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Original Article

Reconstruction of *Plasmodium vivax* outbreaks in a low malaria endemic setting utilizing conventional restriction fragment length polymorphism

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Abstract: Suriname is on track to eliminate local malaria transmission. *P. vivax* malaria reemerged in March and September 2019 in the Amerindian village Palumeu, free of malaria for two years and concurrently, a case was reported in another village Alalaparoe. The outbreaks were contained through targeted interventions including Mass Drug Administration (MDA). Molecular outbreak analysis was performed on 23 dried blood spots (DBS) using combined polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) with *Pvmsp-1* F2 and *Pvmsp-3α* as polymorphic marker genes. Independent controls substantiated the discriminating capacities of the utilized PCR-RFLP method. All isolates from the first and second Palumeu outbreak shared a distinctive haplotype presuming single clonal lineage. An imported case probably triggered the first outbreak, while a delayed episode, prompted by withdrawal of drug pressure at the end of the prophylactic MDA, was suggested as source of the second outbreak. A diverging variant was demonstrated in Alalaparoe, implicating an infection from a different source. PCR-RFLP proved to be a useful molecular tool for *P. vivax* outbreak management in low endemic malaria settings.

Keywords: Suriname, vivax malaria, outbreak origin, *Pvmsp-3α* and *Pvmsp-1 F2* PCR-RFLP, sequence diversity, restriction pattern, mass drug administration

Introduction

Plasmodium vivax (*P. vivax)* is geographically the most widespread of malaria species, causing 75% of malaria cases in the Americas in 2018 [1].

Prior to 2006, Suriname was regarded as the country in the Americas, with the highest concentration of *Plasmodium falciparum* malaria, but stringent control measures markedly decreased the malaria burden, particularly for *P. falciparum*. Since 2007, *P. vivax* became the predominant species and Suriname is on track to reach zero indigenous malaria cases [2]. The majority of current cases is imported and can be attributed to cross-border movement of migrant gold-miners, mostly Brazilians through French Guiana. In March 2019, an outbreak of *P. vivax* malaria was reported for Palumeu, an Amerindian village, which had been free of malaria for two years. During this outbreak, resurgence of malaria was reported after eleven years in another Amerindian village Alalaparoe, located south of Palumeu, albeit restricted to one case. The Palumeu outbreak was contained through various interventions, but the village experienced a second outbreak in September 2019, after a bible conference with several guest from other localities.

The aim of the study was to determine the genetic origin and relation between the three events. In Suriname, molecular applications for *P. vivax* isolates had been restricted to polymerase chain reaction (PCR) in combination with the restriction fragment length polymorphism (RFLP) technique to discriminate between relapse, recrudescence and new infections. Globally, advanced molecular-based tools have been optimized for *P. vivax* genetic research. Whole genome sequencing to screen

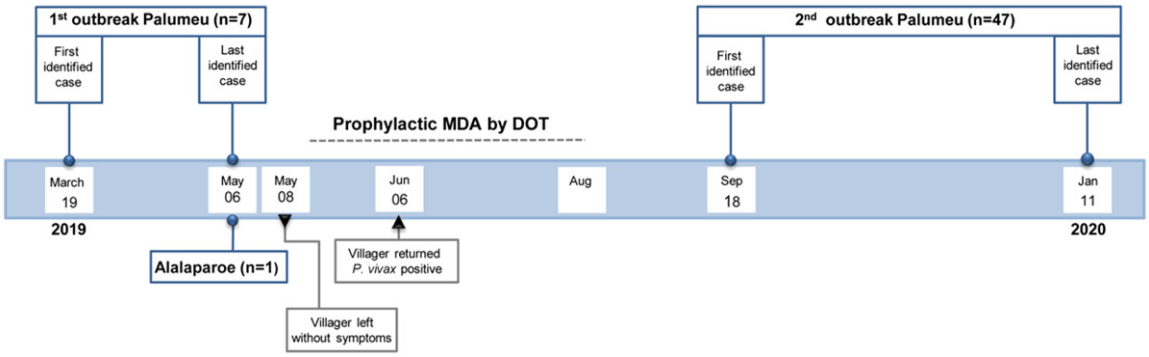

Figure 1. Timeline of *P. vivax* outbreaks in Palumeu and Alalaparoe case.

for potential molecular markers for drug resistance [3], microsatellite genotyping [4] and molecular barcoding [5] to determine genetic relatedness of parasites in outbreak analysis, or to track the geographic origin of infections [6].

In this study, conventional PCR-RFLP was innovatively utilized for *P. vivax* outbreak reconstruction. A total of 23 dried blood spots (DBS) were analyzed for genetic variation, using the *P. vivax* polymorphic molecular marker genes *Pvmsp-1* and *Pvmsp-3α,* encoding the merozoite surface protein-1 (Pvmsp-1) fragment F2 and merozoite surface protein 3α (Pvmsp-3α), respectively.

Material and methods

Study settings

P. vivax re-emerged in Palumeu, an Amerindian village of 420 persons free of malaria for the previous two years. This village is known for high malaria endemicity before 2007 and a high density of *A. darlingi* mosquitoes, related to the presence of nearby marshes. Seven cases of *P. vivax* malaria were reported from March through May 2019, while none of these persons had traveled outside the village in the previous months, indicating that the index case was an unregistered visitor, probably from neighboring Brazil.

In addition, an isolated case was reported concurrently in another village, Alalaparoe.

Malaria cases were treated with 30 mg/kg Chloroquine (CQ) in a 3-day course and Primaquine (PQ) 0.50 mg/kg for 14 days via directly observed therapy (DOT). Absence of glucose-6-phosphate dehydrogenase (G6PD) deficiency was evaluated through Carestart™ G6PD testing. The outbreak response consisted of a series of targeted interventions including biweekly Active Case Detection (ACD). Despite the absence in the country of sufficient rapid tests for G6PD deficiency at that specific time, mass drug administration (MDA) was implemented in May in order to at least contain further onward spread of the disease. Prophylactic treatment of CQ (300 mg) was administered (DOT) to all asymptomatic villagers with a negative blood smear, followed by weekly CQ (150 mg) during 8 weeks.

A villager without malaria symptoms left Palumeu before the start of MDA and returned in June 2019, microscopically positive for *P. vivax* (**Figure 1**).

A second outbreak was observed in Palumeu, right after an influx of bible conference visitors, resulting in 47 *P. vivax* cases from September 2019 to January 2020 (**Figure 1**).

Four patients from the first outbreak also experienced a malaria episode during the second outbreak, of which two persons were even diagnosed in the first week.

Interventions included ACDs and the implementation of a second MDA by DOT for all eligible villagers with a full *P. vivax* therapeutic course of CQ plus PQ (for persons without G6PD deficiency).

Sample collection

All samples included in this study were DBS on filter paper (3 MM, Whatman Inc.) stored in the National Malaria Gene Bank (NMGB). The

Table 1. Characteristics of study samples and PCR-RFLP characterization

Sample No.	Gender	Age (yrs)	Collection date	Geographic origin	PCR-RFLP haplotype[a]
Controls: unrelated field isolates					
#01	M	9	2018-11-01	Tampoki (French Guiana)	Be4-Ad4
#02	M	23	2018-11-08	North-West (Guyana)	Bc2-Ab2
#03	M	41	2018-11-22	Kawemhakan (Suriname)	Aa1-Bc5
#04	F	30	2018-11-27	Yaw Pasi (Suriname)	Be5-Ae3
#05	F	32	2019-04-26	Sophie (French Guiana)	Bb2-Ab2
#06	F	32	2019-06-06	Venezuela	Bf2-Af7
Controls: related field isolate (to #01)					
#07[b]	F	5	2018-11-01	Tampoki (French Guiana)	●●●-Ad4
Palumeu 1st outbreak					
#08	M	13	2019-03-19	Palumeu (Suriname)	Aa1-Aa1
#09	M	46	2019-03-22	Palumeu (Suriname)	Aa1-Aa1
#10[c]	M	52	2019-04-21	Palumeu (Suriname)	Aa1-Aa1
#11[b]	M	20	2019-04-22	Palumeu (Suriname)	Aa1-A●1
#12[b]	M	0.5	2019-04-29	Palumeu (Suriname)	Aa1-A●1
#13	F	26	2019-04-30	Palumeu (Suriname)	Aa1-Aa1/Ag6[d]
#14	F	62	2019-05-06	Palumeu (Suriname)	Aa1-Aa1
Palumeu travelling villager					
#16	M	19	2019-06-06	Palumeu (Suriname)	Aa1-Aa1
Alalaparoe concurrent case					
#15	M	41	2019-05-06	Alalaparoe (Suriname)	Bd3-Bc5
Palumeu 2nd outbreak					
#17	M	24	2019-09-18	Palumeu (Suriname)	Aa1-Aa1
#18	F	20	2019-09-18	Palumeu (Suriname)	Aa1-Aa1
#19	M	4	2019-09-19	Palumeu (Suriname)	Aa1-Aa1
#20[c]	M	52	2019-09-23	Palumeu (Suriname)	Aa1-Aa1
#21	F	42	2019-09-30	Palumeu (Suriname)	Aa1-Aa1
#22	M	12	2019-10-08	Palumeu (Suriname)	Aa1-Aa1
#23	M	46	2019-10-18	Palumeu (Suriname)	Aa1-Aa1

[a]: Allelic patterns for both genes of each isolate were characterized by labeling the size type with letters (caps), while different restriction patterns were labeled with numbers and letters, rendering each isolate with a six-character code. The first three characters reflect the pattern for the *Pvmsp-1 F2* gene (size polymorphism, AluI and MnlI pattern), while the remaining three characters denote the *Pvmsp-3α* gene profile (size polymorphism, AluI and HhaI pattern). [b]: Missing data (●), due to insufficient sample and/or poor amplification. [c]: Same person experiencing a malaria episode in both outbreaks. [d]: Mixed infection.

selected study samples (n=23) included all patients from the first outbreak in Palumeu (n=7), the concurrent case in Alalaparoe, seven randomly chosen samples from the second outbreak, next to a sample from a travelling villager returning to Palumeu in between the outbreaks (**Table 1**).

Also, unrelated field isolates (n=6) from geographically diverse areas/countries and a different time period and one related field isolate were selected as controls to assess the discriminating capacity of PCR-RFLP (**Table 1**).

Patient's malaria history data was provided by the National Malaria Program.

Informed consent for molecular malaria research was provided by all patients upon enrollment for the NMGB. The national ethics committee within the Ministry of Health approved the NMGB project (VG007-08).

Assessment of size and sequence polymorphisms in Pvmsp-1 F2 and Pvmsp-3α genes

DNA was extracted with a modified Saponin-Chelex extraction [7]. Conventional nested PCRs targeting *Pvmsp-1 F2* and the *Pvmsp-3α* genes were performed according to protocols described earlier [8, 9]. Size polymorphisms were detected by horizontal agarose electrophoresis and sequence polymorphisms were

analyzed from restriction patterns attained on polyacrylamide gels by exerting two restriction enzymes for each gene; *Alu*I, *Mnl*I for *Pvmsp-1 F2* and *Alu*I, *Hha*I for *Pvmsp-3α*. Consistency of results was confirmed through duplicate analysis of random samples and positive controls.

To facilitate comparative analysis of the parasite isolates, PCR-RFLP-results were converted to a six-character code. The allelic pattern for each gene was classified with letters and numbers, combining data from the PCR size polymorphism represented as letters (caps) and different RFLP patterns labeled with numbers and letters for each restriction enzyme (**Table 1**). Samples were designated as mixed infections when two or more PCR-products of different size were generated or when the RFLP analysis of a single PCR band exceeded the size of the uncut PCR band. Infections displaying identical restriction profiles or mixed patterns containing a shared haplotype and another genotype were considered as single clonal lineages, originating from the same source, while deviations in one or more of the four restriction patterns were designated as multiple clonal lineages.

The chi-square (χ^2) test (Statistical Packages for Social Sciences (SPSS 21.0)) was used to compare the frequencies of RFLP profiles among the sample sets with a statistical significance level set at $P<0.05$.

Results

Demographic data (Palumeu outbreaks)

The median age of the Palumeu patients (n=14) was 22 years with a range of 6 months-62 years and 71.4% of the patients were males (**Table 1**).

Size polymorphisms

Amplification of the *Pvmsp-1 F2* polymorphic region revealed both known size polymorphisms, designated as A (1150 bp) and B (ca 1087 bp) [8].

In contrast, only the two most common *Pvmsp-3α* size variants corresponding to the locus types A (1.9 kb) and type B (1.5 kb) were observed among these field isolates, with type A as the more common type.

Restriction fragment length polymorphisms

PCR-RFLP genotyping allowed the identification of different distinguishable alleles for all 6 independent controls, demonstrating the discriminating capacities of the RFLP method (**Table 1**). On the other hand, the related control samples originating from Tampoki with identical collection dates displayed matching *Pvmsp-3α* restriction patterns. Due to lack of amplification of the *Pvmsp-1 F2* gene, the *Pvmsp-1 F2* profile could not be considered in the comparison.

PCR-RFLP analysis of all 7 isolates from the first outbreak at Palumeu shared the same haplotype (Aa1-Aa1), although one isolate harbored a mixed infection with the Palumeu haplotype and another strain, only evident in the *Pvmsp-3α* pattern [Aa1 & Ag6]. The distinct haplotype demonstrated in Palumeu was significantly different from the allelic patterns revealed in the control samples (**Table 1**).

PCR-RFLP profiles from all investigated cases from Palumeu's second outbreak (n=7) and the field isolate from the travelling Palumeu villager matched the haplotype from the first outbreak.

The field isolate collected in Alalaparoe harbored a different variant than the Palumeu cohort, and yet another variant with respect to the controls (**Table 1**).

A slightly greater differentiating capacity was revealed for the *Pvmsp-3α* marker with 7 allelic variants detected with each restriction enzyme, while RFLP with the *Pvmsp-1 F2* marker generated 6 and 5 different patterns with *Alu*I and *Mnl*I, respectively. However, the combination of both restriction enzymes resulted in 7 variants for each marker. The combined profile of both markers, each with two restriction enzymes augmented the individual marker results and yielded a total of 9 allelic variants, including the mixed genotype infection.

Discussion

The discriminatory capacity and robustness of PCR-RFLP for *P. vivax* genotyping with *Pvmsp-1 F2* and *Pvmsp-3α* as polymorphic marker genes was substantiated with the highly diverse profiles generated for the unrelated field iso-

lates (controls). The findings also supported the premise that low endemic settings can still have a heterogeneous parasite population [10]. The matching results for the two related controls indicated that geographic isolation may be reflected within different geographical regions in Suriname, similar to the spatial clustering demonstrated within continents [11], countries [12] and endemic areas [10]. However, only a small number of isolates was investigated, thus restricting all conclusions.

The higher occurrence observed for the *Pvmsp-3α* type A size polymorphism was consistent with previous publications, indicating type A as the most prevalent type around the world [9, 10].

According to the expectations, combined RFLP with both polymorphic markers augmented individual results. The presence of a distinctive haplotype in all Palumeu isolates during the first outbreak was consistent with an outbreak of locally acquired secondary cases, contracted via a primary single index case introduced in an area, which had been free from malaria for some years.

The discordant pattern revealed in the isolated malaria case, concurrently diagnosed in Alalaparoe, highlighted the discriminating potential of the utilized RFLP method and suggested no direct relation between the Alalaparoe case and the Palumeu outbreak. Instead, the diverging allele composition of the Alalaparoe isolate pointed towards an infection or relapse from a different lineage, acquired from another source.

The finding that all investigated cases from the second outbreak harbored the haplotype associated with the first outbreak dismissed the notion of a new infection introduced through a conference visitor as source of the outbreak, but rather implicated the first outbreak. The considerable variation observed in the allele composition of the independent controls further substantiated this notion.

It could be hypothesized that the index case and even other cases during the second outbreak were the result of a delayed primary attack from an infection acquired during the first outbreak. Patients, infected within the first outbreak, but still asymptomatic and with a microscopically undetectable parasitaemia at the start of the MDA were regarded as negative patients and received the prophylactic treatment with CQ, thus eliminating the erythrocytic stages. Withdrawal of the CQ drug pressure at the end of the MDA could have allowed hypnozoites to be activated, resulting in a delayed primary *P. vivax* episode.

The presumed single clonal lineage for cases from both outbreaks could also be explained with an index case resulting from a conventional relapse in one or more patients from the first outbreak, since relapses caused by Primaquine failure even with correct treatment administered under supervision, are still common with relapse rates ranging from 2.8-19.0% [13, 14]. The finding that two persons from the first outbreak also experienced a malaria episode during the first week of the second outbreak provided some support for this possibility, although caution is warranted since disparities in infection and disease dynamics prohibit identification of the index case by simply following a timeline, while high mosquito density and close proximity of villagers further diminished the significance of this finding.

The impact of the prophylactic MDA in containing the onward spread of vivax malaria during the first outbreak was exemplified by the travelling villager from Palumeu, who did not receive prophylactic treatment and harbored *P. vivax* parasites upon return.

Genetic relatedness of *P. vivax* strains in outbreak analysis and parasite population studies generally involves advanced techniques as microsatellite genotyping [4], whole genome sequencing [15] and even molecular barcoding [5, 6]. However, utilization of conventional PCR-RFLP in conjunction with epidemiological data provided ample support for the hypothesized correlation between the *P. vivax* outbreaks, despite the technical limitations and constraints of this method imposed by the complexity of parasite strain evolution influenced by host and/or environmental factors. Although it was reported earlier that PCR-RFLP may even surpass sequencing as molecular tool to classify *P. vivax* genotypes [16, 17], RFLP might be inapt in areas with moderate to high transmission, due to a higher genetic diversity in the parasite population, heterolo-

gous hypnozoites activation and an even more challenging interpretation of restriction patterns in the case of multiple-clone infections. Nevertheless, in low endemic malaria settings as Suriname, PCR-RFLP proved to be useful to gain valuable insight in the genetic diversity of *P. vivax* isolates circulating during the outbreaks and can therefore serve as strategic tool to aid in *P. vivax* outbreak management.

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Disclosure of conflict of interest

None.

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