

Re-emergence of *Plasmodium malariae* in Costa Rica

Nidia Calvo¹, Jessica Morera¹, Antony Solórzano-Morales², Marco Vinicio Herrero², Gaby Dolz²

Abstract

In this paper we report the diagnosis of four cases of *Plasmodium malariae* in humans in Costa Rica, using parasitological and molecular techniques. Real-time PCR detected *P. malariae* in all four samples and established absence of other *Plasmodium* spp., whereas *P. malariae* was identified microscopically only in three samples. Sequence analysis of these three samples confirmed the results and identified them as *P. malariae/P. brasilianum*. This represents the first report of *P. malariae* in Central America since 1959. Future investigations are needed to elucidate if *P. malariae* is re-emerging in Costa Rica or if it has always been present but unreported due to lack of specificity and difficulties of *P. malariae* diagnosis by microscopic technique.

The nucleotide sequences of samples 1, 2 and 4 were deposited in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) and received the accession numbers KJ934251, KJ934252 and KJ934253, respectively.

Keywords: *Plasmodium malariae*, humans, Costa Rica, real-time PCR, sequencing

Author and article info:

1) National Reference Center for Parasitology, National Institute for Research and Education in Nutrition and Health (CNRP-INCIENSA), Cartago, Costa Rica

2) Entomology Laboratory, Research Program in Population Medicine, School of Veterinary Medicine, Universidad Nacional (UNA), Heredia, Costa Rica

Received: Nov 28 2014 **Accepted:** Apr 7 2015 **Published:** Apr 30 2015

Citation: Calvo N, Morera J, Solórzano-Morales A, Herrero MV, Dolz G (2015) Re-emergence of *Plasmodium malariae* in Costa Rica. *Science Postprint* 1(2): e00049. doi: 10.14340/spp.2015.04A0004.

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Funding: This work was supported partially by Fondo Especial para el Financiamiento de la Educación Superior Estatal, Consejo Nacional de Rectores (Special Fund for Financing Higher State Education, National Council of Rectors, FEES-CONARE), San José, Costa Rica.

Introduction

The identification of the infecting *Plasmodium* species and sequence analysis from an outbreak in a new region or from imported cases is especially relevant for disease surveillance and decision making to identify possible sources of infection or reservoirs as well as for the therapeutic decision making and the development of control and prevention measures for the disease. For this reason, CNRP-INCIENSA introduced diagnostic methodologies like endpoint polymerase chain reaction (PCR) in 2009 and real-time PCR in 2011, which have contributed to molecular laboratory-based surveillance of malaria in Costa Rica. The use of real-time PCR allows results in approximately 3 hours, ensuring a timely and accurate diagnosis of infection and permits parasite quantification [1].

Plasmodium malariae had not been detected in humans from Costa Rica since 1959 [2], although *Plasmodium brasilianum* was found in blood smears of howler monkeys (*Alouatta palliata*) of Costa Rica [3]. According to Collins and Jeffery [4], South American monkeys are naturally infected with *P. brasilianum*, and the high genetic identity found between *P. malariae* and *P. brasilianum* suggests the occurrence of recent transfers between hosts [5]. Between 2012 and 2013, four human cases caused by *P. malariae* were diagnosed for the first time since 1959 in CNRP-INCIENSA [6, 7]. It has been reported, that blood stages of *P. malariae* persist for extremely long periods, often for the life of the human host. In this respect, there have been reported cases of people, who have left zones of endemicity and, either following donation of blood in which

Competing interest: The Authors declare that there is no conflict of interest.

Ethics: The Ethics Committee (Dirección Técnica) of INCIENSA approved a research carried out as a laboratory-based epidemiological surveillance of malaria. It ensures that international ethical research standards were met, such as confidentiality, principle of individual autonomy (coded samples), and principle of beneficence (analysis and publication of data of diseases of mandatory notification, that are essential for decision-making in health).

Corresponding author: Gaby Dolz

Address: Campus Presbitero Benjamin Nuñez, Lagunilla, Barreal Heredia, Heredia, Costa Rica

E-mail: gaby.dolz.wiedner@una.cr

Data availability: The nucleotide sequences of samples 1, 2 and 4 were deposited in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>; accession numbers: KJ934251, KJ934252 and KJ934253).

Peer Reviewers: Gustavo Fontecha¹ and Reviewer B

¹ Microbiology Research Institute, National Autonomous University of Honduras, Tegucigalpa, Honduras

the recipient developed an infection, or whose infections have recrudesced after many years (up to 50 years) of dormancy under stress [4]. The objective of this paper was to confirm the finding of *P. malariae* in Costa Rica by using microscopic and molecular techniques.

Materials and Methods

Ethics statements

The Ethics Committee (Dirección Técnica) of INCIENSA approved a research carried out as a laboratory-based epidemiological surveillance of malaria, which is a strategic role of the National Reference Center for Parasitology (CNRP-INCIENSA). This committee reviewed the ethical content of the research project generated with the samples that were received from all health services of the country for their respective diagnosis and confirmation. It ensures that international ethical research standards were met, such as confidentiality, principle of individual autonomy (coded samples), and principle of beneficence (analysis and publication of data of diseases of mandatory notification, that are essential for decision-making in health).

Size and type of samples

Three samples of whole blood (samples 1, 2 and 3) were submitted for malaria diagnosis to the CNRP-INCIENSA in the first semester of 2012, and one more sample (sample 4) in the first semester of 2013. Sample 1 belonged to a transfusional case detected in San José, the capital of Costa Rica; sample 2 belonged to a clinical case detected in Talamanca, Limón, a southeastern region of the country; sample 3, belonged to a

chronic asymptomatic case and sample 4 to a clinical case, both detected in San Carlos, Alajuela, located in the northern part of the country.

Microscopic analysis

Thick and thin blood smears were prepared from each sample, stained with modified Romanowsky, and examined microscopically with the 100x immersion objective, to determine parasitological density and to identify species [8]. Density of parasites per microliter (μl) was determined by counting number of *Plasmodium* parasites present in 100 leukocytes in relation to the white blood counts per μl of each patient, in high parasitemias 500 *Plasmodium* parasites were counted in present leukocytes [8]. Once a sample was determined positive by a trained and internationally certified microscopist in a local laboratory, another blood sample was taken within three days, and sent to CNRP-INCIENSA, the Reference Laboratory for Malaria, to carry out another slide reading, these blood samples were also used for molecular analysis.

DNA extraction

DNA extraction was performed using the column extraction kit GenElute (Sigma Aldrich) proceeding according to the manufacturer's instructions. DNA concentration was determined with the Nanodrop 2000.

Real-time PCR

The extracted DNA was analyzed using the real-time PCR protocol described by Veron et al. [1], using species specific primers and species specific probes labeled with fluorochromes, targeting the 18S ribosomal RNA gene of *Plasmodium* (*P. malariae*, *P. vivax*, and *P. falciparum*). DNA of *P. malariae* donated by Center Disease Control (CDC) in Atlanta, USA, was used as positive control. Samples were amplified in the Step One Plus (Applied Biosystems) and amplification curves were analyzed with the software Step One. The number of amplification cycles required to reach the threshold of fluorescence (Ct values) of each sample was determined.

Sequencing and phylogenetic tree

The DNA was subjected to endpoint PCR, to amplify a sequence of the 18S rDNA gene type A of *Plasmodium* spp. [9]. The genus specific primers rPLU6 (5'-TTAAAATTGTTGCAGTTAAAACG-3' and rPLU5 (5'-CCTGTTTGTGCCTTAAACTTC-3') were used [10]. Reactions with 12.5 μl Dream Taq™ PCR Master Mix 2X (Fermentas®), 1.0 μl of each primer (20 pmol/ μl), 5 μl DNA (~50 μg) and 5.5 μl water (molecular biology grade, Fermentas®) were prepared to a final volume of 25 μl . Amplification protocol consisted of an initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation (95°C for 1 minute), alignment (48°C for 1 minute), extension (72°C for 1 minute), and a final extension of 72°C for 5 minutes. DNA of *P. vivax* donated by CNRP-INCIENSA was used as positive control; water (molecular biology grade, Fermentas®) was used as negative control. The PCR products were visualized by agarose gel electrophoresis (1.4%) in TBE (Tris Base, boric acid, EDTA, pH8, 0.5M), and stained with GelRed™ Nucleic Acid Gel Stain (Biotium, 5 $\mu\text{g}/\text{ml}$). GeneRuler 100 bp DNA Ladder Plus (Sm243, Fermentas®) was used as marker. Amplified fragments with a size of 1050 bp were considered positive and sent for its purification and sequencing to Macrogen (Seoul, Korea). Partial sequences were aligned with BioEdit Sequence Alignment Editor® and compared using the BLASTn algorithm with the database of NCBI (National Center for Biotechnology Information) [11]. Afterwards they were imported in MEGA 5 [12], using the algorithm of Jukes and Cantor and the UPGMA method for the design of the phylogenetic tree [13, 14]. Ten thousand repetitions were calculated [15]. Reference sequences of the 18S rDNA genes of *Plasmodium* spp., available in the GenBank database, accession numbers AF145336, AF488000, AF487999 and M54897 (*P. malariae*), AF130735 (*P. brasilianum*), M19172 (*P. falciparum*) and AY579418 (*P. vivax*) were included in the analysis. The tree was compared with the 18S rDNA sequence of *Babesia bovis* (GenBank L19077) belonging to Haematozoa,

Apicomplexa, as *Plasmodium* spp [16].

Results

In three out of four samples analyzed microscopically, *P. malariae* was identified. The concentrations of parasites and DNA in these samples are shown in Table 1. However real-time PCR detected *P. malariae* in all four samples, and established absence of *P. vivax* and *P. falciparum* (Table 1, Figure 1). The number of amplification cycles required to reach the threshold of fluorescence were 24, 28, 37 and 28 for samples 1, 2, 3, and 4, respectively.

In three out of four samples sequence analysis was possible and phylogenetic tree confirmed the results obtained microscopically and by real-time PCR, identifying those samples as *P. malariae/brasilianum* (Figure 2 and 3). BLASTn analysis resulted in a 99% identity between the samples, and 99% identity with *P. malariae* isolated from atypical human cases that occurred in Asia, and 99% identity with a sequence of *P. brasilianum* isolated from a nonhuman primate of Guiana, and that were deposited in GenBank (Table 2, Figure 3 and 4). Nucleotide sequences of samples 1, 2 and 4 were deposited in GenBank and received the accession numbers KJ934251, KJ934252 and KJ934253, respectively.

Discussion

In three samples (1, 2 and 4) *P. malariae* was detected microscopically, and all four samples tested positive for *P. malariae* by real-time PCR, which is in accordance with the detection limit of each technique. Routine clinical microscopy cannot reliably detect very low parasitemias (<5 to 10 parasites/ μl) [17], whereas real-time PCR detects from 2 to 5 parasites/ μl [1].

Future studies, however, have to determine, if *P. malariae* is re-emerging in Costa Rica or has always been present these years. The use of modified Romanowsky as a staining method for malaria diagnosis was recommended by the Pan American Health Organization, World Health Organization and carried out from 1959 until 2013 in Costa Rica, because of its advantage of no need of prior dishemoglobinizing blood samples. However, disadvantages of this non-standard procedure is the poor differentiation of parasitic stages, when compared to Giemsa staining. Furthermore, malaria cases are detected in Costa Rica generally at early parasitological stages using blood smears, when immature trophozoites from different *Plasmodium* species are difficult to differentiate, especially using modified Romanowsky. For this reason, it is recommended to improve the quality of microscopic analysis in Costa Rica. Between 2013 and 2017 laboratories in Costa Rica will be trained in Giemsa staining, to improve the diagnosis and eliminate malaria from the country (Nidia Calvo, personal communication).

Sequence analysis of the three Costa Rican samples established a 99% of identity with two peculiar forms of *P. malariae* reported by Kawamoto et al. [18] whose early trophozoite stages resemble morphologically that of *Plasmodium vivax*, var. *minuta* and *Plasmodium tenue*, and may also explain why *P. malariae* was not diagnosed since 1959 in Costa Rica.

Referring to the samples studied, sample 1 belonged to a transfusional malaria case that presented all parasite stages in the blood smears, which facilitated the diagnosis, especially since this patient was also immunosuppressed because of cancer treatment. Sample 2 presented only immature trophozoite stages in the blood smears, making difficult to diagnose correctly the *Plasmodium* species until real-time PCR was carried out. Sample 3 was positive in real-time PCR but negative in microscopy, presenting a low parasitemia, and sample 4 presented parasitic stages which made possible the microscopic diagnosis.

The fact that the three samples showed 99% identity with

sequences of *P. brasilianum* detected in non-human primates from French Guiana, coincides with what has been reported recently in the literature, that rainforest monkeys may represent a natural reservoir for human malaria [19], and with what has been proposed in recent studies, that *P. malariae* might derive from *P. brasilianum* [5]. *P. brasilianum* was detected in Costa Rica in 2005 in howler monkeys using blood smears [3].

It is important to notice, that the three samples also showed 99% of identity with a *P. malariae* detected in humans in New Guinea [19, 20]. Interestingly, those samples also showed 99% identity with two peculiar forms of *P. malariae* parasites found in Myanmar (Burma), whose morphologies of early trophozoite stages were distinct from that of the typical *P. malariae* [18]. Although PCR-based diagnosis confirmed these parasites as new variant forms of *P. malariae* they were separated into two genetic types that correlated with the two morphological types. The *P. tenue*-like type was closer to a monkey quartan malaria parasite, *P. brasilianum*. These results illustrate that the microscopic appearance of human *P. malariae* parasites may be more varied than previously assumed and emphasizes the value of molecular tools in the evaluation of malaria morphological variants [18].

The sample with a negative result in blood smear (sample 3) had a low parasite count, only detectable with real-time PCR, and did correspond to a person with chronic asymptomatic *P. malariae* infection, who donated blood and transmitted the parasites to the patient of sample 1. According to the medical records, this donor suffered from fevers when young, also mild icterus was reported during those fevers, which made malaria a suspect, however, he never was subjected to a malaria test. The other two cases are not linked epidemiologically to the donor and the recipient, or between them. Sample 2 belonged to an elderly man (75 years old), that lived in Home Creek, Talamanca, that had never left the district and never experienced malaria before, while the latter case was a male, who indicated to have entered the forest and was bitten by mosquitoes few weeks before getting sick. The available data indicate that at least two cases may have been due long-term persistence of *P. malariae* in man (samples 2 and 3), whereas one case seemed to be related to forests and mosquitoes. The importance of these findings resides in that this is the first time that cases of *P. malariae* have appeared in Central America in a long time. Further studies have to determine if these were due to *P. malariae*'s long recrudescence period or new cases.

Elimination of *P. malariae* would require much more stringent parameters than *P. falciparum* or *P. vivax*, for this reason, the following measures were taken: From the year 2013 onwards, blood smears from suspicious clinical cases in Costa Rica are stained with Giemsa and analyzed microscopically. All positive samples and ten percent of negative samples are sent to CNRP-INCIENSA for confirmation with microscopic analysis, and Real Time PCR is carried out to confirm all positive samples.

Conclusions

For the first time since 1959, the present study reports the parasitological and molecular detection and characterization of *P. malariae* in humans in Costa Rica, Central America. It remains to elucidate in future studies, if *P. malariae* is re-emerging in Costa Rica or has always been present, but was not microscopically diagnosed or misdiagnosed as *P. vivax*. It is recommended to improve the quality of microscopic analysis and confirm all microscopically positive *Plasmodium* results with real-time PCR in the future.

Author contributions

Conceived and designed the work: Calvo N, Dolz G
 Acquired the data: Calvo N, Morera J, Solórzano-Morales A, Herrero MV, Dolz G
 Analyzed and/or interpreted the data: Calvo N, Morera J, Solórzano-Morales A, Herrero MV, Dolz G

Drafted the work: Calvo N, Morera J, Solórzano-Morales A, Herrero MV, Dolz G

Revised and approved the work: Calvo N, Herrero MV, Dolz G
 All authors approved the final version to be published.

Table 1. Results of four blood samples from Costa Rica analyzed by blood smears and real-time PCR

Sample	Blood smears (p parasite count/ μ l)	DNA concentration (ng/ μ l)	Real-time PCR		
			<i>P. malariae</i>	<i>P. vivax</i>	<i>P. falciparum</i>
1	<i>P. malariae</i> (19522 parasites/ μ l)	82.0	+	-	-
2	<i>P. malariae</i> (1720 parasites/ μ l)	20.5	+	-	-
3	Negative	29.2	+	-	-
4	<i>P. malariae</i> (1516 parasites/ μ l)	17.7	+	-	-

Table 2. Percentage of identity of partial sequences of the 18S rDNA gene of three positive *P. malariae* samples from Costa Rica with other *Plasmodium* species reported in GenBank

Access Number	Species of <i>Plasmodium</i>	Percentage of identity	Detected in	Country	Reference
AF130735.1	<i>P. brasilianum</i>	99%	<i>Alouatta</i> sp., <i>Ateles</i> sp., <i>Aotus</i> sp., <i>Brachyteles arachnoides</i> , <i>Callicebus</i> sp., <i>Cebus</i> sp., <i>Chiropetes chiropetes</i> , <i>Lagothrix</i> sp., <i>Saimiri</i> sp.	French Guiana	16
AF488000.1	<i>P. cf. malariae</i> type 2	99%	<i>Homo sapiens</i>	Japan	15
AF145336.1	<i>P. malariae</i>	99%	<i>Homo sapiens</i>	New Guinea	18
AF487999.1	<i>P. cf. malariae</i> type 1	99%	<i>Homo sapiens</i>	Japan	15

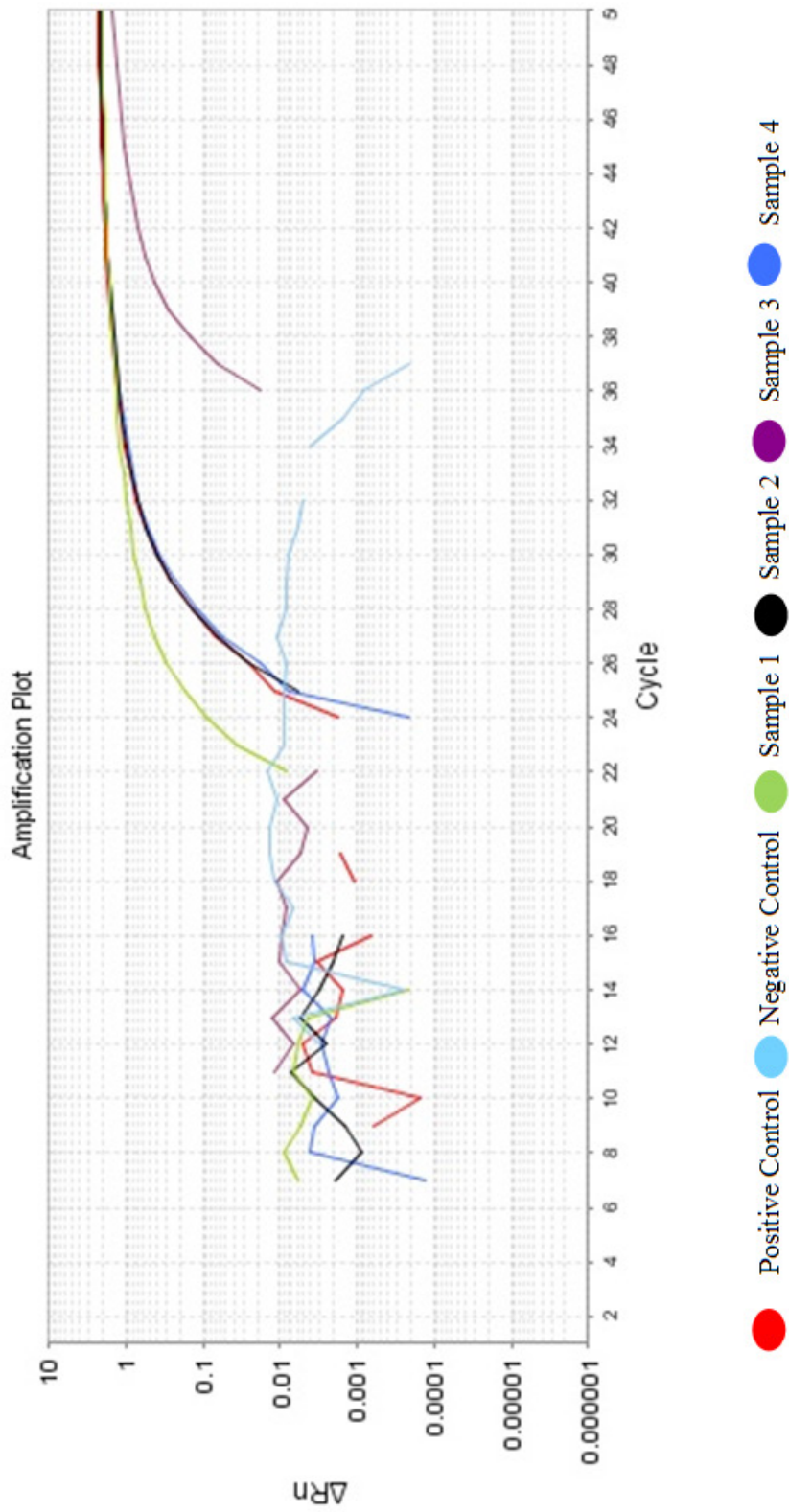


Figure 1. Real-time PCR of sample 1 to 4 with *P. malariae* probe

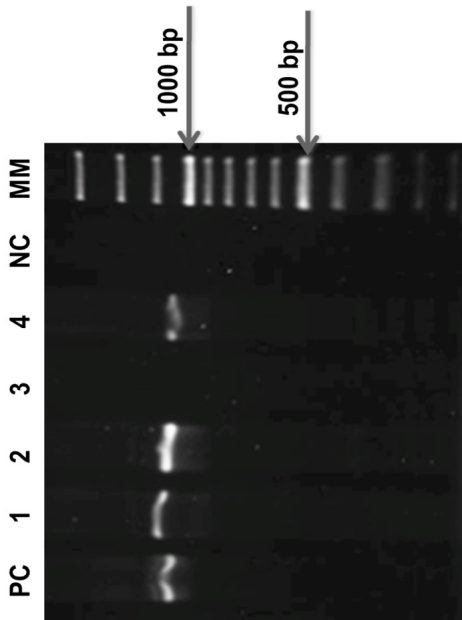


Figure 2. Agarose gel electrophoresis of the amplified PCR products of the ADNr 18S type A gene of *Plasmodium* sp.
 1: Positive Control; 2: Sample 1; 3: Sample 2; 4: Sample 3; 5: Sample 4; 6: Negative Control, MM: Molecular Marker

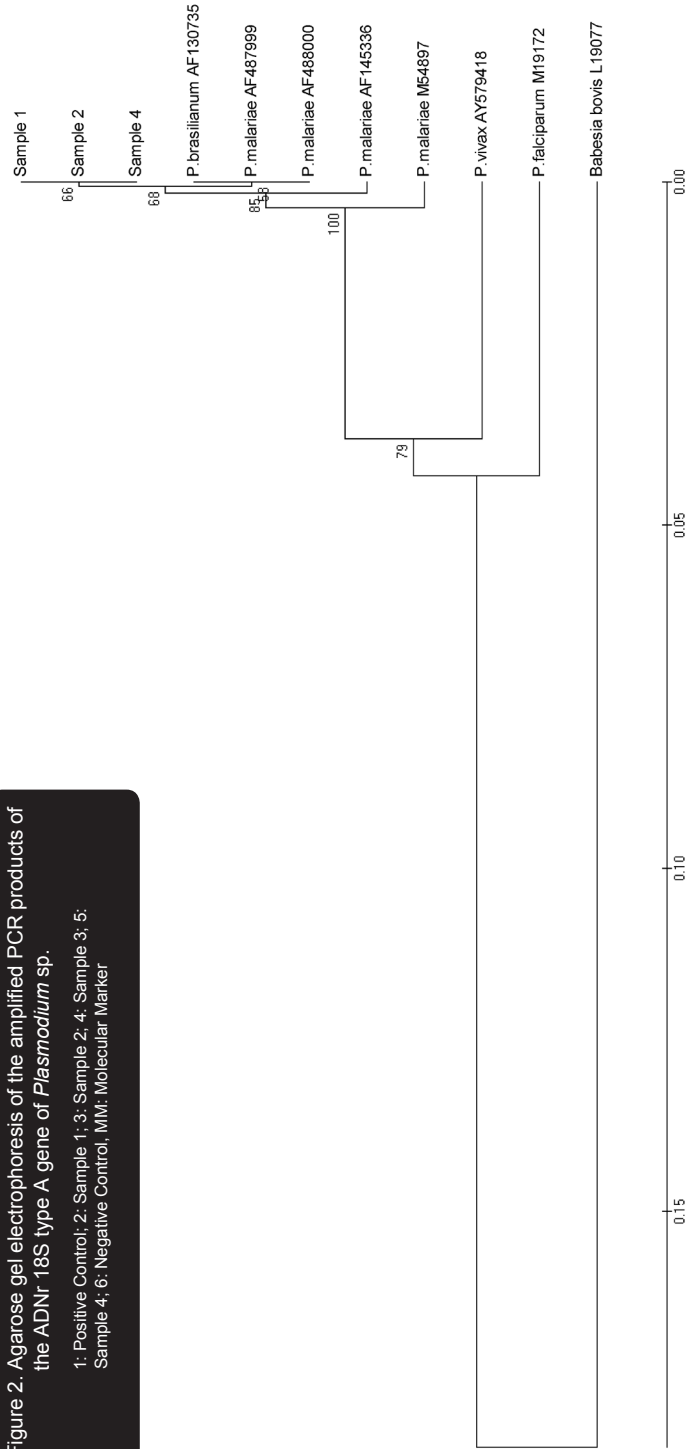


Figure 3. Phylogenetic tree of the 18S rDNA gene sequence of positive patient samples to *P. malariae* in Costa Rica

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-----|-----|-----|-----|-----|
      5       15       25       35       45
Sample 1  ATCAATATTT AGTAATGCTT TGTATATTTA TAACAAAGTT GTACGTTAAG
Sample 2  ATCAATATTT AGTAATGCTT TGTATATTTA TAACAAAGTT GTACGTTAAG
Sample 4  ATCAATATTT AGTAATGCTT TGTATATTTA TAACAAAGTT GTACGTTAAG
P.brasilianum ATCAATATTT AGTAATGCTT TGTATATTTA TAACAAAGTT GTACGTTAAG
P.malariae ATCAATATTT AGTAATGCTT TGTATATTTA TAACAAAGTT GTACATTAAG

-----|-----|-----|-----|-----|
      55       65       75       85       95
Sample 1  AATAAACGCC AAGCGTTATA TTTTCTCTGT TACATTTTGT TTTATTAATA
Sample 2  AATAAACGCC AAGCGTTATA TTTTCTCTGT TACATTTTGT TTTATTAATA
Sample 4  AATAAACGCC AAGCGTTATA TTTTCTCTGT TACATTTTGT TTTATTAATA
P.brasilianum AATAAACGCC AAGCGTTATA TTTTCTCTGT TACATTTTGT TTTATTAATA
P.malariae AATAAACGCC AAGCGTTATA TTTTCTCTGT TACATTTTGT TTTATTAATA

-----|-----|-----|-----|-----|
     105     115     125     135     145
Sample 1  TATATATGCG TTCTTATTAT AAAAATGATT CTTTTTAAAA TTCFTTTGTA
Sample 2  TATATATGCG TTCTTATTAT AAAAATGATT CTTTTTAAAA TTCFTTTGTA
Sample 4  TATATATGCG TTCTTATTAT AAAAATGATT CTTTTTAAAA TTCFTTTGTA
P.brasilianum TATATATGCG TTCTTATTAT AAAAATGATT CTTTTTAAAA TTCFTTTGTA
P.malariae TATATATGCG TTCTTATTAT AAAAATGATT CTTTTTAAAA TTCFTTTGTG

-----|-----|-----|-----|-----|
     155     165     175     185     195
Sample 1  TAATTTTTTA TGCATGGGAA TTTTGTACT TTGAGTAAAT TAGAGTGTTT
Sample 2  TAATTTTTTA TGCATGGGAA TTTTGTACT TTGAGTAAAT TAGAGTGTTT
Sample 4  TAATTTTTTA TGCATGGGAA TTTTGTACT TTGAGTAAAT TAGAGTGTTT
P.brasilianum TAATTTTTTA TGCATGGGAA TTTTGTACT TTGAGTAAAT TAGAGTGTTT
P.malariae TAATTTTTTA TGCATGGGAA TTTTGTACT TTGAGTAAAT TAGAGTGTTT

-----|-----|-----|-----|-----|
     205     215     225     235     245
Sample 1  AAAGCAAACA GTTAAAACAG TTTCTGTGTT TGAATACTAC AGCATGGAAT
Sample 2  AAAGCAAACA GTTAAAACAG TTTCTGTGTT TGAATACTAC AGCATGGAAT
Sample 4  AAAGCAAACA GTTAAAACAG TTTCTGTGTT TGAATACTAC AGCATGGAAT
P.brasilianum AAAGCAAACA GTTAAAACAG TTTCTGTGTT TGAATACTAC AGCATGGAAT
P.malariae AAAGCAAACA GTTAAAACAG TTTCTGTGTT TGAATACTAC AGCATGGAAT

-----|-----|-----|-----|-----|
     255     265     275     285     295
Sample 1  AACAAAATTG AACAAAGTCAG AATTTTGTTT TTTTCTCTTA TTTTGGCTTA
Sample 2  AACAAAATTG AACAAAGTCAG AATTTTGTTT TTTTCTCTTA TTTTGGCTTA
Sample 4  AACAAAATTG AACAAAGTCAG AATTTTGTTT TTTTCTCTTA TTTTGGCTTA
P.brasilianum AACAAAATTG AACAAAGTCAG AATTTTGTTT TTTTCTCTTA TTTTGGCTTA
P.malariae AACAAAATTG AACAAAGTCAG AATTTTGTTT TTTTCTCTTA TTTTGGCTTA

-----|-----|-----|-----|-----|
     305     315     325     335     345
Sample 1  GTTACGATTA ATAGGAGTAG CTTGGGGGCA TTTGTATTCA GATGTCAGAG
Sample 2  GTTACGATTA ATAGGAGTAG CTTGGGGGCA TTTGTATTCA GATGTCAGAG
Sample 4  GTTACGATTA ATAGGAGTAG CTTGGGGGCA TTTGTATTCA GATGTCAGAG
P.brasilianum GTTACGATTA ATAGGAGTAG CTTGGGGGCA TTTGTATTCA GATGTCAGAG
P.malariae GTTACGATTA ATAGGAGTAG CTTGGGGGCA TTTGTATTCA GATGTCAGAG

-----|-----|-----|-----|-----|
     355     365     375     385     395
Sample 1  GTGAAATTCT TAGATTTTCT GGAGACAATC AACTGCGAAA GCATTTGCCT
Sample 2  GTGAAATTCT TAGATTTTCT GGAGACAATC AACTGCGAAA GCATTTGCCT
Sample 4  GTGAAATTCT TAGATTTTCT GGAGACAATC AACTGCGAAA GCATTTGCCT
P.brasilianum GTGAAATTCT TAGATTTTCT GGAGACAAGC AACTGCGAAA GCATTTGCCT
P.malariae GTGAAATTCT TAGATTTTCT GGAGACAAGC AACTGCGAAA GCATTTGCCT

-----|-----|-----|-----|-----|
     405     415     425     435     445
Sample 1  AAAATACTTC CATTAATCAA GAACGAAAGT TAAGGGAGTG AAGACGATCA
Sample 2  AAAATACTTC CATTAATCAA GAACGAAAGT TAAGGGAGTG AAGACGATCA
Sample 4  AAAATACTTC CATTAATCAA GAACGAAAGT TAAGGGAGTG AAGACGATCA
P.brasilianum AAAATACTTC CATTAATCAA GAACGAAAGT TAAGGGAGTG AAGACGATCA
P.malariae AAAATACTTC CATTAATCAA GAACGAAAGT TAAGGGAGTG AAGACGATCA

-----|-----|-----|-----|-----|
     455     465     475     485     495
Sample 1  GATACCGTGC TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTGGATGA
Sample 2  GATACCGTGC TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTGGATGA
Sample 4  GATACCGTGC TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTGGATGA
P.brasilianum GATACCGTGC TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTGGATGA
P.malariae GATACCGTGC TAATCTTAAC CATAAACTAT GCCGACTAGG TGTTGGATGA

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Figure 4a. Alignment of the nucleotide sequences of the 18S rDNA gene sequence of *P. malariae*

Sample 1, Sample 2 and Sample 4: Obtained from three humans of Costa Rica; *P. brasilianum*: Obtained from a monkey (GenBank AF130735); *P. malariae*: Isolated from a human in Southeast Asia (GenBank AF488000).

	505	515	525	535	545
Sample 1	TAGAGTAAAA	AATAAAAAGGG	ACATTCATAT	ATATGAGTGT	TTCTTTTAGA
Sample 2	TAGAGTAAAA	AATAAAAAGGG	ACATTCATAT	ATATGAGTGT	TTCTTTTAGA
Sample 4	TAGAGTAAAA	AATAAAAAGGG	ACATTCATAT	ATATGAGTGT	TTCTTTTAGA
<i>P.brasilianum</i>	TAGAGTAAAA	AATAAAAAGGG	ACATTCATAT	ATATGAGTGT	TTCTTTTAGA
<i>P.malariae</i>	TAGAGTAAAA	AATAAAAAGAG	ACATTCATAT	ATATGAGTGT	TTCTTTTAGA

	555	565	575	585	595
Sample 1	TAGCTTCCTT	CAGTACCTTA	TGAGAAATCA	AAGTCTTTGG	GTTCTGGGGC
Sample 2	TAGCTTCCTT	CAGTACCTTA	TGAGAAATCA	AAGTCTTTGG	GTTCTGGGGC
Sample 4	TAGCTTCCTT	CAGTACCTTA	TGAGAAATCA	AAGTCTTTGG	GTTCTGGGGC
<i>P.brasilianum</i>	TAGCTTCCTT	CAGTACCTTA	TGAGAAATCA	AAGTCTTTGG	GTTCTGGGGC
<i>P.malariae</i>	TAGCTTCCTT	CAGTACCTTA	TGAGAAATCA	AAGTCTTTGG	GTTCTGGGGC

	605	615	625	635	645
Sample 1	GAGTATTTCG	GCAAGCGAGA	AAGTTAAAAG	AATTGACGGA	AGGGCACCAC
Sample 2	GAGTATTTCG	GCAAGCGAGA	AAGTTAAAAG	AATTGACGGA	AGGGCACCAC
Sample 4	GAGTATTTCG	GCAAGCGAGA	AAGTTAAAAG	AATTGACGGA	AGGGCACCAC
<i>P.brasilianum</i>	GAGTATTTCG	GCAAGCGAGA	AAGTTAAAAG	AATTGACGGA	AGGGCACCAC
<i>P.malariae</i>	GAGTATTTCG	GCAAGCGAGA	AAGTTAAAAG	AATTGACGGA	AGGGCACCAC

	655	665	675	685	695
Sample 1	CAGGCGTGGG	GCTTGCGGCT	TAATTTGACT	CAACACGGGG	AAACTCACTA
Sample 2	CAGGCGTGGG	GCTTGCGGCT	TAATTTGACT	CAACACGGGG	AAACTCACTA
Sample 4	CAGGCGTGGG	GCTTGCGGCT	TAATTTGACT	CAACACGGGG	AAACTCACTA
<i>P.brasilianum</i>	CAGGCGTGGG	GCTTGCGGCT	TAATTTGACT	CAACACGGGG	AAACTCACTA
<i>P.malariae</i>	CAGGCGTGGG	GCTTGCGGCT	TAATTTGACT	CAACACGGGG	AAACTCACTA

	705	715	725	735	745
Sample 1	GTTTAAGACA	AGAGTAGGAT	TGACAGATTA	ATAGCTCTTT	CTTGATTTC
Sample 2	GTTTAAGACA	AGAGTAGGAT	TGACAGATTA	ATAGCTCTTT	CTTGATTTC
Sample 4	GTTTAAGACA	AGAGTAGGAT	TGACAGATTA	ATAGCTCTTT	CTTGATTTC
<i>P.brasilianum</i>	GTTTAAGACA	AGAGTAGGAT	TGACAGATTA	ATAGCTCTTT	CTTGATTTC
<i>P.malariae</i>	GTTTAAGACA	AGAGTAGGAT	TGACAGATTA	ATAGCTCTTT	CTTGATTTC

	755	765	775	785	795
Sample 1	TGGATGGTGA	TGCATGGCCG	TTTTTAGTTC	GTGAATATGA	TTTGTCTGGT
Sample 2	TGGATGGTGA	TGCATGGCCG	TTTTTAGTTC	GTGAATATGA	TTTGTCTGGT
Sample 4	TGGATGGTGA	TGCATGGCCG	TTTTTAGTTC	GTGAATATGA	TTTGTCTGGT
<i>P.brasilianum</i>	TGGATGGTGA	TGCATGGCCG	TTTTTAGTTC	GTGAATATGA	TTTGTCTGGT
<i>P.malariae</i>	TGGATGGTGA	TGCATGGCCG	TTTTTAGTTC	GTGAATATGA	TTTGTCTGGT

	805	815	825	835	845
Sample 1	TAATTCGGAT	AACGAACGAG	ATCTTAACCT	GCTAATTAGC	GGTAAATACA
Sample 2	TAATTCGGAT	AACGAACGAG	ATCTTAACCT	GCTAATTAGC	GGTAAATACA
Sample 4	TAATTCGGAT	AACGAACGAG	ATCTTAACCT	GCTAATTAGC	GGTAAATACA
<i>P.brasilianum</i>	TAATTCGGAT	AACGAACGAG	ATCTTAACCT	GCTAATTAGC	GGTAAATACA
<i>P.malariae</i>	TAATTCGGAT	AACGAACGAG	ATCTTAACCT	GCTAATTAGC	GGTAAATACA

	855	865	875	885	895
Sample 1	CTATATTCTT	AAGTGAAATT	AGAATATAGA	TAAATTGTGC	TAATTTTGAT
Sample 2	CTATATTCTT	AAGTGAAATT	AGAATATAGA	TAAATTGTGC	TAATTTTGAT
Sample 4	CTATATTCTT	AAGTGAAATT	AGAATATAGA	TAAATTGTGC	TAATTTTGAT
<i>P.brasilianum</i>	CTATATTCTT	AAGTGAAATT	AGAATATAGA	TAAATTGTGC	TAATTTTGAT
<i>P.malariae</i>	CTATATTCTT	AAGTGAAATT	AGAATATAGA	TAAATTGTGC	TAATTTTGAT

	905	915	925	935	945
Sample 1	TAAAATATTA	GAATGTTTTT	TTTAATAAAA	ACGTTCTTTT	CCCTTTTTTT
Sample 2	TAAAATATTA	GAATGTTTTT	TTTAATAAAA	ACGTTCTTTT	CCCTTTTTTT
Sample 4	TAAAATATTA	GAATGTTTTT	TTTAATAAAA	ACGTTCTTTT	CCCTTTTTTT
<i>P.brasilianum</i>	TAAAATATTA	GAATGTTTTT	TTTAATAAAA	ACGTTCTTTT	CCCTTTTTTT
<i>P.malariae</i>	TAAAATATTA	GAATGTTTTT	TTTAATAAAA	ACGTTCTTTT	CCCTTTTTTT

	955	965	975	985	995
Sample 1	CTTAATTATG	CATATTTATT	CTTTTTCTTT	TTTCGCATAA	GAATGTATTT
Sample 2	CTTAATTATG	CATATTTATT	CTTTTTCTTT	TTTCGCATAA	GAATGTATTT
Sample 4	CTTAATTATG	CATATTTATT	CTTTTTCTTT	TTTCGCATAA	GAATGTATTT
<i>P.brasilianum</i>	CTTAATTATG	CATATTTATT	CTTTTTCTTT	TTTCGCATAA	GAATGTATTT
<i>P.malariae</i>	CTTAATTATG	CATATTTATT	CTTTTTCTTT	TTTCGCATAA	GAATGTATTT
				
	1005				
Sample 1	GCTTAAT				
Sample 2	GCTTAAT				
Sample 4	GCTTAAT				
<i>P.brasilianum</i>	GCTTAAT				
<i>P.malariae</i>	GCTTAAT				

Figure 4b. Alignment of the nucleotide sequences of the 18S rDNA gene sequence of *P. malariae*. Sample 1, Sample 2 and Sample 4: Obtained from three humans of Costa Rica; *P. brasilianum*: Obtained from a monkey (GenBank AF130735); *P. malariae*: Isolated from a human in Southeast Asia (GenBank AF488000).

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