from freshly killed animals that had been hunted in the wild. The material had either been confiscated by the governmental anti-poaching program or came from hunters who had agreed to collaborate and who were educated about conservation of primates, as well as the risks associated with direct contact with primate blood, body fluids or tissues. Whole blood was collected and either stored in tubes at -20°C or spotted onto filter paper (Whatman plc, Kent, UK) or 903 filter paper cards (Schleicher & Schuell, Keene, NH, USA) and stored at ambient temperature. All samples were first subjected to mtDNA analysis to test for DNA integrity and to confirm the species of origin. This was done by amplifying a 386 bp fragment spanning the mitochondrial 12S rRNA gene using primers 12S-L1091 and 12S-H1478 (van der Kuyl et al., 2000). Blood samples from 292 *C. nictitans* were identified among material from 12 different locations across southern Cameroon (Fig. 1). These samples had been obtained between 1999 and 2004, and were collected at various times throughout the year, including 28% obtained in the months with lower rainfall (December to February). One sample site (CS7) comprised markets in the city of Yaounde, so the precise geographic origin of those samples is unknown, although they were most likely brought to the city from south and southeast Cameroon. The other 11sample sites were all located in forested areas.

Samples were screened for the presence of *Plasmodium* and related parasites by standard PCR amplification with primers that amplify a 939 bp mtDNA fragment encoding part of the cytochrome b gene (*cytb*); these primers have previously been shown to amplify *cytb* from a wide range of haemosporidian species (Liu et al., 2010). Briefly, nested PCR was performed, using DW2 and DW4 primers in the first round and Pfcytb1 and Pfcytb2 in the second round. For the first round, 5 µl DNA was used in a 50 ul reaction volume, containing 2 µl dNTPs (10 mM of each dNTP), 20 pmol of each primer (DW2 and DW4), 5 µl of PCR buffer and 0.75 µl Expand Long Template enzyme mix (Roche, Meylan, France). Cycling conditions included an initial denaturation step of 5 min at 94°C, followed by 35 cycles of denaturation (92°C, 20 s), annealing (50°C, 30 s) and elongation (72°C, 90s), and then a final elongation step of 10 min at 72°C. For the second round, 2 µl of the first-round product was used in a 50 µl reaction volume, under the same PCR conditions. Amplicons were gel-purified and sequenced directly. All new sequences were submitted to GenBank, under the accession numbers **JQ070813-JQ070956**.

Haemosporidian *cytb* sequences were amplified from 144 (49.2%) of the 292 *C. nictitans* samples (Table 1). Evidence of haemosporidian infection was found at almost all sampling locations; only sites CS1 and CS11, where in each case the sample size was very small ($n = 2$), yielded no positive samples. The high overall prevalence was not due to one or two specific sites; the fraction of positive samples ranged between 47% and 59% at four of the six sites with larger sample sizes ($n > 10$). The *cytb* sequences were aligned with representative sequences from haemosporidian species and phylogenetic trees were constructed using maximum likelihood methods, with a GTR+I+G nucleotide substitution model, and 1,000 bootstrap replicates, executed in PhyML (Guindon and Gascuel, 2003). All sequences, except one (S2138_CS8, discussed further below), fell clearly within the radiation of *Hepatocystis* parasites known to infect Old World monkey species (Fig. 2). Importantly, none of the new sequences were similar to the previously reported *P.*

falciparum-like *cytb* sequence from a captive *C. nictitans*, and indeed none were found to cluster with the *Laverania* lineage (Fig 2).

To investigate in greater detail the relationship between the *Hepatocystis* parasites infecting *C. nictitans* and those from other Old World monkeys, the new sequences were aligned with all distinct *Hepatocystis cytb* sequences available in GenBank. All of the *Hepatocystis* sequences from Old World monkeys, including those from *C. nictitans*, formed a monophyletic group within the radiation of previously reported *Hepatocystis* spp. from bats (Fig. 3). Among the clade of *Hepatocystis* sequences from monkeys, there was very little evidence of host-species specificity, with the *C. nictitans*-derived sequences completely interspersed among those from other African species (including guenons (*Cercopithecus* spp.), mandrills (*Mandrillus sphinx*) and baboons (*Papio* spp.)) and even Asian monkeys (macaques (*Macaca* spp.)). There was also no evidence for geographic clustering of specific haplotypes; multiple haplotypes were found at each sample site and haplotypes from, for example, Mindourou (CS8), spanned the entire tree. While the level of genetic divergence across this clade of monkey parasites was similar to that seen between *P. falciparum* and *P. reichenowi* (see Fig. 2), it is as yet unclear whether these *Hepatocystis* sequences comprise multiple taxa or a single widely dispersed species. However, the phylogeny shown in Fig. 3 is based on a very short sequence alignment; thus the addition of longer sequences, as well as sequences from the nuclear and apicoplast genomes, together with sequences from parasites from more host species, will be needed to resolve this issue.

The *cytb* sequence from one *C. nictitans* sample (S2138_CS8) fell within a clade of "sauropsid" *Plasmodium* spp., previously found primarily in birds and lizards (Fig. 2). Due to the ubiquitous and high levels of *Hepatocystis* infections in these samples, it is theoretically possible that additional *Plasmodium* infections had been missed in the initial screening because the broad Haemosporidian *cytb* primers were amplifying primarily *Hepatocystis* targets and masking lower levels of infections with other species. To investigate this possibility, as well as to examine the distribution of this unclassified *Plasmodium* among greater spot-nosed monkeys, we re-screened all samples using new *Plasmodium*-specific primers, which do not co-amplify *Hepatocystis* spp. The same primers and conditions, as described above, were used for the first round, but the primers Cytb1SP1 (5′-AGAAAAATAATACAATAAATAAWCGA-3′) and Cytb2SP2 (5′-CWATYGGGTCAACAATGACTTTATTT-3′) were used for the second round. However, with the exception of S2138, none of the samples yielded an amplicon. Parasites falling within this "sauropsid" *Plasmodium* clade have been found infecting bats from both Africa (Madagascar) and Asia (Cambodia) (Duval et al., 2007), but have not been reported in primates. Thus, while parasites from this clade are certainly capable of infecting mammals, since we found only a single monkey sample to be infected with this lineage, it is not yet clear whether they infect primates generally.

In stark contrast to the widespread endemic infection with *Hepatocystis* parasites, we found no evidence of *P. falciparum*-like sequences in any of the 292 samples tested, either by screening with primers targeting all haemosporidian parasites, or with primers specific for *Plasmodium* spp. It is highly unlikely that this lack of detection of *P. falciparum*-like infections in wild *C. nictitans* was due to suboptimal amplification conditions. Using an identical primer set, we previously amplified *Plasmodium cytb* sequences from 286 (21%) of 1,390 chimpanzee and gorilla samples collected at numerous field sites across southern Cameroon and neighboring parts of the Republic of Congo and the Central African Republic (Liu et al., 2010). In those ape samples, these same primers detected six *Plasmodium* spp. within the *Laverania* subgenus, as well as *P. ovale*-like, *P. malariae*-like and *P. vivax*-like parasites (Liu et al., 2010). Moreover, these previous studies were done using faecal

samples, which most likely have a substantially lower parasite burden than the blood samples analysed here.

Greater spot-nosed monkeys inhabit a wide range across western central Africa (Groves 2001). The pet monkey previously reported to be infected with a *P. falciparum*-like parasite was from Gabon. Our sample sites were all in Cameroon, but many were in the south of the country, where it borders Gabon. The mosquito vectors that transmit apicomplexan parasites between monkeys are not known and therefore their potential to transmit parasites between monkeys in Gabon and Cameroon cannot be estimated. However, for *Laverania* infections of wild apes, where the vectors are also unknown, it is clear that such distances and (unsurprisingly) political boundaries do not interrupt the flow of malarial parasites (Liu et al., 2010). Therefore, it is highly unlikely that wild *C. nictitans* in Gabon have a higher prevalence of infection with *P. falciparum*-like parasites than their conspecifics in Cameroon. Also, while prevalence of *Plasmodium* infections may vary across seasons, our samples were collected throughout the year. Thus, we conclude that *P. falciparum*-like infections must be extremely rare, or more likely absent, in wild greater spot-nosed monkeys. For example, if the individuals we sampled are representative, and the true prevalence (as detectable by our methods) was only 1%, there would still be a 95% probability of observing at least one infected individual among 292, whereas we saw none. Nonetheless, it cannot be excluded that greater spot-nosed monkeys are susceptible to *Laverania* parasites in captivity when exposed to mosquitoes not normally encountered in the wild, which appears to be the case for captive chimpanzees and bonobos (Duval et al., 2010; Krief et al, 2010).

In contrast to a recent proposal (Prugnolle et al., 2011a,b), we therefore conclude that greater spot-nosed monkeys are not natural carriers of *P. falciparum*-like parasites, and that there is no evidence to support the speculation that they could have served as the reservoir from which humans became infected. In contrast, *P. falciparum*-like parasites have been documented in wild gorillas at numerous sites in Cameroon (Fig. 1) and neighbouring countries, and these apes thus appear to have been the original host of *P. falciparum* infecting humans (Liu et al., 2010; Rayner et al., 2011; Sharp et al., 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Wild *Cercopithecus nictitans* from Cameroon were screened for *Plasmodium falciparum*-related parasites.

- *Hepatocystis* spp. were detected in 49% of the samples

- None of the 292 wild *C. nictitans* harbored *P. falciparum*-like parasites.

- Greater spot-nosed monkeys do not represent a natural *P. falciparum* reservoir
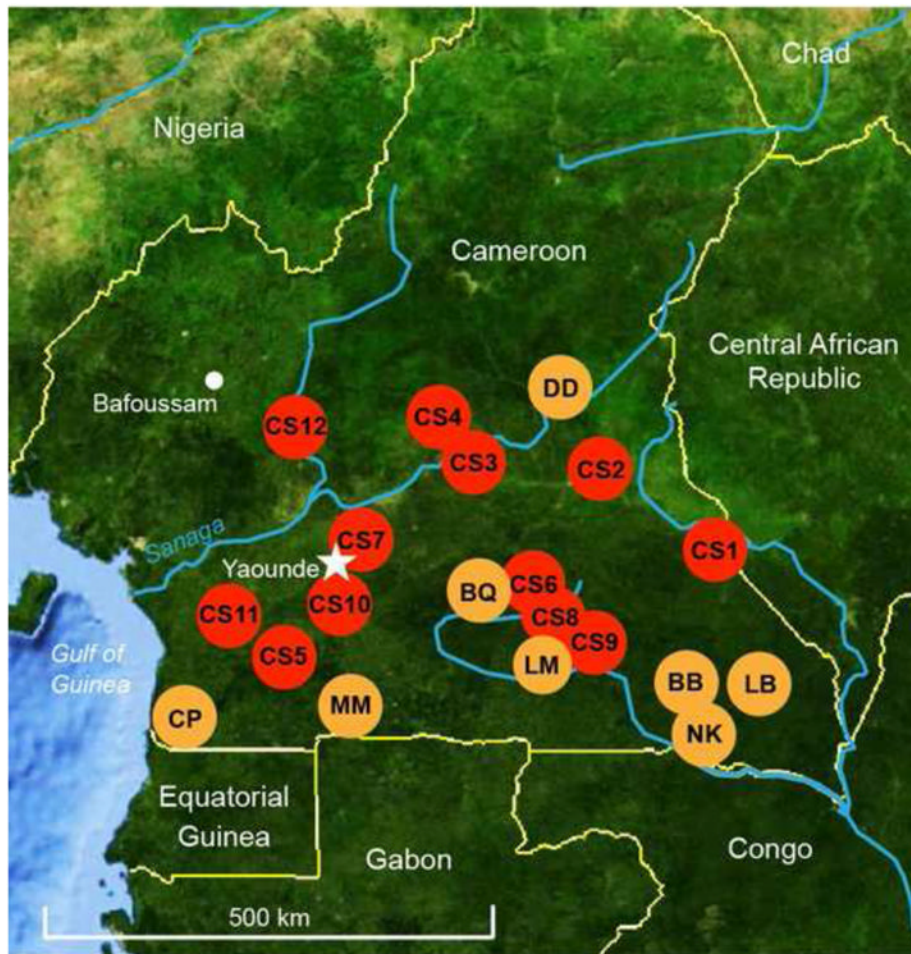
Fig. 1.
Collection sites of blood samples from greater spot-nosed monkeys in Cameroon. Red circles indicate 12 locations (CS1-CS12) where samples were collected from hunted animals. Orange circles denote sites where *Plasmodium falciparum*-like parasites were previously detected in wild gorilla populations (Liu et al., 2010).
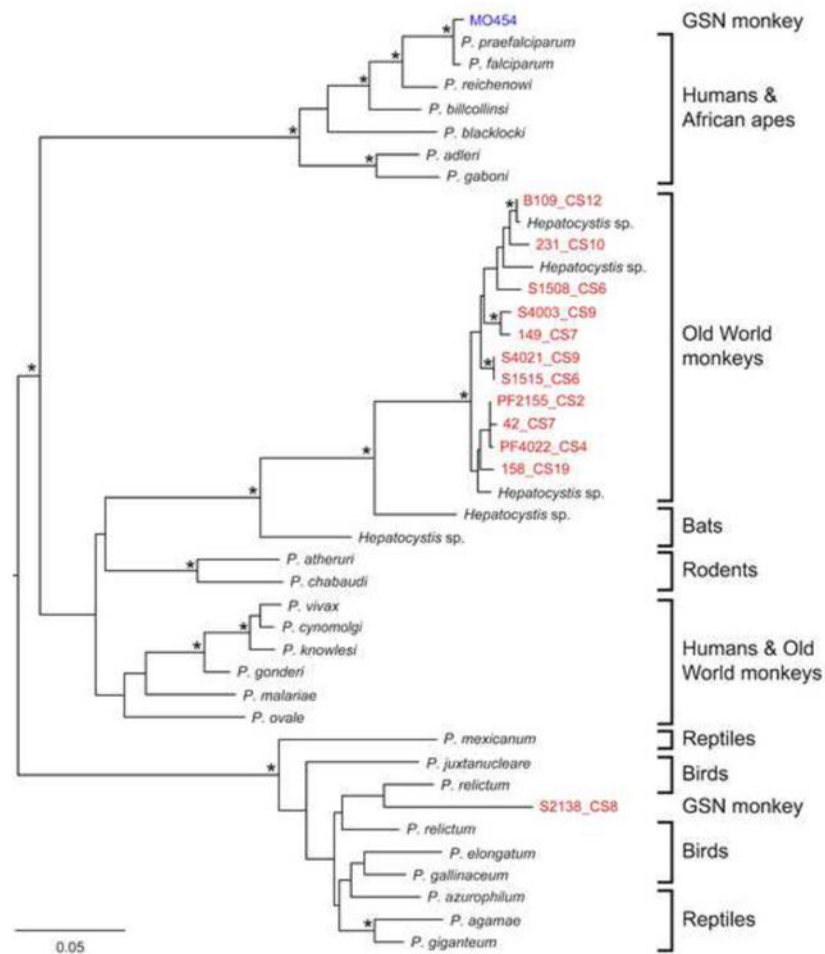
Fig. 2.
Evolutionary relationships of haemosporidian sequences from greater spot-nosed (GSN) monkeys (*Cercopithecus nictitans*). A maximum likelihood tree of mitochondrial *cytb* sequences (726 bp) is shown, with *Plasmodium* and *Hepatocystis* reference sequences in black, newly derived sequences highlighted in red (only a subset of *Hepatocystis* sequences is shown), and one *Plasmodium falciparum*-like sequence (MO454) previously reported from a captive GSN monkey (Prugnolle et al., 2011) in blue. Asterisks on branches indicate bootstrap values ≥ 80%; the scale bar represents 0.05 nucleotide substitutions per site. GenBank accession numbers for all sequences are listed in Supplementary Table S1.

Fig. 3.

Evolutionary relationships of *Hepatocystis* parasites. A maximum likelihood tree of mitochondrial *cytb* sequences (201 bp) is shown. *Hepatocystis* reference sequences are shown in black, with their respective host species indicated in parentheses; newly derived sequences from greater spot-nosed monkey samples are highlighted in red (the number of sequences with the same haplotype is shown in parentheses). Asterisks on branches indicate bootstrap support values 70%; the scale bar represents 0.02 nucleotide substitutions per site. GenBank accession numbers for all sequences are listed in Supplementary Table S1.

Table 1

Numbers and percentages of *cytb* positive samples from greater spot-nosed monkeys (*Cercopithecus nictitans*) at 12 different field sites in Cameroon.

Site	Geographic area	*n* tested	*n* (%) positive for *cytb* PCR
CS1	Gribe	2	0 (0.0%)
CS2	Bertoua	4	2 (50.0%)
CS3	Nanga Eboko	2	2 (100%)
CS4	North Sanaga	1	1 (100%)
CS5	Ebolowa	40	10 (25.0%)
CS6	Eboumetoum	36	20 (55.6%)
CS7	Yaoundé	49	29 (59.2%)
CS8	Mindourou	99	55 (55.6%)
CS9	Messok	30	11 (36.7%)
CS10	Nditam	19	9 (47.4%)
CS11	Mbalmayo	2	0 (0.0%)
CS12	Bipindi	8	5 (62.5%)
Total		292	144 (49.2%)