Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation

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Summary

Background Malaria was eliminated from southern and southeastern Brazil over 50 years ago. However, an increasing number of autochthonous episodes attributed to *Plasmodium vivax* have recently been reported from the Atlantic Forest region of Rio de Janeiro state. As the *P vivax*-like non-human primate malaria parasite species *Plasmodium simium* is locally enzootic, we performed a molecular epidemiological investigation to determine whether zoonotic malaria transmission is occurring.

Methods We examined blood samples from patients presenting with signs or symptoms suggestive of malaria as well as from local howler monkeys by microscopy and PCR. Samples were included from individuals if they had a history of travel to or resided in areas within the Rio de Janeiro Atlantic Forest, but not if they had malaria prophylaxis, blood transfusion or tissue or organ transplantation, or had travelled to known malaria endemic areas in the preceding year. Additionally, we developed a molecular assay based on sequencing of the parasite mitochondrial genome to distinguish between *P vivax* and *P simium*, and applied this assay to 33 cases from outbreaks that occurred in 2015, and 2016.

Findings A total of 49 autochthonous malaria cases were reported in 2015–16. Most patients were male, with a mean age of 44 years (SD 14·6), and 82% lived in urban areas of Rio de Janeiro state and had visited the Atlantic Forest for leisure or work-related activities. 33 cases were used for mitochondrial DNA sequencing. The assay was successfully performed for 28 samples, and all were shown to be *P simium*, indicative of zoonotic transmission of this species to human beings in this region. Sequencing of the whole mitochondrial genome of three of these cases showed that *P simium* is most closely related to *P vivax* parasites from South America. The malaria outbreaks in this region were caused by *P simium*, previously considered to be a monkey-specific malaria parasite, related to but distinct from *P vivax*, and which has never conclusively been shown to infect people before.

Interpretation This unequivocal demonstration of zoonotic transmission, 50 years after the only previous report of *P simium* in people, leads to the possibility that this parasite has always infected people in this region, but that it has been consistently misdiagnosed as *P vivax* because of an absence of molecular typing techniques. Thorough screening of local non-human primates and mosquitoes (*Anopheline*) is required to evaluate the extent of this newly recognised zoonotic threat to public health and malaria elimination in Brazil.

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Introduction

Zoonotic malaria occurs when people become infected with malaria parasite species that more commonly infect non-human primates. Species such as *Plasmodium knowlesi* and *Plasmodium cynomolgi*, both parasites of macaque monkeys (*Macaca*), can infect people via the bites of infected mosquitoes under natural and experimental conditions. *P knowlesi* is responsible for a high proportion of human malaria cases in Southeast Asia, mostly affecting individuals living or working in close contact with forests.[1] Zoonotic malaria poses a unique problem for malaria control efforts and complicates the drive towards eventual elimination of the disease; because of the nature of its reservoir and transmission dynamics, the interruption of its transmission might not be achievable with the available tools in areas of high forest coverage.

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For more on the **cases reported by the Ministry of Health** see www.saude.gov.br/malaria

Research in context

Evidence before this study

Autochthonous malaria infections in people leaving near the Atlantic Forest in Rio de Janeiro, Brazil, were diagnosed as *Plasmodium vivax*, a human malaria parasite. The diagnosis of *P vivax* was on the basis of the morphology of the parasites as observed through microscopy of thin blood smears stained with Giemsa's solution. As malaria was thought to have been eliminated from this area over 50 years ago, it was uncertain where and when this malaria parasite pool had emerged. Cases have been increasing in the past 5 years.

Added value of this study

This study shows that these parasites are, in fact, not *P vivax*, but rather *P simium*, a closely related parasite species whose natural hosts are non-human primates native to the Atlantic Forest. This diagnosis was made by molecular investigation of parasite DNA. Genotyping of malaria parasites from monkeys in this region revealed that the same parasites are infecting both monkeys and human beings in this area.

Implications of all the available evidence

Our study suggests that malaria transmission in the Atlantic Forest region of Rio de Janeiro has a zoonotic component, with parasites shared between human beings and monkeys. The implications of this finding for malaria control and elimination in this region are profound, as zoonotic reservoirs of disease are difficult to target with interventions.

Once prevalent throughout the country, malaria transmission in Brazil now occurs almost entirely within the northern Amazon region. However, a consistent number of autochthonous cases have been reported in southern and southeastern regions of Brazil from where human malaria was eliminated 50 years ago.[2] From 2006 to 2016, 1032 autochthonous cases (Ministry of Health Brazil 2017) were reported at sites scattered along the mountainous valleys covered by the Atlantic Forest in these regions. The Atlantic Forest is rich in bromeliads (Bromeliaceae), which provide a larval habitat for *Anopheles Kertezsia cruzii*, a vector of both human and non-human primate malaria parasites. Most of the malaria cases reported in the Atlantic Forest have been attributed to *Plasmodium vivax* and mainly occur among non-resident visitors, without any identifiable index case that could have introduced the parasite from a malaria endemic region.[3]

It has long been hypothesised that autochthonous human malaria in the Atlantic Forest could be the result of infection by non-human primate parasite species.[4] In 1966, Deane and colleagues[4] proposed that monkeys could serve as reservoirs of *Plasmodium* that could be transmitted to people by *A K cruzii*, because this species is known to bite both monkeys in the forest canopy and people at ground level.

Two malaria parasite species are known to infect new world monkeys (Ceboidea) in the Atlantic Forest of Brazil: *Plasmodium simium* and *Plasmodium brasilianum*.[5] These are similar at the morphological, genetic, and immunological levels to *P vivax* and *Plasmodium malariae*, respectively.[6] *P simium* has been observed to naturally infect howler monkeys of the genera *Alouatta* and *Brachyteles*, and capuchin monkeys of the genera *Cebus* and *Sapajus*.[7] Despite the distribution of the howler monkeys and capuchins across almost all biomes in South and Central America, the distribution of *P simium* is considered to be limited to the Atlantic Forest of south and southeastern Brazil.[5]

Here we describe the parasitological and molecular analyses of parasites causing autochthonous human malaria in the Atlantic Forest region of Rio de Janeiro in 2015 and 2016, with the aim of determining whether zoonotic malaria transmission occurs there.

Methods

Study area, population, and design

Rio de Janeiro state is located in southeast Brazil. It consists of urban areas with high population densities, mostly in the coastal lowlands, and mountainous areas covered by the Atlantic Forest containing small cities and settlements scattered in the valleys. Localities where malaria cases have been reported are situated in valleys between 280 m and 1300 m above sea level.[8]

We performed an epidemiological investigation to characterise the possible location of infection, by classifying each episode as autochthonous or imported. The cases considered here are from patients who attended the Instituto Nacional de Infectologia Evandro Chagas (INI), a reference centre for the diagnosis and treatment of infectious diseases at the Fundação Oswaldo Cruz (Fiocruz), in Rio de Janeiro, Brazil. Blood samples from patients with acute fever symptoms were collected from the Acute Febrile Illness Outpatient Clinic in INI. The INI-Fiocruz Ethical Board approved the study (number 0062.0.009.000-11). All participants provided informed written consent.

Procedures

Individuals were recruited upon presentation of signs or symptoms suggestive of malaria, a history of travel to or habitation in areas within the Rio de Janeiro Atlantic Forest, and a positive test by thick blood smear or PCR, or both. Individuals were excluded if they had malaria prophylaxis, blood transfusion or tissue or organ transplantation, used intravenous drugs, had a needlestick injury, resided or undertook recreation near ports or airports, or travelled to known malaria endemic areas in the preceding year. Following informed consent, venous blood was drawn for clinical laboratory analyses and molecular studies. Additional tests, such as blood

culture, viral serology and G6PD deficiency, were done for all patients at the attending physician's discretion.

Diagnosis by microscopy

Giemsa's solution-stained thin and thick blood smears were examined by bright-field microscopy, with a 100×/1·3 numerical aperture oil immersion objective lens for species identification and parasite density estimations. Blood films were examined for a minimum of 100 fields in the case of the presence of malaria parasites and 500 fields when no parasites could be detected. Parasite numbers were recorded per 200 white blood cells, or 500 white blood cells in the case of low parasitaemia. To estimate parasite density, a standard mean white blood cell count of 6000 white blood cells per µL of blood was assumed. All slides were subsequently examined by an independent Pan American Health Organization or WHO accredited malaria microscopist.

DNA extraction and *P vivax* species-specific PCR

DNA was extracted from whole blood with the QIAamp midi kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA samples were tested for *P vivax* by conventional and real-time PCR (rtPCR), both using the cysteine proteinase gene (GenBank number L26362) as a target.[9] For rtPCR, 2·5 µL of DNA were added to a 47·5 µL mixture containing the 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 300 nM of primer Pv1 (5′ATCAACGAGCAG ATGGAGAAATATA3′), 300 nM of primer Pv5 (5′GCT CTCGAAATCTTTCTTCGA3′), and 150 nM of PVIV probe (5′ FAM AACTTCAAAATGAATTATCTC MGB NFQ 3′) (Applied Biosystems). Amplification conditions involved two holds (50°C for 2 min and 95°C for 10 min) followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). The rtPCR was run at least in duplicate on the ABI PRISM Sequence Detection System 7500 (Applied Biosystems). The results were analysed using ABI Prism 7500, software version 1.1 RQ Study. TaqMan RNaseP Control 20x was used as an endogenous reaction control. To avoid DNA contamination, we used separated workstations for mix preparation and DNA extraction and we applied DNA Away for surface decontamination. Positive (DNA extracted from blood from patients with known *P vivax* infection) and negative (no DNA and DNA extracted from individuals who have never travelled to malaria-endemic areas) controls were used in each round of amplification. This PCR does not discriminate between *P vivax* and *P simium*.

Non-human primate samples

DNA was extracted from samples obtained from four howler monkeys from different sites and times in southeastern Brazil (MB CPRJ from Guapimirim in December, 2013; RJ 30 from Vale das Princesas, Miguel Pereira on March 21, 2016; RJ 59 from Macaé on Sept 22, 2016; and ATCC from São Paulo in 1966).

DNA extracted from the spleen and liver of one brown howler monkey (*Alouatta guariba clamitans*), found dead in Guapimirim (one of the municipalities where human malaria occurs in the Atlantic Forest of Rio de Janeiro), was used for *Plasmodium* species detection by nested-PCR.[10] Samples from both organs were positive for *P vivax* or *P simium* DNA, according to our PCR method.[7]

DNA was also extracted from the blood of two *A g clamitans*; one was captured at Vale das Princesas, Miguel Pereira (a site where human malaria cases have also been reported in Rio de Janeiro) in 2016, and tested positive by PCR analysis for both *P vivax* or *P simium* and *P brasilianum* or *P malariae*, and the other was from Macaé (another locality in Rio de Janeiro with human malaria cases), and was positive for *P vivax* or *P simium*. Additionally, a *P simium* reference sample (American Type Culture Collection [ATCC] 30130), derived from a howler monkey (*Alouatta fusca clamitans*) captured in São Paulo, southeast Brazil, in 1966, was also used. The DNA extracted from these four monkey samples also underwent mitochondrial genome analysis.

For more on the **reference sample** see https://www.atcc.org/~/ps/30130.ashx

Molecular phylogenetic analysis of *P simium* infections

Among samples derived from 39 individuals presenting at INI, 33 were subjected to parasite mitochondrial genome sequencing (20 from 2015 and 13 from 2016): 30 had partial analysis and three full-length mitochondrial genome sequencing. Samples from two monkeys collected from the Atlantic Forest of Rio de Janeiro and one ATCC *P simium* reference sample were also subjected to malaria parasite mitochondrial genome sequencing.

Because of the low amount of high-quality parasite DNA, full-length mitochondrial genome sequence was obtained for only four samples (three cases: AF 1, AF 2, and AF 3 and the ATCC reference sample), following the method reported by Culleton and colleagues,[11] and was compared with 794 *P vivax* mitochondrial genome sequences and three sequences of *P simium* (accession numbers AY800110, NC_007233 and AY722798, all of which have identical sequences) deposited in Genbank.[11–17] Using these sequences, a median-joining haplotype network was produced with NETWORK 4.5.0, as previously described.[11]

The mitochondrial genome of the remaining 30 samples was partially sequenced to distinguish *P simium* from *P vivax*. *P simium* differs from the most closely related *P vivax* isolate at two unique single-nucleotide polymorphisms (SNPs) in the mitochondrial genome, at positions 3535 (T→C) and 3869 (A→G), numbered according to the nucleotide sequences deposited by Culleton and colleagues.[11] These two SNPs are close together, and can be PCR amplified and sequenced with a single set of primers, or with a nested PCR if DNA concentrations are low. Primer pairs for the outer PCR were PsimOUTF 5′CAGGTGGTG TTTTAATGTTATTATCAG3′ and PsimOUTR 5′GCATAG GTAAGAATGTTAATACAACTCC3′, whereas inner

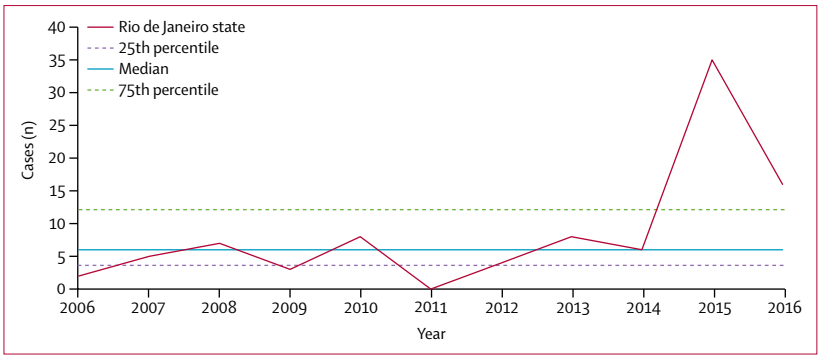

Figure 1: **Historical series of autochthonous malaria cases in the state of Rio de Janeiro, Brazil, from 2006 to 2016**
Historical series of autochthonous malaria cases from 2006 to 2016. In 2015–16, the number of cases exceeding the 75th percentile of maximum expected cases increased sharply, configuring an outbreak.

See Online for appendix

PCR primers were PsimINF 5′GCTGGAGATCCTATT TTATATCAAC3′ and PsimINR 5′ATGTAAACAATCCAA TAATTGCACC3′.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between 2006 and 2014, 43 autochthonous malaria cases were reported in the Atlantic Forest in the state of Rio de Janeiro, an average of 4·8 cases per year (SD 2·8), with an unexpected increase in the number of cases occurring during outbreaks in 2015 (n=33) and 2016 (n=16; figure 1). In 2015, 25 (76%) of the 33 cases were followed and processed at Fiocruz. In 2016 (until Oct 31), 14 of 16 (88%) cases were also investigated at Fiocruz, with a total of 39 (80%) of the 49 cases reported in the state.

Patients followed up at Fiocruz had a mean age of 44 years (SD 14·6) and median age of 50 years (range 7–82; table). Most patients were male (79%; table) and inhabitants of urban areas of Rio de Janeiro state (82%), who visited areas of the Atlantic Forest for leisure or work-related activities, spending a median of 5 days (range 1–30) in vegetation-dense areas and its close surroundings. Transmission occurred either in people who entered regions of dense vegetation coverage or in people who lived in rural areas with low-population density in mountain valleys (figure 2). The presence of monkeys was regularly reported in the neighbouring forest by the inhabitants of all areas.

Case clustering occurred only when individuals travelled together and developed symptoms in the same incubation period. Fever was the main symptom and was present in all malaria cases. Periodic tertian fever was observed in 35 cases (90%). No patient was admitted to hospital and all made full recoveries with complete cessation of symptoms following treatment. It was the first malaria episode for all patients and only one patient was G6PD deficient.

In 37 cases (95%) a diagnosis of *P vivax* was made by microscopy. The highest parasitaemia was 3000 parasites per µL of blood and, in more than 67% of the cases, it was lower than 500 parasites per µL. Two patients had negative tests for the presence of parasites by microscopy. A PCR for *P vivax*-species detection, which does not discriminate between *P vivax* and *P simium*, suggested the presence of *P vivax* in 38 patients (97%).

When compared with *P vivax* from the malaria endemic Amazonian regions, parasites from the Atlantic Forest diagnosed as *P vivax* were morphologically different (appendix). Trophozoites were pleomorphic but less amoeboid than those observed in *P vivax* (appendix). They had a large mass of chromatin and a more compact cytoplasm with malaria pigment (appendix). Usually stippling was mostly observed in infected cells with late developmental forms, but erythrocytes containing early trophozoites were also frequently stippled (figure 3A–F). Furthermore, developing schizonts contained fewer merozoites than in *P vivax* (figure 3G–L). The highest number of merozoites in mature schizonts was 14 (figure 3M). Gametocytes were round with compact cytoplasm and marked pigmentation (figure 3N–P).

Non-infected erythrocytes showed marked anisocytosis and poikilocytosis (figure 3). Poikilocytosis was represented mainly by acanthocytes, dacrocytes, and spherocytes, which occurred together on the same preparations (figure 3).

Analysis of the four usable mitochondrial genome samples from the 33 human cases used for DNA sequencing revealed that they shared identical sequences, and these were in turn identical to the mitochondrial genome sequence of *P simium* deposited at Genbank, which differs from the most closely related isolates of *P vivax* by two SNPs. Analysis of 794 full-length mitochondrial genome sequences from globally acquired *P vivax* samples showed that these SNPs were unique to *P simium*. A haplotype network tree (appendix) was constructed using these sequences, and shows that *P simium* is most closely related to the *P vivax* parasites of human beings isolated from South America.

On the basis of two informative SNPs that differentiate *P vivax* from *P simium*, we were able to diagnose an infection of *P simium* in 28 of 33 samples typed for their species (table). We were unable to achieve PCR amplification for the remaining five samples, because of technical constraints. The same informative SNPs were found in *P simium* infecting three local howler monkeys, MB CPRJ, RJ 30, and R J59 (table).

Discussion

The results of our study have important implications for public health and for the malaria elimination agenda. To our knowledge, this is the first demonstration of

P simium naturally infecting human beings in forest locations in a region considered to have eliminated transmission of malaria at least 50 years ago. The sudden increase in malaria cases in the past 2 years in that area is associated with the Atlantic Forest of Rio de Janeiro. No major environmental modifications appear to have occurred that might have modified the behaviour of *Anopheles* spp or monkeys during this

	Sample collection (year)	Age (years)	Sex	Main activity developed in the area	Visitor or resident	Entry into Atlantic Forest area	Time between onset of symptoms and diagnosis (days)	Triad of malaria*	Highest axillar temperature (°C)	Parasites density (mm³/μL)	*Plasmodium simium* SNPs†
Human samples											
AF 1	2015	34	Male	Photographer	Visitor	Yes	11	Yes	39·5	920	Yes‡
AF 2	2015	58	Male	Engineering	Visitor	No	12	Yes	38·6	560	Yes‡
AF 3	2015	50	Male	Geologist	Visitor	Yes	14	Yes	38·8	112	Yes‡
AF 4	2015	27	Male	Ecotourism	Visitor	Yes	15	Yes	38·1	1200	Yes
AF 5	2015	26	Male	Ecotourism	Visitor	Yes	14	Yes	39·0	64	Yes
AF 6	2015	51	Male	Inhabitant	Resident	Yes	14	No	38·5	480	Yes
AF 7	2015	40	Male	Ecotourism	Visitor	Yes	13	Yes	39·5	800	Yes
AF 8	2015	52	Male	Ecotourism	Visitor	Yes	10	Yes	39·8	416	Yes
AF 9	2015	29	Male	Ecotourism	Resident	Yes	6	Yes	38·0	64	Yes
AF 10	2015	35	Male	Architecture	Visitor	Yes	16	Yes	39·0	576	Yes
AF 11	2015	52	Female	Ecotourism	Visitor	Yes	12	Yes	38·8	320	Yes
AF 12	2015	48	Male	Inhabitant	Visitor	No	9	Yes	NA	208	Yes
AF 13	2015	52	Male	Forestal Garden	Resident	Yes	12	Yes	40·0	624	Yes
AF 14	2015	26	Male	Ecotourism	Visitor	Yes	13	Yes	40·0	128	Not determined§
AF 15	2015	44	Male	Ecotourism	Visitor	Yes	39	Yes	39·0	1296	Yes
AF 16	2015	59	Male	Ecotourism	Visitor	Yes	20	Yes	40·0	336	Yes
AF 17	2015	54	Male	Ecotourism	Visitor	Yes	6	Yes	39·8	96	Not determined§
AF 18	2015	39	Male	Ecotourism	Visitor	Yes	3	No	38·0	112	Yes
AF 19	2015	56	Male	Ecotourism	Visitor	Yes	16	Yes	39·0	480	Yes
AF 20	2015	22	Male	Engineering	Visitor	No	NA	No	39·5	80	Not determined§
AF 21	2016	82	Female	Tourism	Visitor	No	13	Yes	NA	3000	Yes
AF 22	2016	40	Male	Ecotourism	Resident	Yes	10	Yes	39·0	48	Yes
AF 23	2016	35	Female	Inhabitant	Resident	No	12	Yes	39·0	Negative	Yes
AF 24	2016	50	Male	Ecotourism	Visitor	Yes	14	Yes	NA	80	Yes
AF 25	2016	26	Male	Ecotourism	Visitor	Yes	9	Yes	38·5	672	Yes
AF 26	2016	55	Male	Ecotourism	Visitor	Yes	14	No	39·0	1160	Yes
AF 27	2016	54	Female	Ecotourism	Visitor	Yes	18	Yes	40·0	2600	Yes
AF 28	2016	54	Male	Ecotourism	Visitor	Yes	3	No	38·5	592	Yes
AF 29	2016	51	Female	Ecotourism	Resident	Yes	11	Yes	38·0	416	Not determined§
AF 30	2016	52	Male	Ecotourism	Visitor	Yes	16	Yes	41·9	384	Yes
AF 31	2016	54	Female	Ecotourism	Visitor	Yes	10	Yes	NA	80	Yes
AF 32	2016	72	Female	Ecotourism	Visitor	Yes	13	Yes	39·0	704	Yes
AF 33	2016	53	Male	Ecotourism	Visitor	Yes	NA	Yes	NA	144	Not determined§
AF 34	2015	39	Female	Ecotourism	Visitor	Yes	13	No	NA	48	Not done
AF 35	2015	7	Male	Tourism	Visitor	No	12	Yes	39·7	48	Not done
AF 36	2015	47	Male	Ecotourism	Visitor	Yes	18	No	38·5	122	Not done
AF 37	2015	53	Male	Ecotourism	Visitor	Yes	21	Yes	39·7	80	Not done
AF 38	2015	22	Male	Gardener	Resident	Yes	9	Yes	NA	Negative	Not done
AF 39	2016	42	Male	Ecotourism	Visitor	Yes	20	Yes	39·0	256	Not done

(Table continues on next page)

	Sample collection (year)	Age (years)	Sex	Main activity developed in the area	Visitor or resident	Entry into Atlantic Forest area	Time between onset of symptoms and diagnosis (days)	Triad of malaria*	Highest axillar temperature (°C)	Parasites density (mm³/μL)	*Plasmodium simium* SNPs†
(Table continued from previous page)											
Non-human primates											
ATCC 30130	1966	..	..	..	..	..	..	..	..	..	Yes‡
MB CPRJ	2013	..	..	..	..	..	..	..	..	..	Yes
RJ 30	2016	..	..	..	..	..	..	..	..	..	Yes
RJ 59	2016	..	..	..	..	..	..	..	..	..	Yes

SNP=single-nucleotide polymorphisms. NA=not available. ATCC=American Type Culture Collection. *Fever, chills or rigors, and sweating. †SNPs identified by partial mitochondrial genome sequencing. ‡SNPs identified by whole mitochondrial genome sequencing. §Unable to achieve PCR amplification because of technical constraints.

Table: **Clinical, epidemiological, and parasitological characteristics of studied samples and identification of *Plasmodium simium* SNPs through whole or partial mitochondrial genome sequencing**

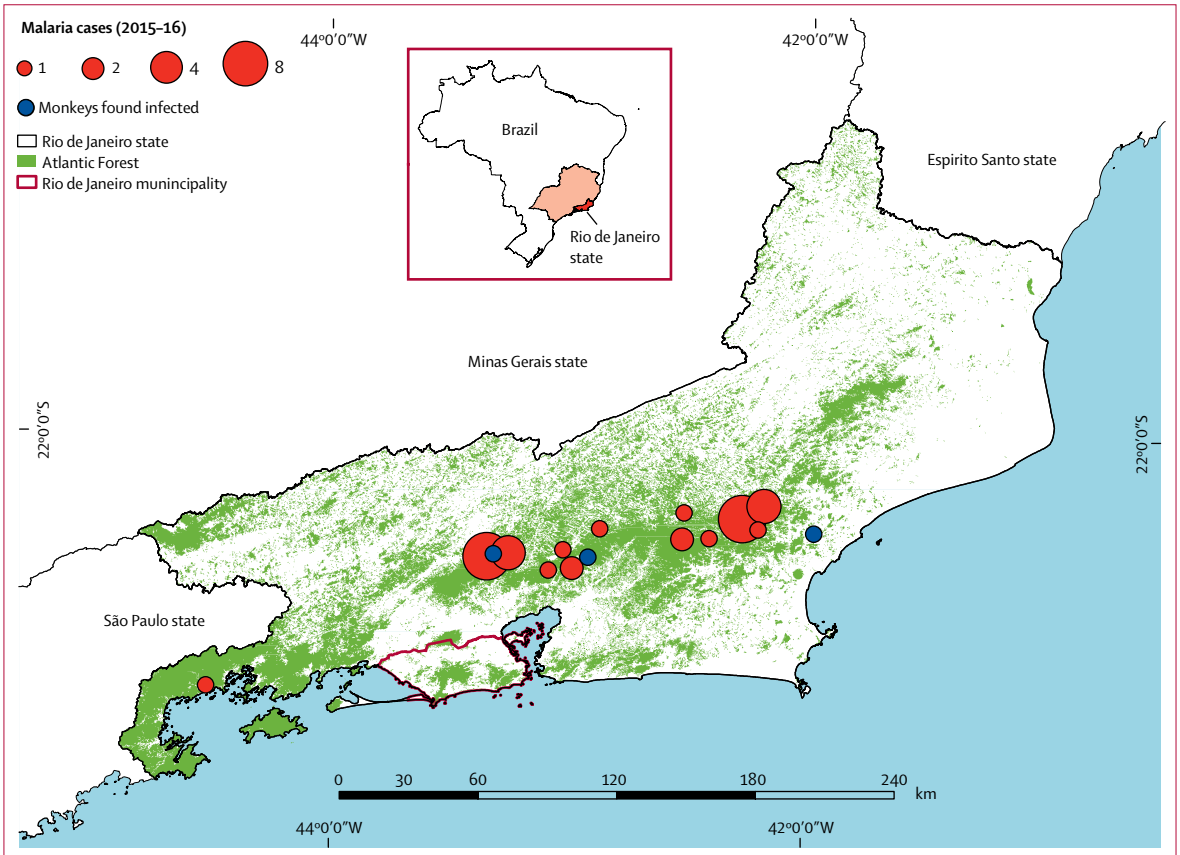

Figure 2: **Map of the Rio de Janeiro state, Brazil, showing the Atlantic Forest and indicating where human malaria cases of simian origin and monkeys infected with *Plasmodium simium* have been detected**

Human cases are represented by red spots of different sizes (symbolising one to eight cases), and the three captured, infected, wild howler monkeys are shown as blue spots. The extension of the area covered by the Atlantic Forest vegetation is indicated in green. All cases were reported in forest fragments located in Serra do Mar, and monkeys carrying *P simium* were found in the vicinity of each area. The municipality of Rio de Janeiro, delimitated with the red bold line, is free of malaria transmission.

time. However, the recent rise of ecotourism and the so-called back to nature movement might increase the opportunities for vector sharing between monkeys and human beings in this region. Despite increasing urbanisation, most of Brazil remains forested, with many human populations living in close contact with forests. The 2017 outbreak of sylvatic yellow fever in southeastern Brazil, a well established zoonosis that

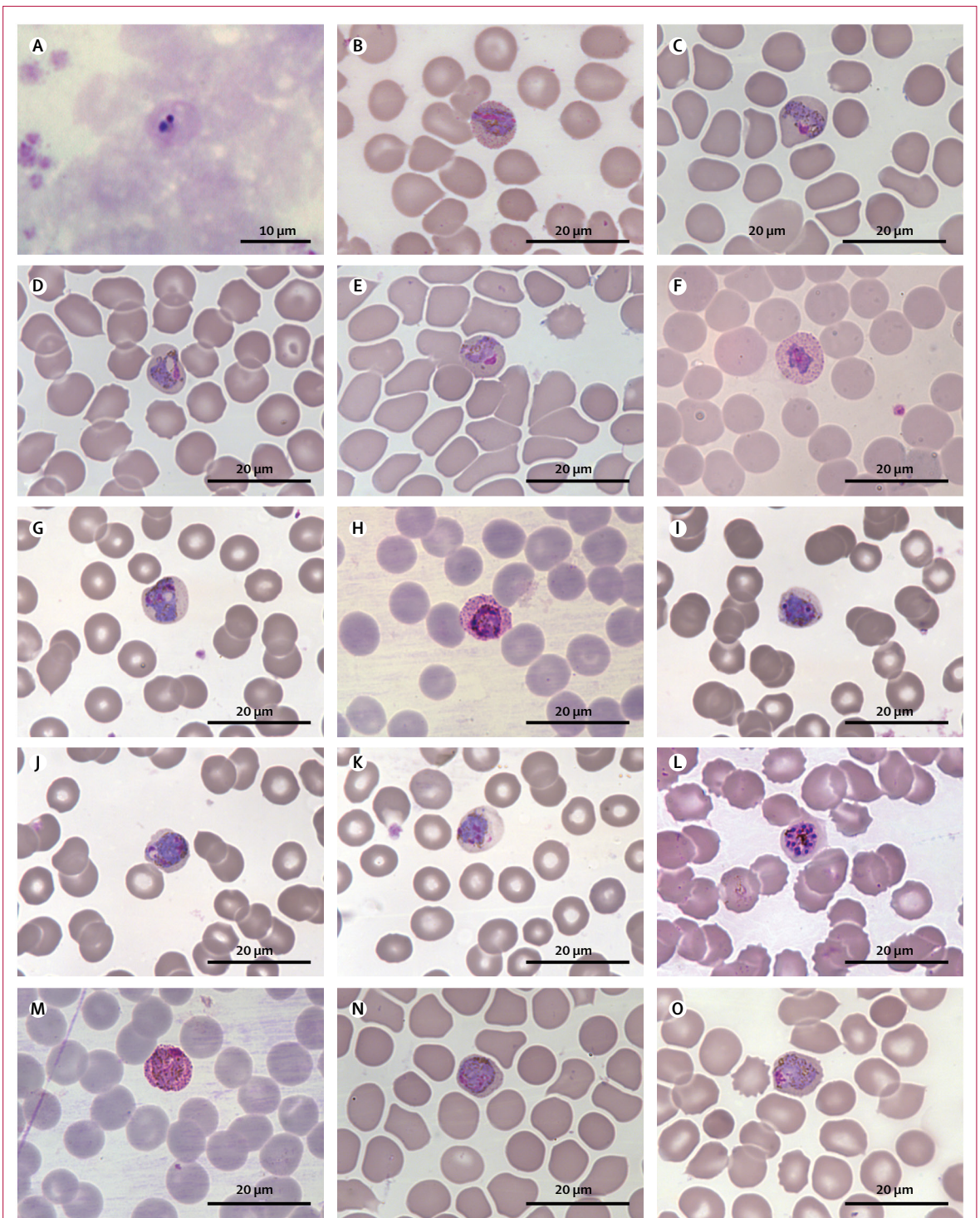

Figure 3: **Giemsa's solution-stained preparations of blood samples from human beings naturally infected with *Plasmodium simium* in Rio de Janeiro state, Brazil**
All preparations are thin blood films, except A (thick blood smear). (A) Early trophozoite. (B–F) Pleomorphic developing trophozoites. (G–L) Immature schizonts. (M) Mature schizont. (N–P) Gametocytes.

affected at least five Brazilian states, should raise concern for the possibility of the extension of occurrence of zoonotic malaria, because of the resemblance of the environmental and demographic characteristics in which both infections occur.[18] Further research is needed to elucidate these aspects.

P simium, a tertian malaria parasite found in New World non-human primates was first identified in 1951 in a monkey from the state of São Paulo and appears to be restricted to the Atlantic Forest regions of southern and southeastern Brazil.[5,19,20] Fonseca (1951),[21] Garnham (1966),[22] and Deane and colleagues (1966)[4] highlighted the morphological differences between *P vivax* and *P simium*; the trophozoites of *P simium* being less amoeboid and with coarser and more precocious and very prominent Schüffner's dots than *P vivax*. Garnham (1966)[22] reported that the detection of stippling in *P simium* early parasitised cells depends on the staining procedures. These morphological characteristics of *P simium* are consistent with those described here for the infections of human beings from the Atlantic Forest.

Although the initial diagnoses for these infections was *P vivax*, molecular evidence has revealed that these parasites are *P simium*. This misdiagnosis of a zoonotic non-human primate malaria parasite as a human parasite species has precedent and parallels the discovery of the large focus of *P knowlesi* in Borneo, which was initially attributed to *P malariae* on the basis of morphological characteristics.[23]

Despite the apparent genetic similarity of *P simium* to *P vivax*, attempts at inducing infections of *P simium* in human beings under laboratory conditions have been unsuccessful.[24] In 1966, however, Deane and colleagues[4] described the infection of a man with a *P vivax*-like parasite that they considered to be *P simium* on the basis of morphological characteristics of the parasite and because infection had occurred in a forest reserve outside São Paulo, where *P simium* was known to be transmitted. This infection remains the only previous case report of a possible human infection with *P simium*.

The clinical and parasitological features of our cases reveal that the pyrogenic threshold of *P simium* infection is considerably low. Whether this low fever threshold is related to the naive status of the individuals or specific parasitic-associated characteristics (eg, GC-content and other inflammatory factors) are yet to be better investigated.[25,26]

Patients who were naturally infected with *P simium* reported clinical symptoms congruent to symptoms of *P vivax* malaria, and responded successfully to chloroquine and primaquine, with no hospital admission, relapses, or deaths. It is not known whether *P simium* is capable of producing hypnozoites in human beings and, thus, relapses, as does *P vivax*. However, one patient (AF 3) who was treated solely with chloroquine because of G6PD deficiency and one other patient (AF 21) who discontinued primaquine treatment due to adverse events did not present any symptomatic relapse and were always negative for *Plasmodium* in all parasitological and molecular tests done during 18 months' follow-up. Further studies will be required to establish if *P simium* is capable of producing hypnozoites.

Whether this parasite can be transmitted from person to person is not known. All patients who presented with disease had entered the forest or visited the forest surroundings inhabited by howler monkeys, the main host of *P simium*. Case clustering occurred only when patients had entered such regions together, and in these cases the same time to onset of disease symptoms was observed. Although gametocytes were detected in blood smears of *P simium*-infected individuals in the present study, the infectivity of human infections of *P simium* mosquitoes is yet to be determined. Vector competence of primatophilic mosquitoes other than *A K cruzii* for *P simium* has not been studied and is a subject that needs to be urgently addressed.

Thorough screening of a large number of the local non-human primate and mosquito (*Anopheline*) populations in this area is required to evaluate the extent of this newly recognised zoonotic threat to public health. Moreover, one limitation of this study is the inclusion of samples from only one state covered by the Atlantic Forest. The analysis of both human and non-human primate samples from other areas that have been collected at different times will clarify whether the SNPs used to distinguish *P vivax* from *P simium* are specific to this region and this specific timeframe. However, the ATCC monkey sample was collected in a different region and time (50 years before) and it contains the same *P simium*-specific SNPs observed in the Rio de Janeiro Atlantic Forest. The small number of sequences from *P simium* hampers further analysis, and precludes drawing any conclusions regarding the evolution, natural history, and species status of this parasite.

This unequivocal demonstration of zoonotic *P simium* transmission leads to the possibility that this parasite, consistently misdiagnosed as *P vivax* because of an absence of molecular typing techniques, has always infected human beings in this region. Alternatively, it might be the case that *P simium* has only recently acquired the ability to frequently infect human beings, and this scenario has extremely important implications in terms of parasite–host relationships and evolution.

In summary, we report that the malaria outbreaks in 2015 and 2016 in the Atlantic Forest of southeastern Brazil were caused by *P simium*, previously considered to be a monkey-specific species of malaria parasite that is related to but distinct from *P vivax*, and which has never conclusively been shown to infect human beings before. Such zoonotic transmission of a malaria parasite from a monkey reservoir to human beings has immediate consequences for public health in this region, and for future attempts to control and eventually eliminate malaria in Brazil. Thorough screening of the local non-human primate and mosquito (*Anopheline*) populations in this area is required to evaluate the extent of this newly recognised zoonotic threat to public health.

Contributors

PB and CTD-R conceived the study. PB and AMS clinically followed-up the patients and AdP-C and CBJ obtained patients' data. FVSdA, RL-d-O, DAMdA, CBJ, AdP-C, and AP worked with the non-human samples. ACFdSS and CLP provided data from the National Program of Malaria Control from the Brazilian Ministry of Health. SS and GMZ examined (and RL-d-O reviewed) the microscopic slides and analysed the parasitological data. RL-d-O and SS contributed to the description of parasite morphological characteristics and SS did the slide photographs. MP-M described the red blood cell morphological characteristics. DAMdA and CFAdB undertook the molecular diagnosis of non-human primate samples. MdFF-d-C undertook the molecular diagnosis of human samples. ALLA carried out the mitochondrial genome sequence. MGZ, DAMdA, CFAdB, PC, MdFF-d-C, and RC did the analysis and interpretation of molecular data, and MGZ and RC did the DNA sequence analysis and the haplotype network in human and non-human primate samples. FVSdA and RL-d-O captured, made parasitological analysis, and interpreted non-human primate data. HGA and MCSM did the geographical description of the Atlantic Forest sites and the maps. PB, AdP-C, AMS, CFAdB, RL-d-O, RC, and CTD-R drafted and finalised the manuscript. All authors read, made suggestions, and approved the final manuscript.

Declaration of interests

We declare no competing interests.

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