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Autochthonous simian malaria in Brazil outside the Amazon: Emergence, zoonotic transmission and implications for disease control

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ABSTRACT

Although human malaria is endemic in the Brazilian Amazonian region, autochthonous cases are registered regularly outside this region in areas under the couverture of the Atlantic Forest biome. The infecting species in the Atlantic Forest was initially believed to be the classical *Plasmodium vivax*. However, these locations have epidemiological characteristics that contribute to maintaining zoonotic monkey malaria, showing a great adaptation to different hosts, and many years later, it was discovered that almost all human malaria cases in the Atlantic Forest correspond to *P. simium* zoonosis. This review reported the history of discovering human infections by parasites originating from non-human primates in Brazil. It also examines epidemiology and underscores the need for specific preventive measures in the malaria elimination era. The data gathered so far have demonstrated that several factors enable zoonotic disease transmission in these areas. Given the facilitating ecological aspects involved and the scarce knowledge of the disease by the populations of the non-endemic area, this scenario adds difficulty to the challenge of eliminating malaria in Brazil.

1. The disease

Considered the most important parasitic disease in the world, malaria has endured for centuries in our history and continues to be a major public health problem. Despite the efforts of authorities to invest in national malaria control and prevention programs in 85 endemic countries, there were around 249 million human cases worldwide in 2022, an increase from the previous year [1].

Malaria is an acute febrile infectious disease caused by protozoa of the genus *Plasmodium*. It is transmitted by the inoculation of *Plasmodium* spp. parasites through the bite of an infected female of an *Anopheles* mosquito [2]. The human disease is manifested by the malaria triad: fever, chills, and sweats, frequently associated to headache. It can also cause general symptoms such as malaise, muscle pain, nausea, and dizziness. The clinical picture can vary, being mild, moderate, or severe, depending on the infecting species and the individual's immunity [3].

2. The malaria diagnosis

The microscopic diagnosis through the thick blood smear method (TBS) is considered the gold standard for malaria diagnosis and assesses the parasite density [3]. In addition, rapid diagnostic tests (RDTs) based on an immunochromatographic method identify specific parasite antigens using antibodies. These tests can be easily used but have lower sensitivity than TBS. PCR is the technique of choice for reference diagnosis, cases of mixed infection, low parasitized individuals, and when a significant number of samples must be processed, such as screening in blood banks and epidemiological surveillance [4,5]. The immunological techniques are not recommended for diagnosis but for epidemiological studies to identify previously infected individuals [3].

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3. *Plasmodium* species

The human *Plasmodia* have a zoonotic origin from our nonhuman primate (NHP) ancestors [6].

More than 200 *Plasmodium* species worldwide infect many animals, including birds, bats, primates, lizards, ungulates, and rodents [7]. Of all of them, five species are considerable causative agents of human diseases since human population sustains their transmission dynamics: *P. malariae* (Laveran, 1881), *P. vivax* (Grassi; Feletti, 1890), *P. falciparum* (Welch, 1897), *P. ovale curtisi* (Stephens, 1922) and *P. ovale walikeri* (Sutherland et al., 2010) with some recent evidence of *P. knowlesi* (Sharp et al., 2020) [8].

It is well-known that *Plasmodium* species are generally host-specific. However, some species that cause the disease in non-human primates (NHP) can infect humans when the distribution of infected NHPs and competent anopheline vectors that of human settlements in enzootic areas [9].

Until now, 27 known *Plasmodium* species infect NHPs. Six out of them have been experimentally transmitted to humans, and some have already been described to naturally infect humans: *P. cynomolgi* (Ta et al., 2014), *P. knowlesi* (White, 2008), *P. inui* (Coatney et al., 1966), *P. brasilianum* (Gonder and Von Berenberg-Gossler, 1908), *P. simium* (Da Fosenca, 1951) and *P. schwetzi* (Contacos et al., 1963) [10]. However, in Brazil, only *P. simium* and *P. brasilianum* were found naturally infecting humans [8].

All mammalian *Plasmodia* are transmitted by *Anopheles* mosquitoes, in which the sexual reproduction of parasites takes place.

4. Brazil and worldwide

P. vivax and *P. falciparum* are responsible for most cases worldwide (WHO, 2021) [11].

P. falciparum is the leading cause of malaria deaths, especially in children under five and pregnant women in Africa. *P. vivax*, on the other hand, is the most widely distributed species worldwide, predominantly in the Americas with 72 % of cases, Southeast Asia with 45.7 %, the Western Pacific with 27.3 %, the Eastern Mediterranean with 29.4 % and Africa (0.5 %). With the disproportionate increase in cases in Africa compared to the other continents, *P. vivax* currently holds 2.8 % of global cases [1].

In Brazil, more than 99 % of malaria cases in the country occur in the Amazon region. *P. vivax* is the most prevalent species, accounting for 82.70 of the country's cases in 2023. This year, there were 140,268 disease cases, and the Indigenous areas were the most affected, representing 39.2 % of the cases, followed by rural populations (32.7 %) and mining areas (14.2 %). [12,13]. This data shows the challenge of controlling the disease, given the difficulty of accessibility in these regions [13].

5. Brazilian extra-amazon region

In addition to the Amazonian context, where the vectors belong to the subgenus *Nyssorhynchus,* whose immature stages develop mainly in large water bodies like streams and dams, autochthonous human cases can also occur in the extra-Amazonian region. These occurrences have been essentially associated with transmission in the Atlantic Forest biome where the vectors are bromeliad breeding anophelines of the *Kerstezia* subgenus, the etiological agent is *P. simium,* and the reservoirs are NHP [14,15]. In these locations, the infection, considered to have a zoonotic origin, is known as “bromeliad malaria” because of the vector larval habitats in the typical and abundant plants of the region (Fig. 1). Moreover, the acrodendrophilic behavior of these vectors facilitates the transmission of the infection between monkeys living mainly in the canopy of trees. Still, they may also bite humans on the ground, in the forest, or nearby, thus maintaining transmission as a zoonosis [16].

Morphologically, *P. simium* is very similar to *P. vivax. P. brasilianum* and *P. malariae,* another New World monkey malaria parasite that is also infective to humans, have been considered a unique species of *Plasmodium.*

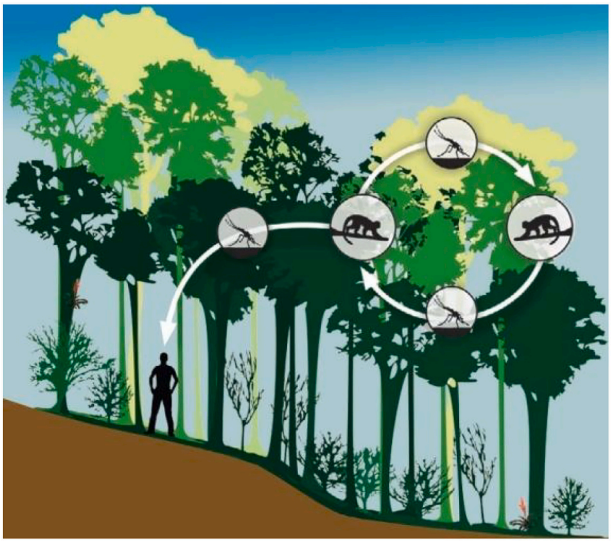

Fig. 1. Zoonotic “bromeliad-malaria” cycle. (Adapted: Pina-Costa et al., 2014).

This remarkable morphological, immunological, and molecular similarity has made accurate diagnosis difficult. Because of this, zoonotic malaria may represent an additional challenge in controlling the disease in Brazil. Thus, understanding this zoonosis is necessary to define appropriate prevention and intervention strategies [17].

Of the 26 Brazilian states and the Federal District, 17 have the Atlantic Forest biome: Alagoas, Bahia, Ceará, Espírito Santo, Goiás, Mato Grosso do Sul, Minas Gerais, Paraíba, Paraná, Pernambuco, Piauí, Rio de Janeiro, Rio Grande do Norte, Rio Grande do Sul, Santa Catarina, São Paulo and Sergipe. Among the reported cases Espírito Santo had the majority figs. (31 municipalities out of 78–39 %) [7;8;9], followed by São Paulo (34 out of 645–5 %) [1;2;3], Paraná (33 out of 399–8.2 %) [12]), Rio de Janeiro (18 out of 92–19.5 %) [12], Minas Gerais (24 out of 853–2.8 % [12]), Santa Catarina (11 out of 295–3.7 %), and Rio Grande do Sul (11 out of 497–2.2 %) [12].

However, using the extra-amazon region data of the Brazilian Notifiable Disease/Malaria Information System (SINAN) and considering as the Atlantic Forest/bromeliad malaria cases only those occurred in municipalities with the Atlantic Forest biome to differentiate the introduced from imported cases of the Amazon region [12], Espírito Santo, São Paulo, Paraná and Rio de Janeiro detain the largest number of cases among the 1120 cases recorded in SINAN from 2007 to 2023 (Fig. 2).

This review summarizes the status of human-derived-simian malaria in Brazil, focusing on *P. simium* and *P. brasilianum* in the non-endemic regions of the country.

THE BEGINNING.

In 1909, the first species of simian *Plasmodium* infecting monkeys in the Americas was discovered. *P. brasilianum* was found infecting the monkey, *Cacajao calvus*, native to the Brazilian Amazon region, and then in different NHP species years later. This first description already reported the tremendous morphological similarity of this parasite with *P. malariae*. Although, at the time, researchers tried unsuccessfully to inoculate this parasite to humans (Gonder and Von Berenberg-Gossler, 1909; Von Berenberg-Gossler, 1909; Seidelin, 1912), it was later confirmed that *P. brasilianum* was experimentally infective to humans either through inoculation of blood forms or by infected mosquitos' bites [18–20].

In malaria cases from remote Yanomami indigenous communities in Venezuela, naturally acquired infections in humans with parasites termed *P. brasilianum* were reported for the first time in the Amazon in

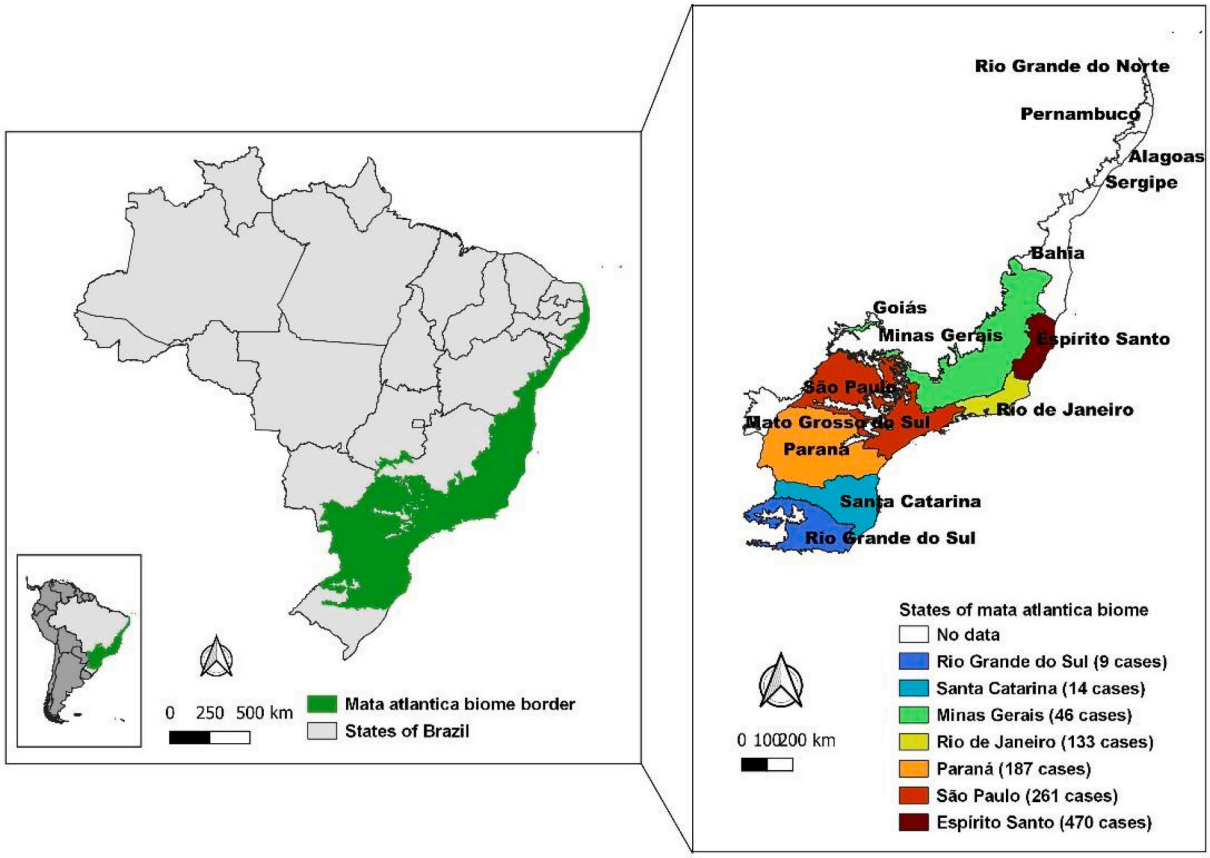

Fig. 2. Number of Atlantic Forest malaria cases from 2007 to 2023 in Brazil's south and southeast regions, according to SINAN.

2015 [21]. Based on 18S rRNA gene sequencing, 12 patients harbored malaria parasites, which were 100 % identical to *P. brasilianum* isolated from the monkey *Alouatta seniculus.* The authors suggest that quartan malaria parasites are easily exchanged between humans and monkeys in Latin America and propose that *P. brasilianum* may have a nomenclatorial revision if further research confirms these findings. Although the *P. brasilianum* can infect many monkey species, data about human cases in Brazil are scarce. Particular efforts should be made in this sense [21].

P. simium was discovered in America in 1951 through the reanalysis of a yellow fever study in NHP in 1939 when one of the animals was *Plasmodium*-infected. At the time, they thought it was a *P. brasilianum* infection. Only 11 years later,in 1950, after re-reading the slides, they realized it was a new species. The infected monkey was an *Alouatta fusca* (Fig. 3) from Itapecerica, in São Paulo. This parasite showed significant morphological similarity to *P. vivax* [22].

Fig. 3. *Alouatta fusca* (Source: Janaina Paula Black– Pontifícia Universidade Católica do Rio Grande do Sul).

In 1966, Deane and colleagues reported the first human infection by *P. simium* [23,24]. The infection occurred during an entomological survey in the Cantareira Forest Reserve in São Paulo. This study was carried out from February 1964 to March 1966 in a location where human malaria had never been recorded. However, simian malaria was enzootic, and 80 % of howler monkeys presented malarial pigment in their spleen or parasite in blood smears. Since the aim was to investigate how the disease was transmitted between native monkeys of the region, mosquitoes were collected on platforms set up at different heights in the forest. The mosquitoes were collected at ground level and the canopy of trees, using humans as bait. In total, 3887 mosquitoes were captured; 30 % were *Anopheles* (Kerteszia) *cruzii* (Fig. 4), considered the main vector of bromeliad malaria; 698 *An. cruzii* were dissected, and 12 contained sporozoites. The sporozoites were inoculated into *Saimiri sciureus, an Amazonian* monkey, but the monkeys did not become infected [24].

Meanwhile, one of the local inhabitants who used to collect mosquitoes at the forest canopy presented the malarial triad and tertian fever, which were associated with vivax malaria cases. A parasite similar to *P. vivax* was detected by microscopy in blood smears. But, considering the epidemiological aspects of the region, the presence of the mosquito vector *An. cruzii* and the high prevalence of howler monkeys in the area, the first case of zoonotic malaria in Brazil and the second naturally transmitted all over the world, were described [24].

Since then, reports of autochthonous human cases in the Atlantic Forest biome were done as suspected of zoonotic origin. For example, it was reported that in the summer of 1993, three mosquito collectors contracted malaria during an entomological survey in the Serra do Mar region of the São Paulo state. The collections took place over three days

Fig. 4. *Anopheles Kersteszia cruzii* (By Genilton Vieira. Source: Pina-Costa et al., 2014).

and totaled 1170 *An. cruzii*, the only anopheline present in the area. The individuals had low parasitemia and were diagnosed as being infected with *P. vivax* using a TBS test [25].

Coincidentally, in the same summer of 1993, an outbreak of malaria was reported affecting seven people among visitors and residents of different small settlements in the Atlantic Forest in Nova Friburgo, Rio de Janeiro, where there had never been any record of human malaria. The zoonotic transmission was suspected because *An. cruzii* was predominant in the area. No imported index case was detected, the patients had not traveled to endemic areas, and they presented very low parasitemia microscopically identified as *P. vivax* [26].

More than 20 years later, an unexpected outbreak occurred in Rio de Janeiro in 2015–2016, affecting nine municipalities surrounded by Atlantic Forest. In total, 49 people were infected, a figure exceeding the total number of cases reported in the past decade. At the time, it was diagnosed as *P. vivax / P. simium*, and later, through a specific protocol, it was confirmed to be an outbreak of *P. simium* zoonosis [17].

In 2021, it was reported that one autochthonous human case was diagnosed in Três Forquilhas, Rio Grande do Sul, in the Atlantic Forest. The diagnosis was made by TBS and PCR and was positive for *P. vivax/P. simium.* This finding was an alert signal, as there had been no report of autochthonous human malaria in the state for over 40 years. The patient reported never having been to a malaria-endemic area. The coexistence of the vector *An. cruzii* and howler monkeys in the region led to the diagnosis of *P. simium* zoonotic malaria [27].

6. Entomological studies

Several studies have been carried out over the years to characterize the anopheline's potential vectors of simian, human, and zoonotic malaria in the Atlantic Forest.

In 1984, Deane and co-workers conducted one study to analyze the vertical dispersion of *An. cruzii* in Tapera Atlantic Forest, of Santa Catarina. Mosquitoes were collected at ground level and in the forest canopy, colored with specific colors according to the height, and released back into the forest, with those collected in the canopy stained yellow and those on the ground red. A few days later, examining the colors of the recaptured anophelines, it was possible to trace the species' vertical dispersion in that region where the mosquitoes can move from the canopy to the ground and vice versa. This result reveals that monkey *Plasmodia* could be transmitted to humans by *An. cruzii* in the Atlantic Forest [28].

More than ten years later, in 1997, a study to investigate anopheline infection in the state of São Paulo was conducted in the municipalities of Juquitiba and São Vicente, where sporadic autochthonous human cases, usually associated with mild or asymptomatic clinical profiles, were diagnosed. A total of 2278 mosquitoes (1717 from São Vicente and 1161 from Juquitiba) were collected, and all were identified as *An. cruzii*. Two mosquito samples from São Vicente were found to be infected by *P. vivax* and one from Juquitiba by *P. vivax* VK247. Despite the apparent low anopheline infection rates, three people who captured the mosquitoes became infected during the study [25,29].

In a study, 2290 mosquitoes were collected from March 2004 to February 2006 in the mountainous region of Espírito Santo in 2009. The most prevalent species was *An. cruzii,* accounting for 46.9 % of collected specimens. Other potential vector species of the *Nyssorhynchus* subgenus were also found during the study. The frequency of *An. cruzii* was higher in the forest canopy, while on the ground the predominant species was *An. (Nyssorhynchus) strodei* [30]. PCR-infected mosquito detection identifies that *An. cruzii* is the main vector in the region, and since the infected females were caught in the canopy, the probability of human zoonotic malaria was raised. Species of the subgenus *Nyssorhynchus* could also participate in transmitting the disease because they were infected by *P. vivax/P.simium* in places near human dwellings [30].

Years later, in 2013, the role of *An. cruzii* was reinforced as the main vector, and some *Nyssorhynchus* species were found to be secondary in the transmission. They were infected with *P. vivax / simium* and/or *P. malariae / brasilianum*. The entomological study gathered 6703 anophelines in urban, transition, and forest zones (on the ground and top) in the Parelheiros area of São Paulo state, which had recorded 64 autochthonous cases in the past five years. Although some locations were rural communities, the region was undergoing constant urban expansion, which could increase the spread of asymptomatic individuals. Comparing malaria cases registered in the area with the entomological findings, silent asymptomatic transmission was suggested in the region, and the hypothesis of human zoonotic transmission with monkeys as reservoirs was raised once again [31].

An. cruzii mosquitoes also predominate (99.8 %) in a survey carried out in Juquitiba, São Paulo, in 2014, which aim was to evaluate the feeding and infectivity of anophelines in peridomicile sites of homes with autochthonous human malaria cases. *An. cruzii* mosquitoes were found infected with *P. vivax* and *P. malariae*. Intriguingly, no captured mosquito was fed on monkeys because only human blood was detected, suggesting that asymptomatic patients may be a source of anopheline infection [32]. On the other hand, the chance of catching mosquitoes eventually feeding on monkeys in the surrounding area of houses is meager since the main reservoir, howler monkeys, usually live deep in the forest. What would drive an anopheline recently fed on a howler deep in the forest rich in bromeliads to disperse and rest in the peridomicile area? (Fig. 5)

Fig. 5. Bromeliads from the Atlantic Forest on the slopes of Serra do Mar on the north coast of Rio Grande do Sul (Source: Luiz Filipe Varella, 2010).

In the municipality of Santa Teresa, Atlantic Forest in Espírito Santo, in 2018, once again, *An. cruzii* was considered the primary malaria vector. It was the more frequent mosquito in the forest canopy than in places near the edges of the forest and dwellings. In closer places to human areas, the *An. (N.) strodei* and *An. (N.) triannulatus* were the most common. *An. cruzii* (77 %) and *Nyssorhynchus* (23 %) mosquitoes were infected by *P. vivax/P. simium* [33].

More recently, in a study carried out in a forest reserve in the Serra do Mar, São Paulo, where two autochthonous tourist cases were recorded in 2018 and 2022, 97 % of the mosquitoes collected were *An. cruzii*, a mosquito was infected by *P. vivax/P. simium,* while a mosquito pool was infected by *P. malariae/P. brasilianum*. Surprisingly, although a genome fragment compatible with *P. falciparum-like* parasites had been detected in two pools, no human infection by this species was detected in the area. The mosquito infection rates were lower than expected (0.15 %), possibly related to howler monkeys' density reduction, the main reservoirs for simian malaria, due to yellow fever 2016–2019 outbreaks [34]. But, since infected mosquitoes were still found in this scenario, other NHP reservoirs were suspected, such as Southern Muriqui and Capuchin monkeys, because they were frequent in the region and had already been infected by *P. simium* and *P. brasilianum* [15,34].

7. Serological studies

Over the years, researchers have conducted studies to analyze the prevalence of antibodies in the resident population to gain a more in-depth understanding of disease transmission in the locations.

In 1988, a survey was reported in Peruíbe, São Paulo, where human cases were associated with bromeliad malaria due to the presence of vectors, monkeys, and bromeliads. By indirect immunofluorescence (IFAT) against *P. vivax*, 1.3 % of the 11,051 non-infected residents had anti-*P vivax* IgG antibodies [36].

Years later, in 1997, in locations with recurrent suspected zoonotic autochthonous cases in São Paulo State (Vale do Ribeira and Serra do Mar), a high prevalence of antibodies by IFAT associated with a low occurrence of symptoms and parasitemia in the individuals strongly suggested the transmission of simian parasites to the resident population (Fig. 6) [37].

Later, the same group reported another serological investigation, this time in two parks of Vale do Ribeira: Parque Estadual Intervales and Parque Estadual Turístico do Alto Ribeira (PETAR), in 2006. The expressive prevalence of antibodies against the asexual forms of *P. falciparum* and *P. vivax* in the residents and no known introduced or imported malaria case suggested that monkeys could be reservoirs for focus transmission maintenance [38].

An investigation in this same line, between April 2001 and March 2004, involving 65 patients diagnosed with malaria and 1777 residents, was conducted in Espírito Santo state. At least 50 % of the patients and 36 %of the 1702 residents reacted with one plasmodial antigen by ELISA, and 40 % responded to one *P. vivax* variant and with *P. malariae* simultaneously, demonstrating that many residents have already had contact with both species. These findings, together with the presence of NHP and anophelines species common in bromeliad malaria regions, raised, once more, the possibility of the simian origin of the infection [39].

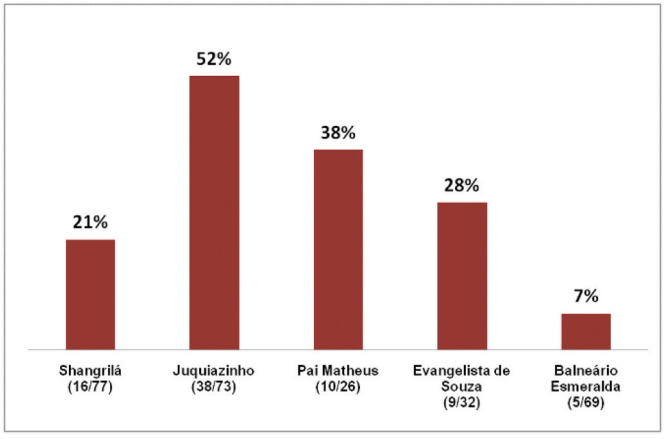

Fig. 6. Percentage of individuals positive for IgG against *P. vivax* by indirect immunofluorescence. (Adapted from Curado et al., 1997).

The first report of *P. vivax* and *P. malariae* autochthonous extra-Amazon cases in pregnant women living in low-endemicity areas was done in Juquitiba, an Atlantic Forest region in the São Paulo state, in 2014. Among the participants, low *P. vivax* or *P. malariae* parasitemia was detected by microscopic examination (TBS). By PCR, more individuals had positive results: two for *P. vivax* and three for *P. malariae*. In ELISA, 44 % of the participants had IgG antibodies against *P. vivax* and 6.4 % against *P. falciparum*, and in IFAT, 18 % revealed IgG antibodies against *P. malariae*. The importance of using molecular diagnostic methods in regions of low endemicity was stressed and pointed to the continuous exposure of pregnant women to the parasite [40].

In Rio de Janeiro state, residents from the municipality of Guapimirim were tested by ME, PCR, IFAT and ELISA. Asymptomatic autochthonous *Plasmodium* infections, not detected by TBS, were diagnosed in 2.8 % of the 324 individuals by PCR, with one case of *P. falciparum* (0.3 %), two cases of *P. vivax* (0.6 %), and six cases of *P. malariae* (1.9 %). Serological tests performed on 314 individuals showed participants reacting with *P. vivax* (7.7 %) and *P. falciparum* (1.3 %). Due to operational problems, only 42 samples were tested for *P. malariae* (30.9 %). Notably, 73 % of the participants did not know about the disease's transmission and prevention [41].

8. Studies on non-human primates

P. simium is known to naturally infect New World monkeys of the *Atelidae* and *Cebidae* families, of the genera *Alouatta* (howler monkeys), *Brachyletes* (muriquis), *Cebus* and *Sapajus* (capuchin monkeys). The infection is restricted to NHP living in the country's south and southeast regions, covered mainly by the Atlantic Forest biome [15,42] (Fig. 7). On the other hand, *P. brasilianum* infects around 35 species and has been detected in southeastern and northern Brazil, essentially in areas under the influence of the Atlantic and Amazon Forests, respectively. It also occurs in other Latin American countries [43].

In the 1960s, Deane began a series of surveys nationwide searching for simian *Plasmodia*. This large-scale project took place between 1964 and 1973, in addition to other complementary works carried out by different investigators at the end of the 1980s [35,44–53].

The latest studies were conducted during hydroelectric plant constructions, which allowed many animals to be examined quickly [50,53]. Arruda, for example, managed to analyze around two thousand NHP of nine species in just three months in the state of Pará. This was possible because the animals that previously lived in these locations had to be relocated to non-flooded areas. Malaria parasites morphologically similar or identical to *P. brasilianum* were detected in 10 % of animals of seven NHP species, including tamarins (*Saguinus midas niger*) (3.7 %), but with the highest prevalence found among howler species (12–22.2 %) [50].

A review by Deane [35] summarized the studies undertaken between 1976 and 1990 [49,50,52,53]. Overall, the research on NHP took place in 118 locations across the country, and of the 26 Brazilian states, only two were left out — Rio Grande do Norte and Paraíba. Overall, 4585 NHP were examined, of which 655 were infected with *Plasmodium*. The infection rate of these animals was quite variable between regions, as shown in Table 1 [42].

Still benefiting from the construction of hydroelectric in the Brazilian Amazon, Lourenço-de-Oliveira & Deane in 1989 examined by microscopy the blood of 378 NHP from 14 species of 10 genera, 126 of which

Fig. 7. Atlantic Forest Area in Rio de Janeiro, Parque Nacional da Serra dos Órgãos. (Source: Instituto Chico Mendes de Conservação da Biodiversidade).

Table 1
Simian *Plasmodium* in Brazil: frequency according to regions detected by blood smears. (Adapted from Deane, 1992).

1937–1990 *							
Regions	Non-Human Primates (n)	*Plasmodium*	*P. brasilianum*	*P.simium*	*P.brasilianum* + *P. simium*	*P. brasilianum* + *Plasmodium*sp	*Plasmodium* sp
Amazon	3472	350	347	–	–	3	–
Northeastern	146	–	–	–	–	–	–
Southeastern	764	272	126	102	26	–	18
Southern	184	33	11	14	6	–	2
West-Central	19	–	–	–	–	–	–
Total	4585	655	484	116	32	3	20

* :fromDeane (1976) [49], Arruda (1985) [50], Lourenço-de-Oliveira (1988, 1990, 1995) [51–53].

were captured in Balbina, State of Amazonas, and 252 from Samuel, Rondônia, in 1988 and 1989. *P. brasilianum* was the only detected malaria parasite infecting 15.8 % and 9.9 % of animals, respectively. The highest prevalence was observed in bearded sakis (*Chiropotes*) and spider (*Ateles*) monkeys (50 %) and howlers (33.3 %) from Balbina, followed by spider (28.6 %) and squirrel (21 %) monkeys from Samuel. No marmosets, tamarins, or owl monkeys were infected [52].

These studies proved at the time that the only species in the Amazon was *P. brasilianum*, while only *P. simium* was found in the southern region. Both species were present in the southeast, with *P. brasilianum* having the highest number of infections [42].

An update of the records of Dunn and Lambrecht (1963), Deane (1976), Brack (1987) and WHO (1987) of the New World NHP detected with natural infection were listed by country [51 - Table III].

In 2006, a seven-year survey was published reporting results of malaria serology in 777 wild monkey samples. The research was conducted in three different Brazilian ecological regions: the Cerrado (51/Goiás), the Atlantic Forest (114/São Paulo), and the Atlantic Semideciduous Forest (612/São Paulo and Mato Grosso do Sul). Even though the regions studied were non-endemic, there was a high prevalence of antibodies in the monkeys in all areas studied, indicating that they were exposed to infected mosquitoes [54].

Another study on NHP was published in 2013 to assess *Plasmodium* infection in monkeys in Rondônia state (Amazon region). A total of 184 animals were evaluated, 135 wild and 49 captives. The samples were submitted for microscopy and PCR to identify *P. falciparum, P. vivax / P. simium,* and *P. malariae / P. brasilianum*. Only three samples tested positive on microscopy, while 20 tested positive on PCR. Of these, 16 were positive for *P. brasilianum* and 2 for *P. falciparum*. No sample tested positive for *P. vivax /P. simium*, reinforcing Deane's previous findings [55]. Besides these findings, the authors reported a monkey species from the family *Aotidae – Aotus nigriceps-* infected with *P. brasilianum* for the first time. This discovery indicates that probably other monkey species that we still do not know are susceptible to the parasites and reinforces the importance of studies on NHP [5]. In fact, in 1985, a closer monkey species – *Aotus vociferans* – was already infected with *P. brasilianum* in Peru, a country near Rondônia state [56].

Also, in 2013, another study regarding *Plasmodium* infection in New World monkeys in regions under anthropogenic actions of the Amazon region was published. The researchers wanted to know the potential epidemiological consequences of human interference in the monkey's habitat. Samples from these animals were obtained from January 2010 to February 2011, resulting in 19 samples from two states: Amazonas (10) and Rondônia (9). These samples were submitted to thin and thick blood smears and PCR: two from Amazonas and two from Rondônia were positive for *P. brasilianum* in microscopy and PCR [57]. This rate (4 out of 19 monkeys) of *P. brasilianum-positive* samples signifies that NHPs of these regions are harboring *P. brasilianum / P. malariae*. These results draw attention to the risk of introducing monkeys from the endemic Amazon region into regions with another biome and that if, for any reason, this happens, the monkeys should be tested for *Plasmodium* beforehand to mitigate simian parasite dispersion and the risk of introducing malaria into non-enzootic and non-endemic regions where competent vectors are present.

A year later, in 2014, a study conducted in Indaial, Santa Catarina state, found that among the 65 examined red howlers (*Alouatta guariba clamitans*) (20 free-living and 45 captives), 4 % were infected with *Plasmodium*: two captives and seven free-living [58]. ELISA results to detect IgG antibodies against *P. vivax* antigens (PvDBPII, PvMSP-1, and PvAMA-1) ranged from 64 to 83 % for each antigen, confirming the usual infection in these NHP [58]. The authors suggested that malaria could become a public health problem in these regions due to the close contact between humans and monkeys.

In the Primate Centre of Rio de Janeiro (CPRJ), *Plasmodium* infection in monkeys was investigated in 30 captive animals, and nine were found to be infected: five with *P. brasilianum*, three with *P. simium*, and one with *P. brasilianum* and *P. simium*. For the first time, the infection in capuchin monkeys by *P. simium* was reported in the extra-Amazon region of Rio de Janeiro [15].

In 2017, now in the Amazon region of the Maranhão state, 161 monkeys (141 captives and 20 wilds) were qPCR tested for *Plasmodium*. The 48 *Plasmodium* positive were subjected to species-specific PCR to identify *P. malariae, P. vivax,* and *P. falciparum,* and 47/48 of the monkeys were positive for *P. malariae/ P. brasilianum.* Of these, eight were free-living NHP. Interestingly, the remaining monkey that was positive for *Plasmodium* was not identified as *P. vivax, P. falciparum,* or *P. malariae* [59]. Although *P. vivax* (the equivalent species in humans) is prevalent in the studied area, no sample was positive for *P. simium*, confirming to date that this *Plasmodium* species is restricted to the Atlantic Forest.

Meanwhile, due to the 2017–2019 yellow fever outbreak in southeastern Brazil, 134 free-living monkeys and marmosets could be examined in a short period from Rio de Janeiro, six from Minas Gerais, one from São Paulo, and five from Espírito Santo. Six species - *Alouatta g. clamitans* (48), *Brachyteles hypoxanthus*(1), *Callicebus nigrifrons* (1), *Callithrix jacchus* (66), *Leontopithecus rosalia* (5) and *Sapajus nigritus* (25) - were investigated for *Plasmodium* infection by microscopy and PCR. Among the 146 NHPs, only howler monkeys were found infected (12/48), 11 from Rio de Janeiro, and one from Espírito Santo. The sample from Espírito Santo was infected with *P. brasilianum*, while in Rio de Janeiro, five were infected by *P. simium,* four by *P. brasilianum* and two by *P. brasilianum* and *P. simium*. These data indicated that the howler monkeys are the main reservoir of malaria in Atlantic Forest sites [60].

In 2019, the prevalence of *Plasmodium* infection in NHP was assessed in Joinville, Santa Catarina in 40 captured monkeys. PCR detected *Plasmodium* in 70 % of the monkeys: *P. simium* (22.5 %), *P. brasilianum* (7.5 %) and *P. simium* + *P. brasilianum* mixed infections (40 %). The unexpectedly high number of infected monkeys showed a worrying scenario through the increased possibility of autochthonous cases in the region, given the contact between humans and monkeys [61].

By 2022, a study on NHP in the Amazon region investigated 68 wild monkeys who lived in forest areas and captive monkeys from a local zoo in the Amazonas state. Only 3 monkeys were *Plasmodium* positive by PCR (4.4 %). DNA sequencing and a restriction enzyme assay proved that these monkeys were infected by *P. vivax* instead of *P. simium*, reinforcing the evidence that *P. simium* is restricted to Atlantic Forest regions [62].

More recently, in 2023, monkey malaria transmission was evaluated by identifying gametocyte transcripts and estimating gametocyte density. Overall, 35 animals were analyzed: 32 free-living animals from Joinville, Santa Catarina, and three captives from Rio de Janeiro. The NHP - 33 brown howler monkeys (*Alouatta guariba clamitans*) and two black-headed uakari (*Cacajao melanocephalus*) were previously diagnosed as: *P. simium* (11), *P. brasilianum* (5) and mixed infection (13), besides six non-infected. As a result, a 74 % rate of gametocyte transcripts was observed, but the gametocyte density estimation per microliter was very low [63]. Once again, it was demonstrated that howler monkeys are the main reservoir of the disease in the Atlantic Forest since more than 60 % of these infected monkeys carry gametocyte markers, maintaining transmission in these regions.

The presence of *Plasmodium* in NHP should not be neglected since there's lateral transmission between monkeys and humans, which can lead to more difficulties in malaria elimination. Thus, there must be strategies to raise awareness among the local population and tourists to prevent and eventually control the disease in these areas.

9. The under-reporting problem

It is well known that in the extra-Amazon region, autochthonous malaria human cases tend to have a low parasite load in the blood. Despite presenting with typical symptoms (chills, fever, sweating), a prolonged course exceeding three weeks can delay malaria diagnosis, potentially leading to underreporting cases. Additionally, the lack of knowledge about malaria in this context among residents, visitors, and health professionals makes it challenging for patients to seek a diagnosis [41,64,65,66,67].

10. Confirming zoonotic malaria - Molecular studies

As mentioned before, *P. simium* and *P. brasilianum* resemble *P. vivax* and *P. malariae* morphologically, immunologically and genetically. Some phylogenetic studies based on the genetic sequences of 18S rDNA, cytochrome B mtDNA, and the circumsporozoite protein of these species suggested that the species may not be distinct but indeed the same, at least, or mainly for *P. brasilianum / P. malariae* [68–70].

The first molecular analysis of *P. malariae* and *P. brasilianum* from Brazil was published in 2012. The data obtained from human, monkey, and mosquito samples were used to assess the genetic diversity concerning their places of origin. The authors concluded that the greater genetic diversity of *P. brasilianum* compared to *P. malariae* suggests the evolutionary hypothesis that *P. malariae* would have originated from an adaptation of *P. brasilianum* parasites to humans [71]. This hypothesis is refuted because *P. malariae* does not exist in Asia, it is scarce in African monkeys and there are no monkeys in Europe. So how could there have been quartan malaria in ancient Rome and Greece before man arrived in America, the land of monkeys infected by *P. brasilianum*? In 2015, the same group suggested the contrary, e.g., the simian sequences could have derived from the human ones [72].

Also in 2015, in a tentative attempt to differentiate *P. malariae* from *P. brasilianum*, in Brazil / Venezuela border samples, the authors sequenced the 18S gene of *P. malariae.* They compared it with a monkey's DNA sequence of a *P. brasilianum* strain. They concluded that the two species are indistinguishable and infect the same hosts. It was also emphasized that the transmission of anthropozoonotic quartan malaria occurs in areas where the habitats of humans and NHP meet. Since quartan malaria successfully infects multiple mammalian NHP hosts, this may facilitate its evasion, thus hindering the control and elimination of the disease [21].

In 2017, in a study made in the Espírito Santo state, *P. vivax / P. simium* samples from humans and monkeys were DNA sequenced. By haplotype network comparisons, it was shown that humans and monkeys of the region were infected by parasites having the same haplotype, demonstrating that humans and NHP have shared parasites molecularly similar [73].

Additionally, in 2017, with outbreaks on the rise between 2015 and 2016 in the state of Rio de Janeiro, to confirm whether these episodes were caused by a zoonosis, in addition to diagnosing these patients using *P.vivax* PCR, a molecular protocol was developed considering that the genome of *P. simium* differs in only 2 SNPs from those of *P. vivax*, at positions 3535 (T → C) and 3869 (A → G) [73]. In this way, 28 human and 3 NHP samples were diagnosed as *P. simium,* demonstrating also molecularly the zoonotic transmission of *P. simium* infection to humans in Rio de Janeiro [17,74]. This protocol based on mitochondrial genome sequences through a nested PCR identified the species in humans and NHP and can significantly assist in zoonosis surveillance [75].

In 2018, asymptomatic *P. vivax*, *P. malariae,* and mixed infection

were detected by molecular techniques in individuals living in Atlantic Forest, where there had been previous reports of the disease in Espírito Santo state [30,39,73]. The asymptomatic cases represent 3.4 % of the population investigated. Thus, human hosts alone would not be sufficient for the circulation of the parasite, and NHP had been suggested as reservoirs in the region. [73,76].

In the same year, population genomic approaches to understanding the ancestry of *P. simium* generated 11 genome sequences. *P. simium* sequences from NHP were shown to be notably less diverse than those from human hosts. A cluster of closely related parasites in some human and monkey samples suggests that the *P. simium* population might have a trim level of stratification through space and time [77]. There is a minimal genetic difference between *P. simium* and *P. vivax* populations from the Americas. Based on the data analysis, the Asian origin of *P. simium* is more probable. It was hypothesized that *P. vivax* came from southern Europe and Africa to Brazil during the colonial era, on the Atlantic Coast, and evolved to infect anophelines of *Kerteszia* subgenus, occurring reverse zoonosis from infecting humans to monkeys. The authors concluded that *P. simium* has genomic signatures of adaptation to infect NHP, especially in the vital erythrocyte ligand [74].

A recent study concerning the *P. simium* genome analysis confirmed a close phylogenetic relationship between P. simium and *P. vivax,* suggesting a recent American origin for *P. simium*. The presence of the DBP1 deletion in all human-derived isolates tested suggests that this deletion may facilitate the invasion of human red blood cells and may explain, at least in part, the basis of the recent zoonotic infections [78]. The recent transfer of parasites from humans to non-human primates, representing a reverse zoonosis of public health significance, was also signalized by population genomics studies [79].

11. Final remarks

The zoonotic malaria dynamics are rooted in non-human primates acting as reservoirs of human infectios. This happens when the geographical and ecological conditions (human, NHP, vector, and parasite) permit contact between both hosts, favoring cross-infections [7]. The extra-amazon region of Brazil, especially the Atlantic Forest, has all the conditions that favor the occurrence of this phenomenon.

Although many years passed between 1966 and the molecular confirmation of the zoonotic infection, several reports of autochthonous cases in the Extra Amazon region, particularly in areas under the influence of the Atlantic Forest biome, were published during this period.

Undeniably, zoonotic malaria is a reality, especially in the southern and southeastern regions of the country. Eliminating the disease in these areas raises an alert, given the variables in maintaining the zoonosis. Although they represent a small proportion of malaria cases in Brazil, autochthonous cases in these regions represent transmission hotspots, which, if not monitored regularly, may potentially increase the risk of the disease reappearing in places where malaria has already been eliminated.

One of the WHO's goals by 2035 is to prevent the re-establishment of malaria in places free of the disease. With increased destruction of biodiversity and global warming, which favor contact between humans and wild cycles of the parasite and impact the behavior, abundance and distribution of competent mosquito vectors, it is expected that the contact between NHP and humans will grow closer, which can be an essential factor for zoonotic malaria occurrence in the future. To this end, it is essential to monitor NHP and mosquitoes in the Atlantic Forest regions to determine the extent of the zoonosis using both serological and molecular techniques [80].

Moreover, enhanced training for local healthcare professionals and public awareness campaigns targeting residents and tourists are crucial to reducing the underreporting of malaria in these areas.

CRediT authorship contribution statement

Beatriz Pires da Silva: Writing – original draft, Methodology. **Ricardo Lourenço-de-Oliveira:** Writing – review & editing. **Jacqueline de Aguiar Barros:** Visualization. **Patrícia Brasil:** Writing – review & editing. **Cláudio Tadeu Daniel-Ribeiro:** Writing – review & editing. **Maria de Fátima Ferreira da Cruz:** Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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