Finding connections in the unexpected detection of *Plasmodium vivax* and *Plasmodium falciparum* DNA in asymptomatic blood donors: a fact in the Atlantic Forest

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Abstract

A recent paper in *Malaria Journal* reported the observation of unexpected prevalence rates of healthy individuals carrying *Plasmodium falciparum* (5.14%) or *Plasmodium vivax* (2.26%) DNA among blood donors from the main transfusion centre in the metropolitan São Paulo, a non-endemic area for malaria. The article has been challenged by a group of authors who argued that the percentages reported were higher than those found in blood banks of the endemic Amazon Region and also that that paper had not considered the literature on the classical dynamics of malaria transmission in the Atlantic Forest, which involves *Anopheles (Kerteszia) cruzii* and bromeliad malaria, due to *P. vivax* and *Plasmodium malariae* parasites, but not *P. falciparum*. The present commentary paper responds to this challenge and brings evidence and literature data supporting that the observed prevalence ratios may indicate a proportion of individuals that are exposed to *Plasmodium* transmission in permissive environments; that blood carrying parasite DNA may not be necessarily infective if used in transfusion; and that in the literature, there are examples supporting the circulation of *P. falciparum* in the area.

Keywords: Malaria, *Plasmodium falciparum*, *Plasmodium vivax*, Subclinical infection, Blood donors, Atlantic Forest

Background

The dynamics of malaria in the Brazilian Atlantic Forest seems to represent ideal, low transmission settings that involve patterns of evolution of *Plasmodium* parasites, its hosts and its vectors. Evidence of subclinical plasmodial infection in inhabitants and visitors of forest areas indicate that the dynamics of malaria may have reached a sustainable transmission level, without causing morbidity and affecting the survival of hosts, reservoirs and vectors. Following the intensive and successful malaria-eliminating programme from the 1950s onwards, *Plasmodium falciparum* and *Plasmodium vivax* reservoirs and mosquito vectors may have remained clustered in small geographical areas that maintain the circulation of the *Laverania* protozoan without the costs of malaria burden. This ideal evolution scenery needs further investigations in order to find connections in the unexpected high prevalence ratio of asymptomatic infection in humans exposed to forest environments. The study by Maselli *et al.* [1] points to the maintenance of *P. falciparum* in the Atlantic Forest in silent cycles that may involve non-human primates, humans and mosquito vectors with distinct biology and ecological features, beyond the classical *Kerteszia/bromeliad* malaria transmission dynamics.

Fully understanding malaria transmission represents a challenge for the disease control and the maintenance of the effectiveness of interventions. In countries where malaria was successfully eliminated, the epidemiology of the disease becomes more complex [2]. It is, therefore, possible to assume that after an exhaustive, successful
control, the residual malaria in the Atlantic Forest may encompass distinct dynamics of transmission. In this biome, the epidemiology of malaria seems to be determined by biologically distinct vectors, wild non-human primates and humans, in a conducive, heterogeneous environment.

Among more than 400 anophelines, approximately 70 species [3] can transmit the six Apicomplexan protozoan of the genus Plasmodium that can infect and cause malaria in humans, *P. falciparum*, *P. vivax*, *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* [4]. Sinka [3] clearly shows that in Brazil, in addition to *Anopheles cruzii* other species of the subgenera *Anopheles*, *Kerteszia* and *Nyssorhynchus* are potential vectors of *Plasmodium* protozoans. Considering that members of the three subgenera are biologically and ecologically distinct, the dynamics of malaria in Brazilian ecosystem seems to encompass complex dynamics.

According to Begon [5], a parasite is an “organism that obtains its nutrients from one or a very few hosts”, whereas a pathogen is “any parasite that gives rises to a disease”. Additionally, Begon argues that both a parasite and a host population have their own interacting dynamics. Consequently, the knowledge of epidemiology of malaria may address the determinants associated to humans, mosquitoes, *Plasmodium* protozoan (that can behave either as parasite or pathogen) and other potential host/reservoir populations.

The origin and evolutionary history of *P. falciparum* has been subject of recent studies and a seminal work on *Plasmodium* changed the paradox of the origin of the human *P. falciparum*. By testing faeces samples from wild-living apes throughout central Africa, Liu et al. [6] showed that *Plasmodium* infections were highly prevalent and widely distributed in the region. Moreover, western gorillas (*Gorilla gorilla*) were infected with a *Plasmodium* nearly identical to *P. falciparum*. Results of phylogenetic analyses using mitochondrial genome sequences showed that human *P. falciparum* clustered within gorilla *P. falciparum* lineage, indicating that the parasite is of gorilla origin. Rayner et al. [7] proposed the name G1 for the phylogenetic lineage that included *Plasmodium* parasites from the subgenus *Laverania*, i.e., human *P. falciparum* and *P. praefalciparum* for the parasites from gorillas as precursor of human *P. falciparum*. Based on evidence that the gorilla *P. falciparum* has been transferred to humans, it is possible that additional cross-species transfer has occurred in other regions where non-human primates live in close association with other potential reservoirs. Garamszegi [8] suggested that parasites may preferentially infect hosts that provide adequate conditions for their reproduction. Furthermore, Sharp et al. [9] predicted that the emergence of *P. falciparum* was likely caused by changes in human demography and behaviour that opened new niches that cause the emergence of the parasite in humans. In agreement, Silva et al. [10] suggested that ape infections with *P. falciparum*-like parasites are a consequence of deforestation and land use. These intensive ecological changes brought humans in close contact with wild-living apes, facilitating the transmission of parasites among infected apes and susceptible humans, and the other way around.

In Brazil, the Atlantic Forest biome has undergone massive and intensive ecological changes since the beginning of European colonization in the Sixteenth Century [11]. Currently, it is estimated that only 11.4–16.0% of the original forest cover remains, most as fragments [12]. As a consequence of the fragmentation, humans and wild non-human primates may be in contact. This situation seems to occur in forest-fragmented regions of the Atlantic Forest, for instance in Juquitiba municipality, in areas of the northeastern coast of the São Paulo state, and in the southern of the São Paulo municipality. In the Parelheiros subdistrict of the São Paulo municipality, there are small fragments of Atlantic Forest, where autochthonous *P. vivax* malaria were notified (see [13]) and a few mosquito species other than *An. cruzii* were tested positive for the parasite [14].

In cross sectional study by Maselli et al. [1], the blood donors that had plasmodial infection either lived in areas of the east region of São Paulo state or had visited localities situated on the coast where most of the forest fragment remains. In striking contrast with the classical bromeliad-malaria model [15], most of the subclinical infections were associated with *P. falciparum*. Likely, the positivity observed was a consequence of a single template TaqMan-based real-time PCR with specific probes for each *Plasmodium* species adopted as the standard protocol for testing blood samples. The single template real time PCR that tested positive for *P. falciparum* (prevalence of Pf = 5.14) of the blood donors is indicating that a *P. falciparum* or *P. falciparum*-like parasite is infecting humans without causing disease. Three evidences corroborate this hypothesis. The first one is a single case of *P. falciparum* malaria among 14 autochthonous malarias patients from areas of the Atlantic Forest that were diagnosed and treated at the Instituto Nacional de Infectologia Evandro Chagas in Fiocruz (from 2008 to 2013). All 14 patients had acute vivax malaria, and a single patient was found infected by both *P. falciparum* and *P. vivax*. This makes possible that the *P. falciparum* infection was detected coincidentally only as a consequence of the concomitant (symptom-inducing) *P. vivax* infection (Pina-Costa, Doctoral Thesis, Instituto Nacional de Infectologia, Fiocruz, August 2014). The second evidence is provided by a recent publication by Abkallo
et al. [16], showing that Plasmodium DNA from a pre-
erthrocytic stage can be detected both in blood and 
faeces from infected non-human primates in the ab-
sence of blood stage parasites. This event, indicating 
that not all infected blood from non-human primates 
are infectious, could also occur in humans and is par-
ticularly important because it could partially explain 
the contrast between the inobservance of P. falciparum 
transfusional malaria cases in an area where an unex-
pected frequency of PCR positive for this parasite was 
registered. Finally, the third evidence comes from 
the confirmation of twenty-four randomly drawn P. falciparum 
positive blood samples retested by nested-PCR at the 
Laboratório de Pesquisa em Malária, Instituto Oswaldo 
Cruz, Fiocruz, according to the protocol of Zalis et al. [17]. 
Consequently, in view of the results reported by Maselli 
et al. [1] and the recent data quoted above, it seems evident 
that the dynamics of malaria in the Atlantic Forest is poorly 
known and needs further investigations, including studies 
relative to mosquito vectors and other determinants of 
the transmission, i.e., infectiveness and pathogenicity of the 
Plasmodium lineages that are circulating in the re-
grion, duration of infection in untreated individuals, dur-
ation of sporogony and gametocytogenesis, as discussed 
by Wernsdorfer [18].

Maselli et al. [1] have taken into account both the 
classical (bromeliad-malaria) and alternative patterns of 
the dynamics of malaria in the Atlantic Forest. However, 
the finding of P. falciparum DNA in blood donors in 
São Paulo suggests that the traditional dynamics of 
bromeliad-malaria is not the only one that can explain 
the presence of Plasmodium in areas of the Atlantic 
Forest in the state of São Paulo. Two major determinants 
of the bromeliad-malaria are the Anopheles (Kerteszia) 
mosquitoes and other Plasmodium species that do not be-
long to the subgenus Laverania. Cerutti et al. [19] ad-
ressed aspects of malaria in mountainous areas of the 
Espírito Santo state and hypothesized that, in the region, 
species of the subgenus Nyssorhynchus of Anopheles may 
be also involved in cycle of Plasmodium transmission. 
Thus, the results reported by Cerutti et al. [20] indicate, 
agreement with the data of Maselli et al. [1], that a distinct 
dynamics of Plasmodium transmission could also occur in 
addition to the classical bromeliad-malaria.

Still considering the arguments against the presence of 
P. falciparum in Atlantic Forest by Mendrone et al. [20], 
it is worth noting that antibodies specific to the circum-
sporozoite protein (CSP) of P. falciparum have been de-
tected in sinians from the Atlantic Forest in São Paulo 
state [21]. Moreover, Yamasaki et al. [22] surveyed mon-
keys from Atlantic Forest for Plasmodium infection, and 
concluded that the high proportion of positive sera 
against the CSP of P. falciparum was uncommon. It is 
interesting to point that Malafronte, a coauthor of the 
study by Yamasaki et al., also coauthored the paper by 
Mendrone et al. [20]. Additional support for the presence 
of P. falciparum in areas of Atlantic Forest, have 
been provided by Duarte et al. [23], who showed by PCR 
amplification that 1.4% of monkeys that tested positive 
for plasmodial infection had P. falciparum DNA. More 
important, the authors also surveyed monkeys both from 
urban areas and forest fragments in the vicinities of 
the municipality of São Paulo. Consequently, the argu-
ments by Mendrone et al. [20] against the presence of 
P. falciparum are not supported by the data published 
in the literature.

The observation of asymptomatic individuals carrying 
P. falciparum DNA in higher prevalence among blood 
donors of a São Paulo Transfusion Center than in blood 
banks from the Amazon region is as intriguing as diffi-
cult to explain. However, if P. falciparum is circulating in 
areas of the Atlantic Forest in non-human primates and 
humans, it is plausible to assume that there are also mos-
quitos infected with P. falciparum. The reasons for the 
lack of report of mosquitoes naturally-infected with 
P. falciparum are unknown; however, it is possible that the 
absence is, at least partially, explained by the methods 
employed to test mosquitoes, usually in pools [14,24].

Cerutti et al. [19] employed immunofluorescence anti-
body test (IFAT) to observe a high percentage of human 
habitants of the Atlantic Forest of Espirito Santo state 
positive for IgM (13.5%) and IgG (13%) antibodies to 
P. falciparum. In addition, the authors reported nine in-
dividuals positive for P. falciparum by multiplex-PCR, 
and concluded that “the puzzling finding of P. falcip-
arum DNA by multiplex PCR in asymptomatic individu-
als” and, more important, quoted that “the possibility of 
false positive results is remote as no other samples in-
fected by P. falciparum were processed by PCR in the 
laboratory at the time of the study, thus ruling out the 
possibility of any cross-contamination”. Consequently, 
the argument by Mendrone et al. [20] that “DNA supposed 
be from P. falciparum... did not usually resist to a second 
amplification by another method” is not supported by data 
presented by team of his coauthor.

Mendrone et al. [20] also challenged the results of 
Maselli et al. [1] by arguing that in a PhD thesis associated 
with the article, an ELISA assay was utilized for testing the 
blood donors and the results were not included in the 
paper. This information is not incorrect. The thesis is 
authored by Aline Monteiro, one of the coauthors of the 
paper by Maselli et al. [1]. Although described as more 
sensitive than IFAT, the gold standard to estimate malarial 
antibody titers, the analytical sensitivity of ELISA-Malaria 
antibodies test kit from DiaMed (product 46460) was 40% 
with specificity of 98.3%, when tested in 923 malaria risk 
donors. According to Dodere et al. [25], the large number 
of non-concordant results between ELISA and IFAT
impaired the performance of this DiaMed kit. Oh et al. [26] also reported that this test was insufficiently sensitive for blood screening for P. vivax. In addition to the described low performance of the DiaMed malarial kit, it has not been systematically tested regarding its sensitivity in low parasitaemia infection, it has not been approved by the Brazilian Agência de Vigilância Sanitária (Anvisa) and the kit production was interrupted. All these points were discussed in the thesis. Therefore, taking into account the negative features of the DiaMed kit, including its low sensitivity and inadequacy to detect subclinical infection, the results of the ELISA assays were considered inconsistent for publication.

Regarding mistakes in the references, the authors were aware of them and submitted an Erratum comment to the journal immediately after publication, which remains attached to the paper. This Erratum consists of a full list of corrections, including the correct number of autochthonous cases in São Paulo, and the fact that the Perandin et al. [27] citation replaces Gama et al. [28].

Conclusions
The article by Maselli et al. [1] poses quite different challenges and has the merit of showing the presence of P. falciparum among asymptomatic blood donors and to emphasize that the current knowledge of malaria transmission in the Atlantic Forest domain is far too limited. The natural history of P. falciparum malaria in the Atlantic Forest is either poorly known or even unknown, including its vectors, hosts and reservoirs, both in spatial and temporal scales. There is an urgent need to improve the knowledge of the biotic and abiotic determinants as well as the natural history of the disease, before speculating and proposing any strict epidemiological profile for the dynamics of the P. falciparum in the biome. Most of the arguments by Mendrone et al. [20] regarding the discoveries of Maselli et al. [1] are based on the rationale that the results published are not supported by previous publications. On the contrary, it has been shown here that part of the previous published literature showed the same evidence. In agreeing with Thierry Maulnier, who says that “it would be unwise to believe that what never happened is impossible”, the reasoning by Mendrone et al. may be considered weak. Indeed, defending a stable and unchangeable pattern of bromeliad malaria, which was proposed in the 1940s and 1960s, to explain the malaria transmission in the Atlantic Forest now, is to deny any evolutionary processes that can drive the dynamics of the transmission of Plasmodium in a changing world.

Competing interests
The authors declared that they have no competing interests.

Authors’ contributions
MAMS, CTD, and SPB conceived the ideas and wrote the manuscript with the help from GZL, MFFC, LMFM and DL. All authors read and approved the final version of the manuscript.

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