EBioMedicine 2 (2015) 1186–1192

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Natural infection of *Plasmodium brasilianum* in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon

Albert Lalremruata [a], Magda Magris [b], Sarai Vivas-Martínez [b,d], Maike Koehler [a], Meral Esen [a], Prakasha Kempaiah [c], Sankarganesh Jeyaraj [a], Douglas Jay Perkins [c], Benjamin Mordmüller [a], Wolfram G. Metzger [a,b,*]

[a] Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany
[b] Servicio Autónomo Centro Amazónico para la Investigación y Control de Enfermedades Tropicales 'Simón Bolívar' (SACAICET), Puerto Ayacucho, Estado Amazonas, Venezuela
[c] Center for Global Health, Department of Internal Medicine, University of New Mexico School of Medicine, Albuquerque, NM, USA
[d] Cátedra de Salud Pública, Escuela de Medicina Luis Razetti, Universidad Central de Venezuela, Caracas, Venezuela

ARTICLE INFO

Article history:
Received 5 May 2015
Received in revised form 15 July 2015
Accepted 24 July 2015
Available online 29 July 2015

Keywords:
Quartan malaria
Yanomami
Venezuela
Plasmodium malariae
Plasmodium brasilianum
New World monkey
Anthropozoonosis
Anthroponosis
Zoonosis
Circumsporozoite protein
CSP
Small subunit ribosomal RNA
18S rRNA
Polymerase change reaction
Sequencing
PCR

ABSTRACT

Background: The quartan malaria parasite *Plasmodium malariae* is the widest spread and best adapted human malaria parasite. The simian *Plasmodium brasilianum* causes quartan fever in New World monkeys and resembles *P. malariae* morphologically. Since the genetics of the two parasites are nearly identical, differing only in a range of mutations expected within a species, it has long been speculated that the two are the same. However, no naturally acquired infection with parasites termed as *P. brasilianum* has been found in humans until now.
Methods: We investigated malaria cases from remote Yanomami indigenous communities of the Venezuelan Amazon and analyzed the genes coding for the circumsporozoite protein (CSP) and the small subunit of ribosomes (18S) by species-specific PCR and capillary based-DNA sequencing.
Findings: Based on 18S rRNA gene sequencing, we identified 12 patients harboring malaria parasites which were 100% identical with *P. brasilianum* isolated from the monkey, *Alouatta seniculus*. Translated amino acid sequences of the CS protein gene showed identical immunodominant repeat units between quartan malaria parasites isolated from both humans and monkeys.
Interpretation: This study reports, for the first time, naturally acquired infections in humans with parasites termed as *P. brasilianum*. We conclude that quartan malaria parasites are easily exchanged between humans and monkeys in Latin America. We hypothesize a lack of host specificity in mammalian hosts and consider quartan malaria to be a true anthropozoonosis. Since the name *P. brasilianum* suggests a malaria species distinct from *P. malariae*, we propose that *P. brasilianum* should have a nomenclatorial revision in case further research confirms our findings. The expansive reservoir of mammalian hosts discriminates quartan malaria from other *Plasmodium* spp. and requires particular research efforts.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Since malaria eradication is on the global health agenda again, non-human primates as source for *Plasmodium* infections in humans have received increased attention (Ramasamy, 2014). In this context, the simian *Plasmodium brasilianum* is particularly interesting. In 1908, a quartan malaria parasite was identified by Gonder and von Berenberg-Gossler in an imported 'bald-headed uakari' (*Cacajao calvus*) and named *P. brasilianum*, the quartan malaria parasite of New World monkeys in Latin America (Gonder and Von Berenberg-Gossler, 1908). *P. brasilianum* resembles the human quartan parasite *Plasmodium malariae* under the microscope, but early cross-species experimental infections by subcutaneous transfer of parasitized blood from black spider monkeys in the 1930s were unsuccessful. Hence, the names of two distinct parasites were maintained (Coatney et al., 2003).

Later investigations in the 1960s demonstrated that humans could very well be experimentally infected with *P. brasilianum* from monkeys, and, vice versa, New World monkeys could be experimentally infected with *P. malariae* from humans (Coatney et al., 2003; Geiman and Siddiqui, 1969). Moreover, studies in the 1980s showed that monoclonal antibodies against the circumsporozoite protein (CSP) of *P. malariae* cross-reacted and even neutralized the infectivity of

* Corresponding author at: Institute of Tropical Medicine, University of Tübingen, Wilhelmstrasse 27, D-72074 Tübingen, Germany.
E-mail address: wolfram.metzger@uni-tuebingen.de (W.G. Metzger).

http://dx.doi.org/10.1016/j.ebiom.2015.07.033
2352-3964/© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

P. brasilianum sporozoites to monkeys and vice versa (Cochrane et al., 1985). Sequencing of the gene coding for CSP confirmed the identity of this otherwise species-specific epitope in the two parasites (Lal et al., 1988).

Another common tool for the molecular species identification of malaria parasites is the gene for the small subunit (18S) of ribosomes (Snounou et al., 1993). In 1999, Fandeur et al. analyzed the 18S gene sequences from quartan malaria parasites (*P. brasilianum*) found in four monkey species from French Guiana with the highest prevalence in *Alouatta* monkeys (Fandeur et al., 2000). The similarity between 18S sequences from *P. brasilianum* and *P. malariae* is more than 99% differing only in single nucleotide polymorphisms (SNPs). SNPs are distributed at random like in the genetic pool of one single species, and no distinctive marker has been identified so far.

Unlike other human *Plasmodium* species, no whole genome sequence is available for quartan malaria parasites. We reviewed other published gene targets (msp-1, dhfr, cytochrome b, microsatellite DNA markers) and found striking homologies in all the markers without any specific identifying SNPs between the two parasite types (Fandeur et al., 2000; Guimarães et al., 2012; Tanomsing et al., 2007). The two parasites are nowadays perceived as variants of the same species, which had specialized on different hosts. Or, in practical terms, when quartan malaria parasites were identified in monkeys, they were designated as *P. brasilianum*. Conversely, when quartan malaria parasites were detected in humans, they were classified as *P. malariae*. Thus, the infected host determined the *Plasmodium* designation.

At the time of writing, altogether thirteen 18S sequences of *P. brasilianum* and thirty-four 18S sequences of *P. malariae* were registered in the NCBI GenBank nucleotide database. Sero-epidemiological studies in Brazil and French Guyana already suggested that non-human primates might constitute a natural reservoir for human malaria, and may contribute to the maintenance of foci for *P. malariae* (Volney et al., 2002). However, as naturally-acquired infections with parasites termed as *P. brasilianum* were never described in humans (Baird, 2009), the idea of host specificity was upheld and the classification of *P. brasilianum* as an independent *Plasmodium* species was retained.

Amazonas, the most southern federal state of Venezuela, is bordering Colombia to the west and Brazil to the east. Half of the population belongs to one of eighteen indigenous ethnic groups with the Yanomami representing one of the largest Amerindian communities. These seminomadic Indians live on both sides of the frontier between Venezuela and Brazil. On the Venezuelan side, about 12,000 Yanomami inhabit the vast forest area where the Orinoco originates and the Casiquiare river bifurcates towards the southern Amazon (Metzger et al., 2008; Humboldt, 1812).

In a traditional Yanomami village, all persons live under one common roof, *shabono*, consisting of a circular open wooden construction that accommodates up to 400 people. Daily life takes place “open air” and the night is spent in hammocks. Many *shabonos* are difficult to reach and are several days walking distance to the nearest health post. It is estimated that around 5000 Yanomami have retreated into the deep jungle with little or no contact to Western culture (Metzger et al., 2008). As forest-dwelling people, Yanomami hunt monkeys as a food source and incorporate them as household's pets.

Overall, *P. vivax* is predominant in Amazonas with roughly 85% of all detected parasites, but the distribution pattern of the malaria species is variable and contingent upon the geographic settlement of the ethnic groups. For example, a pilot study conducted in Yanomami communities from the Upper Orinoco revealed that nearly half of the malaria positive samples were *P. malariae* (Metzger et al., 2008). In contrast, no infections caused by *P. malariae* were detected among indigenous Piaroa from the Middle Orinoco basin (Rodulfo et al., 2007). Interestingly, Yanomami communities have also the highest *P. falciparum* rates (40.3%) compared to other ethnic groups in the region (8.7–22.4%) (Metzger et al., 2009).

The current study was carried out to identify and characterize *Plasmodium* species in the Venezuelan Amazon. Specifically, we investigated quartan malaria cases in Yanomami communities living in remote areas of the Alto Orinoco Casiquiare Biosphere Reserve where humans and non-humans live in such close vicinity that they could be concurrent reservoirs of transmission.

2. Materials and methods

2.1. Samples

Samples for this study originate from surveys in the Yanomami communities of Ocamo, Mavaca, Koyowe, and Platanal situated in the Upper Orinoco area near the Brazilian border, which were carried out as part of governmental malaria and onchocerciasis control activities in the region between 2005 and 2007. Ethical approval was obtained by the Ethical Committees of the *Servicio Autonomo Centro Amazónico de Investigación y Control de Enfermedades Tropicales 'Simon Bolivar'* (SA-CAICET), Puerto Ayacucho, Venezuela, and the London School of Hygiene & Tropical Medicine, London (LSHTM), UK.

When the team—consisting of medical doctors, scientists and health workers—arrived in a *shabono*, people were invited for a gathering. Malaria control and research activities were explained with the help of translators. Special importance was given to the presence of elders and leaders of the community. Informed consent was obtained orally. All individuals who felt sick were examined, diagnosed and treated for malaria, or the respective disease, according to the guidelines of the Venezuelan Health Ministry (Metzger et al., 2008). The ages of the patients were estimated as the Yanomami have no counting system.

Thick and thin blood smears were taken from individuals who presented with a history of fever and/or headache and/or malaise. Blood samples were collected by finger prick and stored on filter papers. 633 samples were used for the retrospective screening to investigate the molecular genetics of *P. malariae* parasites.

2.2. DNA extraction and PCR diagnosis

Parasite DNA from field samples was extracted from dried blood spots on filter paper using a commercial extraction kit (QIAamp DNA Blood Mini Kit, Qiagen). Screening for *Plasmodium* spp. infection was carried out by conventional nested-PCR assay with genus and species-specific primers based on the small subunit ribosomal RNA genes (18S) described previously by Snounou et al. (1993).

Genomic DNA of the *P. brasilianum* Peruvian III strain (MR4-349) was obtained from the Malaria Research and Reference Resource Center (MR4) to generate reference sequences for analyses at the University of New Mexico School of Medicine, Albuquerque, NM, USA.

2.3. 18S: development of new primers and sequencing

Though a *P. malariae*-specific PCR assay by Snounou et al. (1993) is sensitive and typically employed in the differential diagnosis of species, the 145 bp product of this primer set is too short for extensive sequence analysis. Therefore, we designed new sequencing primers targeting an amplicon spanning the entire variable region 5 (V5), one of the eight highly variable regions in the 18S gene which has considerable sequence variations among *Plasmodium* species. In addition we amplified three variable domains (V4, V5, V7) from the genomic DNA of *P. brasilianum* Peruvian III strain obtained from MR4.

Primer sequences were selected from unique and common regions for *P. malariae* and *P. brasilianum* species. The first primer pair (Pm18S Outer-F and Pm18S Outer-R) amplifies an 808-bp fragment. The second set of nested primers (Pm18S Inner-F and Pm18S Outer-R) amplifies a 763-bp fragment. All amplified samples were purified using Exo-SAP It Kit (USB) and sequenced bi-directionally using forward and reverse primers. Details of primer sequences, PCR amplification, and sequencing methods are shown in the appendix.

2.4. CSP: primers and sequencing

For CSP gene amplification, primers were used from the conserved regions flanking the central repeat region that contains immunodominant epitopes. The amplified CSP gene fragment from each isolate was purified using Exo-Sap It kit (USB), cloned using a TOPO TA cloning kit (Invitrogen), and transformed into TOP10 competent *Escherichia coli* cells (Invitrogen). We sequenced at least 3 clones from each isolate using M13 primers and gene specific internal primers. Details of the methods are given in the appendix.

2.5. Sequence analysis of 18S and CSP gene

For the 18S gene, sequences were aligned and edited using the Vector NTI ContigExpress program version 10 (Invitrogen). Nucleotide sequences generated from each isolate were queried against the NCBI GenBank nucleotide database using BLASTN for similarity search. Out of the total 47 published sequences of *P. malariae* and *P. brasilianum*, 23 sequences overlapped with the amplified target. The remaining 24 sequences were amplified from regions different from Snounou et al. primer site. Detailed information about source, origin, and accession number of all published 18S gene sequences is listed in the supplementary (appendix). A *Plasmodium* phylogenetic tree based on the 18S gene was constructed using the Kimura 3-parameter implemented in MEGA software ver. 6.0 (Tamura et al., 2013). *Theileria* sp. (GenBank Ac. AF162432) was used as outgroup. The reliability of the tree was assessed by the bootstrap significance test with 1000 replications. The final tree was refined using the program FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The nucleotide diversities were calculated using DnaSP (Librado and Rozas, 2009). The maximum likelihood genetic distances based on Kimura 3-parameter, modeled with gamma distribution (shape parameter = 0.21) were calculated with MEGA ver. 6.0. For distance calculation, the best suited maximum likelihood model was obtained by using Modeltest implemented in MEGA ver.6.0. The comparable 18S gene (A-type) sequences of other *Plasmodium* spp. for the analyses were obtained from NCBI GenBank and listed in the appendix. The sequence alignments that were used to infer phylogenetic relationships are available from the authors on request.

For the CSP gene, each plasmid sequence containing the CSP gene fragment was aligned using the Vector NTI ContigExpress program version 10 (Invitrogen). The edited sequences of the CSP gene from each isolate were translated to corresponding amino acid sequences using the ExPASy Translate tool (http://web.expasy.org/translate/). Deduced amino acid sequences were compared to CS protein sequences of *P. malariae* and *P. brasilianum* isolates available in the database.

3. Results

3.1. Diagnosis of P. malariae infection

The determination of malaria by conventional nested-PCR detected the presence of *P. malariae* DNA in 75 of 633 samples collected from different individuals in Yanomami villages, constituting an 11.8% carrier rate in this survey. 25 of 75 samples (33%) were co-infected with *P. vivax* (n = 7), *P. falciparum* (n = 12), or triple infections (n = 6) while the remaining 50 had mono-infections with quartan malaria parasites.

3.2. Differentiating P. brasilianum from P. malariae

Out of the 75 samples PCR-positive for *P. malariae*, the 18S gene from 33 samples (27 mono-infections and 6 mixed infections) was successfully amplified and the resulting 763 bp product was analyzed by sequencing. Upon sequence analysis, 12 of the 33 samples had 18S gene sequences that were 100% identical with a *P. brasilianum* strain (GenBank AF130735) isolated from an infected monkey (*Alouatta seniculus*) in French Guiana (Fandeur et al., 2000). The twelve *P. brasilianum* infected individuals were from five different shabonos. The estimated ages of the patients were from 6 to 60 years.

In addition, isolates from four patients (n = 4) were 100% identical with the *P. malariae* Myanmar strain 1 (GenBank AF487999); six isolates (n = 6) were 100% identical with the *P. malariae* Myanmar strain 2 (GenBank AF488000); and one isolate (n = 1) was 100% identical with the *P. malariae* PNG strain (GenBank AF145336). The remaining ten isolates (n = 10) were 99% identical to either *P. malariae* Myanmar strain 2 or *P. malariae* PNG strain. Variation in the ten samples was represented by nucleotide polymorphisms (i.e., substitutions, insertions and/or deletions). Among these ten isolates, four new variants were identified (appendix).

Alignment of the twenty-three published 18S sequences and newly generated sequences of quartan malaria parasites showed 27 polymorphic sites in the 496-nucleotide sequences spanning V5 region. The overall nucleotide diversity (Pi) was 6.5×10^{-3} (SD 0.001), with an average number of nucleotide differences (k) of 2.935.

Table 1 shows the extent of divergence in the genetic distances between *Plasmodium* species, as calculated from the 18S gene spanning variable domain 5. Although the quartan parasites were genetically distinct from the other *Plasmodium* species, there was no genetic differentiation between the *P. malariae* and *P. brasilianum* isolates (distance, d = 0.005). The average genetic distance between all quartan isolates from human and monkey is comparable to intra-species genetic distance in other *Plasmodium* spp. (Table 1).

The phylogenetic analysis by the Neighbor joining method confirmed the similarity of all quartan malaria parasites (irrespective of the source of isolation, *P. brasilianum* or *P. malariae*) by clustering into a single monophyletic clade with a high bootstrap support of 100% (Fig. 1).

No identifying SNPs specific to either *P. malariae* or *P. brasilianum* was identified in the gene locus conventionally employed for differential diagnosis of malaria species. For example, all published *P. brasilianum* sequences isolated from monkeys and the twelve *P. brasilianum* sequences isolated from humans and sequenced in this study displayed G at position 565. However, the *P. brasilianum* isolate from Peru (MR4) sequenced in this study displayed an A, and three recently sequenced *P. malariae* isolates from humans in Costa Rica displayed a G at position 565 (Table 2).

The new sequences identified in this study were submitted to the NCBI GenBank with accession numbers KJ619941–KJ619947 and KM016331–KM016338.

3.3. Analysis of the CSP gene

The central immunodominant repeat region of the CSP gene was amplified and sequenced from three isolates, each representing *P. brasilianum*, *P. malariae* Myanmar strain 1, and *P. malariae* Myanmar strain 2, identified by 18S gene sequencing. Translated amino acid sequences showed that all three isolates constituted the minor tandem tetrapeptide repeat unit NDAG (N, asparagine; D, aspartic acid; A, alanine; G, glycine) and the major unit NAAG (N, asparagine; A, alanine; G, glycine) varying only by the number of repeat units.

All published isolates from South America, including *P. brasilianum* and *P. malariae*, from this and other studies, started the repeat region with the tetrapeptide NDEG, which is similar to *P. malariae* isolates from Asia. In contrast, African isolates from Uganda, Cameroon, and Cote d'Ivoire started the repeat region with NDAG (Table 3).

4. Discussion

All quartan malaria parasites analyzed in this study would be *P. malariae*, if they had been found fifteen years earlier. However, as Fandeur et al. (2000) detected some 100% identical strains in monkeys in 1999, twelve of the 33 parasites had to be named *P. brasilianum*.

Table 1
Estimates of the average genetic distance between and within *Plasmodium* spp.
The table shows average genetic distances based on variable domain 5 (V5) of the 18S gene sequences between (black) and within (red) *Plasmodium* spp. Standard errors (SE) for interspecies values (black) are shown above the diagonal (blue). The number of sequences (n) in each group of *Plasmodium* spp. is indicated in the second column. All V5 sequences generated in this study (including the MR4 sequence) and all comparable sequences from GenBank were included into the analysis. Sequences for *P. malariae* and *P. brasilianum* are listed in the supplementary appendix. Data show that genetic divergence between *P. brasilianum* and *P. malariae* is not more than within a species (gray boxes). Pmal, *P. malariae*; Pbra, *P. brasilianum*; Pfal, *P. falciparum*; Pviv, *P. vivax*; Pkno, *P. knowlesi*; Pcyn, *P. cynomolgi*; Poc, *P. ovale curtisi*; Pow, *P. ovale wallikeri*.

Species	n	Pbra	Pmal	Pfal	Pviv	Pcyn	Pkno	Poc	Pow
Pbra	14	0.004	0.002	0.011	0.017	0.018	0.020	0.017	0.017
Pmal	18	0.007	0.008	0.011	0.016	0.018	0.020	0.017	0.016
Pfal	8	0.043	0.043	0.001	0.017	0.018	0.019	0.016	0.015
Pviv	11	0.071	0.069	0.073	0.002	0.002	0.005	0.011	0.011
Pcyn	5	0.077	0.076	0.077	0.004	0.002	0.006	0.011	0.011
Pkno	19	0.093	0.091	0.080	0.012	0.017	0.004	0.013	0.013
Poc	10	0.077	0.076	0.068	0.040	0.044	0.054	0.012	0.005
Pow	19	0.071	0.070	0.059	0.039	0.042	0.053	0.017	0.009

Based on phylogenetic analysis standards, *P. malariae* and *P. brasilianum* are one species (Fig. 1), and the punctual differences are not more than differences between strains of other *Plasmodium* species (Tables 1 and 2). So far, the host made the difference, as the infected host has been the classification determinant for *P. malariae* and *P. brasilianum*. Our findings show that this distinction criterion no longer applies.

Malaria history surmises that 500 years ago, Old World Humans introduced *P. malariae* to the New World; some of the parasites crossed the species barrier, adapted to New World monkeys and became *P. brasilianum*, the simian quartan malaria parasite of Latin America (Coatney et al., 2003). This hypothesis was challenged later; based on genetic diversity assumptions, it was reasoned that quartan parasites jumped from monkey to man and became human *P. malariae* (Tazi and Ayala, 2011). Though directions of the cross-species transfer are opposing, the two hypotheses share the common element of "host switching" which implies host specificity of *P. brasilianum* and *P. malariae* to monkey and man, respectively.

Our results allow an alternative view. For the first time, quartan malaria parasites, which are identical to those found in naturally infected primates of Latin America, were detected in naturally infected humans. Thus, our results provide evidence that quartan parasites are able to cross host species boundaries with impunity and that humans and non-human primates—in conditions of close contact—share quartan parasites without host specificity. Moreover, it can be speculated that *P. brasilianum* and *P. malariae* neither are distinct species, as the name suggests, nor are they distinct variants of one species, which became specialized on different hosts after switching, but are rather the same quartan malaria parasite species—an anthropozoonosis—circulating freely between monkeys and humans.

This confirmation of host sharing characteristics has long been pending Coatney, 1971; Escalante et al., 1995 and might give a reason to re-appraise quartan malaria, a largely neglected disease thus far, with the qualities to become an emerging infection.

In 1890, *P. malariae* was the first malaria species to get a scientific name (Collins and Jeffery, 2007). To date, the causative agent of quartan malaria has been largely understudied, mainly because—due to low levels of parasitaemia—in most epidemiological surveys only a few infections were detected (Mueller et al., 2007). Nonetheless, in studies with improved detection limits, *P. malariae* has been demonstrated in all malaria-endemic regions of the world Autino et al., 2012 and therefore—strictly speaking—might be the malaria parasite with the widest geographical distribution.

Furthermore, quartan malaria parasites might represent the best adapted malaria parasites. The adaption of these protozoans to its hosts results in a mainly chronic clinical outcome with many carriers suffering no symptoms. It is well-known that quartan parasites can persist in dormancy for decades in the host without causing symptoms (Collins and Jeffery, 2007). For example, two chimpanzees, Takaboh and

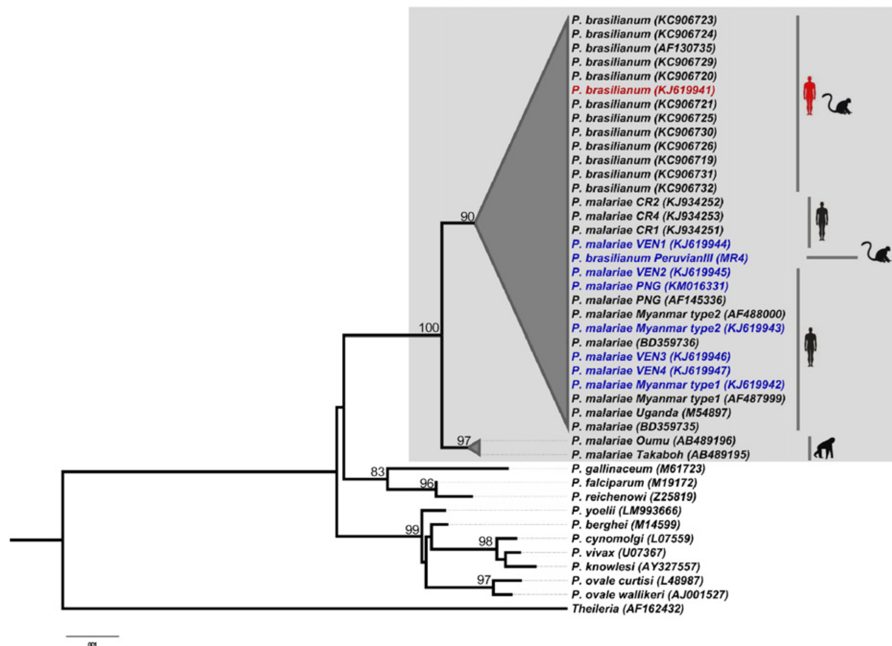

Fig. 1. Neighbor Joining Tree based on 18S gene sequences of *Plasmodium* species. The tree shows that all quartan malaria parasites from humans and monkeys cluster into a monophyletic clade supported by a high bootstrap value of 99%. *P. brasilianum* and *P. malariae* sequences from this study are shown in color (red and blue). Hosts (non-human primate, human) are indicated by graphic symbols beside the taxa names. The sequence highlighted in red was found by Fandeur et al. in Alouatta monkeys, and in this study in humans. The two additional *P. malariae* isolates from human infections from Bangladesh (GenBank Ac. KF906514 & GenBank Ac. KF906514); Fuehrer et al. showed that these two isolates were 100% identical with a *P. malariae*-like isolate from the Chimpanzee Takaboh (GenBank Ac. AB489195).

Table 2
Nucleotide polymorphisms in 18S gene sequences of *P. brasilianum* and *P. malariae* isolates.
The table shows that no distinctive marker for *P. brasilianum* could be identified. The SNP positions given vertically above are numbered according to the nucleotide sequences of *P. brasilianum* (GenBank AF130735). Dots represent identical residues; dashes represent deletions. *P. brasilianum* and *P. malariae* sequences from this study are shown in red and blue, respectively. The numbers in brackets denote the number of identical isolates found in this study. Apart of the four *P. malariae* strains VEN1, VEN2, VEN3, and VEN4, all isolates identified in this study are identical to pre-existing *P. malariae*/*P. brasilianum* sequences.

		Nucleotide positions based on GenBank accession AF130735																											
Isolates	GenBank accession	319	338	384	393	419	425	428	441	444	461	512	550	565	566	568	573	577	578	585	586	596	608	673	679	680	691	696	728
P. brasilianum	AF130735	T	T	T	A	A	G	A	T	C	A	T	A	G	G	C	A	A	T	G	T	A	A	A	T	T	C	C	A
P. brasilianum (n = 12)	KJ619941	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906719	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
P. brasilianum	KC906720	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906721	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906723	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906724	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906725	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906726	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906729	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906730	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. brasilianum	KC906731	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.
P. brasilianum	KC906732	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	T
P. brasilianum Peruvian III (n = 1) (MR4)	KT266778	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae CR1	KJ934251	.	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae CR2	KJ934252	.	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae CR4	KJ934253	.	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Myanmar type1	AF487999	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Myanmar type1 (n = 4)	KJ619942	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Myanmar type2	AF488000	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Myanmar type2 (n = 6)	KJ619943	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae PNG	AF145336	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae PNG (n = 1)	KM016331	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae VEN1 (n = 3)	KJ619944	.	.	C	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.
P. malariae VEN2 (n = 2)	KJ619945	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae VEN3 (n = 1)	KJ619946	.	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae VEN4 (n = 4)	KJ619947	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Uganda-I	M54897	G	.	.	.	.	.	.	A	.	.	.	T	A	.	.	T	.	.	.	.	.	.	.	C	C	G	A	.
P. malariae	BD359736	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae	BD359735	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
P. malariae Takaboh	AB489195	.	.	.	.	.	.	.	.	.	.	.	.	A	A	T	.	T	A	A	.	.	.	.	.	.	.	.	.
P. malariae Oumu	AB489196	.	.	.	.	.	.	.	.	.	.	.	.	A	A	T	.	T	A	A	A	.	.	.	.	.	.	.	.

Oumu, acquired quartan *P. malariae*-like parasites in the African rain forest when they were babies, and the parasites remained undiscovered until detection 30 years after their first day of confinement in a Japanese zoo (Hayakawa et al., 2009). This is perplexing because "hypnozoites"—as in *P. vivax* and *P. ovale*—have never been discovered or have not been well investigated.

What discriminates quartan malaria parasites from other *Plasmodium* spp. is their expansive reservoir of mammalian hosts. Besides the human

Table 3
Comparison and characterization of the circumsporozoite protein (CSP) central repeat region among quartan malaria species.
The table shows that the otherwise species-specific immunodominant repeat region of the CS protein is the same for all quartan malaria parasites. It consists of the major repeat unit NAAG and the minor repeat unit NDAG. Numbers of the repeat unit can change between isolates; this has been shown for all *Plasmodium* spp. Clones of PCR amplified CSP alleles from three isolates are displayed each representing *P. brasilianum*, *P. malariae* Myanmar strain 1, and *P. malariae* Myanmar strain 2, identified by 18S gene sequencing in this study. African isolates start with a NDAG unit, whereas isolates from Asia and South America (*P. malariae* and *P. brasilianum*) start with NDEG.

Central immunodominant repeat units								
Protein ID	Species (strain)	Origins (authors)	Repeat start	Minor unit	No.	Major unit	No.	Repeat size
KM016332	*P. brasilianum*	Venezuela (this study)	NDEG	NDAG	4	NAAG	50	54
KM016333	*P. brasilianum*	Venezuela (this study)	NDEG	NDAG	4	NAAG	50	54
KM016334	*P. brasilianum*	Venezuela (this study)	NDEG	NDAG	4	NAAG	50	54
KM016337	*P. malariae* (Myanmar type 1)	Venezuela (this study)	NDEG	NDAG	4	NAAG	50	54
KM016338	*P. malariae* (Myanmar type 1)	Venezuela (this study)	NDEG	NDAG	4	NAAG	49	53
KM016335	*P. malariae* (Myanmar type 2)	Venezuela (this study)	NDEG	NDAG	5	NAAG	50	55
KM016336	*P. malariae* (Myanmar type 2)	Venezuela (this study)	NDEG	NDAG	5	NAAG	51	56
AAA29553	*P. brasilianum*	Unknown (Lal et al., 1988)	NDEG	NDAG	5	NAAG	58	63
AGO33295	*P. brasilianum*	Brazil (Araújo et.al. 2013)	NDEG	NDAG	2	NAAG	21	23
AAA18618	*P. malariae* (China-1 CDC)	China (Qari et.al. 1994)	NDEG	NDAG	7	NAAG	46	53
AAA29557	*P. malariae* (Uganda-1 CDC)	Uganda (Lal et al., 1988)	NDAG	NDAG	6	NAAG	45	51
CAA04809	*P. malariae*	Cameroon (Tahar et al., 1998)	NDAG	NDAG	7	NAAG	44	51
CAA04812	*P. malariae*	Cote d'Ivoire (Tahar et al., 1998)	NDAG	NDAG	6	NAAG	46	52

host, there have been studies showing quartan malaria parasites in several dozens of monkey species. Merely in South America, quartan parasites were described in 35 monkey species (as *P. brasilianum*) (Fandeur et al., 2000). In Asia, monkeys are infected by the quartan *Plasmodium inui* (Coatney et al., 2003), and in the African rain forest, great apes are infected by quartan parasites (as *Plasmodium rodhaini* or *P. malariae*, and *P. malariae*-like) (Ramasamy, 2014). To our knowledge, this is by far the widest host reservoir compared with malaria of other periodicity.

Clinically, quartan malaria is considered as relatively harmless, but sufficient data are lacking to substantiate this assessment. Many investigators assume that *P. malariae* is the causal factor—perhaps with co-factors—for renal pathologies (Ehrich and Eke, 2007). Generally, it can be assumed that the clinical outcome of *P. malariae* infections is misinterpreted (McKenzie et al., 2001). Due to the chronic nature of the infection, capturing the true burden of the disease would require large longitudinal studies to assess the impact of the infection on occupational and social life of an individual.

Globally, the disease burden of quartan malaria is difficult to assess, because data on the incidence of *P. malariae* are faulty. The main reason is that *P. malariae* is principally underdiagnosed, because it thrives with a few hard-to-detect parasites, which are indistinguishable from *P. vivax* on the thick blood smear and which can also be misinterpreted as *P. falciparum*. Therefore, time-consuming reading of a thin smear or molecular methods would be necessary to identify this species. This explains why in the 1980s the Brazilian Health Ministry had “eradicated” *P. malariae* from Brazil by simply switching the official method of diagnosis from thin to thick blood smear (Oliveira-Ferreira et al., 2010).

In areas with marked variation in seasonal climate, *P. malariae* may account for 50% of the malaria episodes during the low-transmission season (Greenwood et al., 1987). As *P. malariae* is commonly found in sympatry with other *Plasmodium* species of humans, better understanding of species-interaction is necessary. Especially the interactions with *P. falciparum* in mixed infections is a controversially discussed topic (Mueller et al., 2007). Recent seroepidemiological and biomolecular surveys indicated that the prevalence of *P. malariae* are underestimated Mueller et al., 2007; Autino et al., 2012 and high prevalences of *P. malariae* have been reported from Africa (Doderer-Lang et al., 2014), Asia (Bharti et al., 2013), and Latin America (Volney et al., 2002). These findings correspond well with results of our pilot study of the Upper Orinoco Metzger et al., 2008 which prompted us to undertake the current investigation.

Initially, we hypothesized that *P. brasilianum* and *P. malariae* might be discernible by 18S gene sequencing since we noticed that the SNP at position 565 was shared by all hitherto published *P. brasilianum* isolates. Consequently, we identified this SNP as a possible distinctive marker. However, when we sequenced the *P. brasilianum* Peruvian III strain (provided by MR4), our hypothesis was rebutted. It was rebutted a second time, when three *P. malariae* isolates from humans in Costa Rica were published during the revision process of this article displaying a G at position 565. It appeared that SNPs were distributed at random, and none was specific for *P. malariae* or *P. brasilianum* (see Table 2).

To further ensure what was found in the 18S sequence, we analyzed the CS protein sequence, which also is used for species distinction, because the amino acid composition of the CSP gene is an adaptation for eliciting species specific antibody response. For example *P. vivax*, *P. falciparum* and *P. knowlesi* differ considerably in the CSP repeat region: *P. vivax*: DRAGGQPAG, *P. falciparum*: NANP, and *P. knowlesi*: GQPQAQGDGANA (Verra and Hughes, 1999). The CS protein sequence was identical between *P. brasilianum* and *P. malariae*, consistent with results by other investigators (see Table 3) (Escalante et al., 1995; Tahar et al., 1998).

So, unlike the zoonotic parasite *P. knowlesi*, which is genetically different from other *Plasmodium* spp. in several typical marker genes such as CSP and 18S genes (see Table 1) (Escalante et al., 1995; Escalante and Ayala, 1994), *P. brasilianum* is indistinguishable from *P. malariae* and infects the same hosts. And other than the two recently distinguished sympatric *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland et al., 2010), the quartan parasites *P. malariae* and *P. brasilianum* do not segregate into distinct types in all genomic markers used so far (see Fig. 1) (Fandeur et al., 2000; Guimarães et al., 2012; Tanomsing et al., 2007).

It has to be adverted, that logic does not allow demonstration of identity, or non-difference, even if a few whole genome sequences of the parasites were available. Hence, the best approach to show that the parasites are indeed one species would be to do cross-mating experiments. However, if no distinctive markers are known whereby the off-spring can be identified, these experiments are very difficult to design and it is questionable if they are ethically justified.

So far, the evidence of naturally acquired human infections with parasites termed as *P. brasilianum* was a missing piece in the puzzle of the identity of this quartan parasite (Baird, 2009). Based on the results presented here, we conclude that anthropozoonotic transmission of quartan malaria occurs in areas where the habitat of man and monkey overlap. Similar transmission scenarios wit simian participation have been reported in areas outside the Amazonian region, for example in the Atlantic Forest of Brazil (De Pina-Costa et al., 2014).

We wonder if *P. brasilianum* will undergo a nomenclatorial revision, if further research confirms our findings. This would not be the first time that the name of a quartan malaria parasite is revised: In the 1940s, when *P. rodhaini*, the quartan parasite of apes in Africa, was identified to be *P. malariae*, the former name was soon “sinking into synonymy with *P. malariae*” (Coatney et al., 2003) and is not anymore in use today. This was justified just recently: Alignment of the 18S sequences of *P. malariae* from humans in Bangladesh with the parasite sequences of one of the two simian immigrants in the Japanese zoo, Takaboh (GenBank Ac. AB489195), resulted in 100% identity (see also legend of Fig. 1) (Fuehrer et al., 2014).

Malariologists know for some time that simian malaria will play an important role when human malaria eradication is envisaged (Bruce-Chwatt, 1968). As quartan parasites are the only global human malaria parasites successfully infecting multiple mammalian hosts, they could evade control measures that do not account for the animal reservoir. These hidden and possibly emerging parasites could, therefore, represent an important area for future research efforts.

Contributors

WGM, MM, and SVM carried out the primary data collection and supervised field and laboratory work. AL, MK, and PK performed the molecular experiments. BM, ME, SJ, and DJP supervised the laboratory work, facilitated the collaboration and gave overall input. WGM, MM, and AL conceived the study and designed the experiments. WGM and AL did the analysis, drafted and revised the manuscript. All authors interpreted the results, revised and approved the final manuscript.

Declaration of interests

We declare no competing interests.

Funding

The study was funded by WHO (grant No. HQ/05/131401) and the Ministry of Sciences and Technology of Venezuela (grant No. PC125-018) and institutional core funding of the Institute of Tropical Medicine of the University of Tübingen.

Acknowledgement

The study was funded by WHO (grant no. HQ/05/131401), the Ministry of Sciences and Technology of Venezuela (grant no. PC125-018), and institutional core funding of the Institute of Tropical Medicine

of the University of Tübingen. The following reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: MRA-349G, *P. brasilianum* Peruvian III genomic DNA (deposited by William Collins). We acknowledge the support by the Deutsche Forschungsgemeinschaft and Open Access Publishing fund of the University of Tuebingen. We thank the regional health authorities for their logistic support and the Yanomami communities of Ocamo, Mavaca, Koyowe, and Platanal.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.07.033.

References

Araújo, M.S., et al., 2013. Natural Plasmodium infection in monkeys in the state of Rondônia (Brazilian Western Amazon). Malar. J. 12, 180.

Autino, B., Noris, A., Russo, R., Castelli, F., 2012. Epidemiology of malaria in endemic areas. Mediterr. J. Hematol. Infect. Dis. 4, e2012060.

Baird, J.K., 2009. Malaria zoonoses. Travel Med. Infect. Dis. 7, 269–277.

Bharti, P.K., et al., 2013. Emergence of a new focus of *Plasmodium malariae* in forest villages of district Balaghat, Central India: implications for the diagnosis of malaria and its control. Tropical Med. Int. Health TM IH 18, 12–17.

Bruce-Chwatt, L.J., 1968. Malaria zoonosis in relation to malaria eradication. Trop. Geogr. Med. 20, 50–87.

Coatney, G.R., 1971. The simian malarias: zoonoses, anthroponoses, or both? Am. J. Trop. Med. Hyg. 20, 795–803.

Coatney, G.R., Collins, W.E., Warren, M., Contacos, P.G., 2003. CD-ROM. The primate malarias [Original book published 1971]. Division of Parasitic Disease, Producers. Version 1.0. CDC, Atlanta, GA.

Cochrane, A.H., Barnwell, J.W., Collins, W.E., Nussenzweig, R.S., 1985. Monoclonal antibodies produced against sporozoites of the human parasite *Plasmodium malariae* abolish infectivity of sporozoites of the simian parasite *Plasmodium brasilianum*. Infect. Immun. 50, 58–61.

Collins, W.E., Jeffery, G.M., 2007. *Plasmodium malariae*: parasite and disease. Clin. Microbiol. Rev. 20, 579–592.

De Pina-Costa, A., et al., 2014. Malaria in Brazil: what happens outside the Amazonian endemic region. Mem. Inst. Oswaldo Cruz 109, 618–633.

Doderer-Lang, C., et al., 2014. The ears of the African elephant: unexpected high seroprevalence of *Plasmodium ovale* and *Plasmodium malariae* in healthy populations in Western Africa. Malar. J. 13, 240.

Ehrich, J.H.H., Eke, F.U., 2007. Malaria-induced renal damage: facts and myths. Pediatr. Nephrol. Berl. Ger. 22, 626–637.

Escalante, A.A., Ayala, F.J., 1994. Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequences. Proc. Natl. Acad. Sci. U. S. A. 91, 11373–11377.

Escalante, A.A., Barrio, E., Ayala, F.J., 1995. Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene. Mol. Biol. Evol. 12, 616–626.

Fandeur, T., Volney, B., Peneau, C., de Thoisy, B., 2000. Monkeys of the rainforest in French Guiana are natural reservoirs for *P. brasilianum*/*P. malariae* malaria. Parasitology 120 (Pt 1), 11–21.

Fuehrer, H.-P., et al., 2014. High prevalence and genetic diversity of *Plasmodium malariae* and no evidence of *Plasmodium knowlesi* in Bangladesh. Parasitol. Res. 113, 1537–1543.

Geiman, Q.M., Siddiqui, W.A., 1969. Susceptibility of a New World monkey to *Plasmodium malariae* from aman. Am. J. Trop. Med. Hyg. 18, 351–354.

Gonder, R., Von Berenberg-Gossler, 1908. Untersuchungen über malaria-plasmodien der affen. Malar. Int. Arch. Leipzig 1, 47–50.

Greenwood, B.M., et al., 1987. Mortality and morbidity from malaria among children in a rural area of The Gambia, West Africa. Trans. R. Soc. Trop. Med. Hyg. 81, 478–486.

Guimarães, L.O., et al., 2012. The genetic diversity of *Plasmodium malariae* and *Plasmodium brasilianum* from human, simian and mosquito hosts in Brazil. Acta Trop. 124, 27–32.

Hayakawa, T., et al., 2009. Identification of *Plasmodium malariae*, a human malaria parasite, in imported chimpanzees. PLoS One 4, e7412.

Humboldt, A. v, 1812. Über die Verbindung zwischen dem Orinoko und dem Amazonenfluss. Mon. Corresp. Zur Beförd. Erd- Himmels-Kunde 26, S.230–S.235.

Lal, A.A., et al., 1988. Circumsporozoite protein gene from *Plasmodium brasilianum*. Animal reservoirs for human malaria parasites? J. Biol. Chem. 263, 5495–5498.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinforma. Oxf. Engl. 25, 1451–1452.

McKenzie, F.E., Jeffery, G.M., Collins, W.E., 2001. Plasmodium malariae blood-stage dynamics. J. Parasitol. 87, 626–637.

Metzger, W.G., et al., 2008. Malaria diagnosis under field conditions in the Venezuelan Amazon. Trans. R. Soc. Trop. Med. Hyg. 102, 20–24.

Metzger, W.G., et al., 2009. A rapid malaria appraisal in the Venezuelan Amazon. Malar. J. 8, 291.

Mueller, I., Zimmerman, P.A., Reeder, J.C., 2007. *Plasmodium malariae* and *Plasmodium ovale*—the 'bashful' malaria parasites. Trends Parasitol. 23, 278–283.

Oliveira-Ferreira, J., et al., 2010. Malaria in Brazil: an overview. Malar. J. 9, 115.

Qari, S.H., Collins, W.E., Lobel, H.O., Taylor, F., Lal, A.A., 1994. A study of polymorphism in the circumsporozoite protein of human malaria parasites. Am. J. Trop. Med. Hyg. 50 (1), 45–51.c.

Ramasamy, R., 2014. Zoonotic malaria — global overview and research and policy needs. Front. Public Health 2, 123.

Rodulfo, H., De Donato, M., Mora, R., González, L., Contreras, C.E., 2007. Comparison of the diagnosis of malaria by microscopy, immunochromatography and PCR in endemic areas of Venezuela. Braz. J. Med. Biol. Res. Rev. Bras. Pesqui. Médicas E Biológicas Soc. Bras. Biofísica Al 40, 535–543.

Snounou, G., et al., 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol. Biochem. Parasitol. 61, 315–320.

Sutherland, C.J., et al., 2010. Two nonrecombining sympatric forms of the human malaria parasite *Plasmodium ovale* occur globally. J. Infect. Dis. 201, 1544–1550.

Tahar, R., Ringwald, P., Basco, L.K., 1998. Heterogeneity in the circumsporozoite protein gene of *Plasmodium malariae* isolates from sub-Saharan Africa. Mol. Biochem. Parasitol. 92, 71–78.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Tanomsing, N., et al., 2007. Genetic analysis of the dihydrofolate reductase–thymidylate synthase gene from geographically diverse isolates of *Plasmodium malariae*. Antimicrob. Agents Chemother. 51, 3523–3530.

Tazi, L., Ayala, F.J., 2011. Unresolved direction of host transfer of *Plasmodium vivax* v. *P. simium* and *P. malariae* v. *P. brasilianum*. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 11, 209–221.

Verra, F., Hughes, A.L., 1999. Biased amino acid composition in repeat regions of *Plasmodium antigens*. Mol. Biol. Evol. 16, 627–633.

Volney, B., Pouliquen, J.-F., De Thoisy, B., Fandeur, T., 2002. A sero-epidemiological study of malaria in human and monkey populations in French Guiana. Acta Trop. 82, 11–23.