

Molecular Investigation into a Malaria Outbreak in Cusco, Peru: *Plasmodium falciparum* B_{V1} Lineage is Linked to a Second Outbreak in Recent Times

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Abstract. In November 2013, a *Plasmodium falciparum* malaria outbreak of 11 cases occurred in Cusco, southern Peru, where falciparum malaria had not been reported since 1946. Although initial microscopic diagnosis reported only *Plasmodium vivax* infection in each of the specimens, subsequent examination by the national reference laboratory confirmed *P. falciparum* infection in all samples. Molecular typing of four available isolates revealed identity as the B-variant (B_{V1}) strain that was responsible for a malaria outbreak in Tumbes, northern Peru, between 2010 and 2012. The *P. falciparum* B_{V1} strain is multidrug resistant, can escape detection by PfHRP2-based rapid diagnostic tests, and has contributed to two malaria outbreaks in Peru. This investigation highlights the importance of accurate species diagnosis given the potential for *P. falciparum* to be reintroduced to regions where it may have been absent. Similar molecular epidemiological investigations can track the probable source(s) of outbreak parasite strains for malaria surveillance and control purposes.

Peru reported 43,468 malaria cases in 2013, 84% of which were attributed to *Plasmodium vivax* infection while 16% (7,371) were due to *Plasmodium falciparum*.¹ As Peru and other South American countries progress toward the malaria pre-elimination phase, the utilization of appropriate parasite detection tools will facilitate effective disease surveillance and case management. *Plasmodium falciparum* transmission in Peru is mainly found in the Amazonian region; three departments (Loreto, San Martin, and Ucayali) reported *P. falciparum* malaria cases between January and the first week of December 2013.² Between 2010 and 2012, a *P. falciparum* malaria outbreak of 210 cases occurred in the north coastal department of Tumbes, where falciparum transmission had been interrupted for 5 years.³ Using molecular tools, it was determined that a multidrug resistant, *Pfhrp2* gene-deleted lineage (B_{V1}) originating from the Peruvian Amazon caused this outbreak.³

Between November 1 and 20, 2013, 11 malaria cases occurred in two communities—Rosalina and Ivanqui (Palma Real)—in Echarate District, La Convención Province, Cusco Department, which is located in southern Peru.² Information about the 11 malaria-infected patients, eight males and three females, is presented in Table 1. The community of Rosalina, where six malaria cases were detected, consisted of 70 families residing in 54 households whereas the community of Ivanqui, where five of the malaria-infected patients resided, had 92 families in 32 homes at the time of the malaria outbreak.² All 11 patients were initially found to be infected with *P. vivax* based on microscopic analysis and received a 7-day chloroquine (CQ) and primaquine (PQ) treatment regimen.

However, symptoms persisted in four of the patients, who then received CQ and PQ for 14 more days. The four patients continued to experience symptoms for over a month post-treatment until one of them visited a private practitioner on December 12, 2013. Microscopic examination of the patient's thick blood smear revealed *P. falciparum* infection.² Between

December 16 and 17, 2013, blood samples were obtained from the remaining three patients in whom symptoms had persisted after the initial *P. vivax*-targeted treatment. Giemsa-stained blood films were read by microscopy at which time the patients were confirmed to have *P. falciparum* malaria and treated with artesunate plus mefloquine.²

Since 1946, only *P. vivax* malaria transmission had been reported in Cusco Department; all 608 malaria cases reported in 2013 were attributed to *P. vivax*.² For this reason, the *P. falciparum* diagnoses prompted active surveillance for febrile cases in Rosalina and Ivanqui. In addition, the 11 smears confirmed to be positive for *P. vivax* were reexamined and by December 2013, the Cusco Regional Directorate of Health confirmed that the malaria cases from Palma Real corresponded to a *P. falciparum* outbreak.²

Blood samples from the four patients with persistent symptoms were sent to the Centers for Disease Control and Prevention malaria laboratory for further characterization. We determined the molecular profile (by genotyping microsatellites and drug-resistance-associated genes) of these four isolates and evaluated the possible origin(s) and/or relatedness to other *P. falciparum* parasite lineages previously reported in Peru.⁴ Information about the four patients from whom the samples were collected is shown in Table 1.

Neutral microsatellite markers were analyzed to delineate the genetic background of the parasites. These seven markers (TA1 [chromosome 6]; Poly-α [chromosome 4]; PfPK2 [chromosome 12]; TA109 [chromosome 6], 2490 [chromosome 10]; C2M34 [chromosome 2]; and C3M69 [chromosome 3]) have been previously used to characterize *P. falciparum* clonal lineages in Peruvian isolates.^{5,6} Moreover, they were used to determine the source of a *P. falciparum* malaria outbreak in Tumbes.³

Our analyses revealed that all four parasite isolates from Cusco had an identical microsatellite background consistent with the B_{V1} lineage previously reported in at least two other departments of Peru: Tumbes (malaria outbreak of 2012³) and Loreto (specimens collected in Yurimaguas and Requena in 2009–2010; D. Gamboa, J. Bendezu, K. Torres, and others, unpublished data) (Figure 1). To further verify the extent of similarity of the Cusco parasites to the B_{V1} parasite lineage,

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TABLE 1

Characteristics of the 11 patients that presented with malaria during the 2013 outbreak in Palma Real, La Convención Province, Cusco Department, Peru

Sample ID	Age	Sex	Parasitemia	Health center	Diagnosis date	Residence
854*	26	Male	F§	Palma real	November 14, 2013	Rosalina
1885*	32	Male	F	Palma real	November 14, 2013	Rosalina
1886*	30	Male	F	Hospital Quillabamba	November 14, 2013	Rosalina
1930*	33	Male	F	Palma real	November 15, 2013	Rosalina
1913	17	Male	F§	Palma real	November 15, 2013	Ivanqui
1952	25	Female	F§	Palma real	November 16, 2013	Ivanqui
2033	32	Male	F§	Palma real	November 17, 2013	Ivanqui
2109	52	Male	F§	Palma real	November 18, 2013	Rosalina
2139	2	Female	F§	Palma real	November 18, 2013	Ivanqui
2303	24	Male	F‡	Palma real	November 22, 2013	Rosalina
2443	28	Female	No slide available†	Palma real	November 25, 2013	Ivanqui

F = *Plasmodium falciparum*.
 * Patient specimens available for molecular analysis.
 † *Plasmodium falciparum* malaria was positively diagnosed (but undocumented) locally at the Palma Real Health Center. Blood film was not available to the Cusco Department of Health for confirmatory diagnosis.
 ‡ One parasite per field in 100 fields.
 § Between 2 and 20 parasites per field in 100 fields.
 || Between 21 and 200 parasites per field in 100 fields.

four drug resistance-associated genes; *Pfprt* (CQ resistance), *Pfmdr1* (multidrug resistance), *Pfdhfr* (pyrimethamine resistance), and *Pfdhps* (sulfadoxine resistance) were polymerase chain reaction amplified and sequenced as described previously.³ Finally, we genotyped histidine-rich protein 2 (*Pfhrp2*) and *Pfhrp3* as reported by Akinyi and others.⁶

All four isolates had drug resistance profiles identical to the B_{V1} lineage, with an S₇₂V₇₃M₇₄N₇₅T₇₆ haplotype in the *Pfprt* gene, N₈₆D₁₄₄F₁₈₄C₁₀₃₄D₁₀₄₂Y₁₂₄₆ in *Pfmdr1*, R₅₀I₅₁C₅₉N₁₀₈I₁₆₄ in *Pfdhfr*, and S₄₃₆G₄₃₇E₅₄₀G₅₈₁A₆₁₃ in *Pfdhps* (bold font represents the mutant amino acid at each respective position). Also, similar to B_{V1}, all four isolates had deleted *Pfhrp2* and

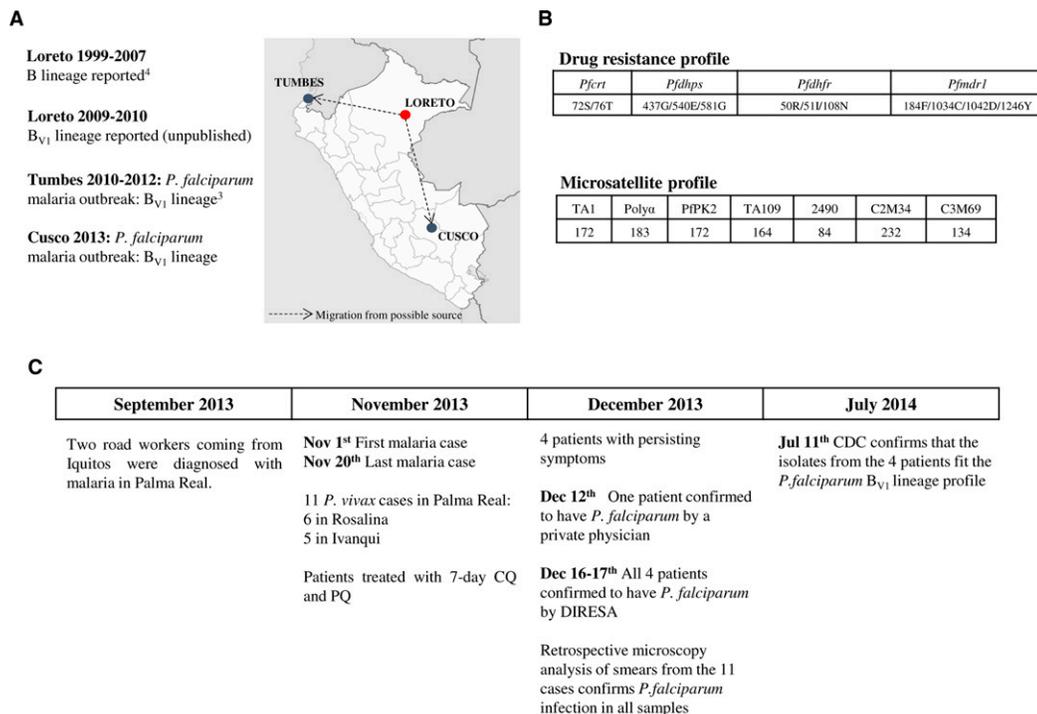


FIGURE 1. (A) Proposed origin and observed migration pattern of *Plasmodium falciparum* B_{V1} lineage parasites in Peru. The dotted arrows in the map indicate human migration from Loreto Department, which is proposed to be the potential origin of the *P. falciparum* B_{V1} clonal lineage in Peru, to Tumbes³ and Cusco departments, resulting in two separate malaria outbreaks. (B) Molecular profile of *P. falciparum* isolates from the malaria outbreak in Cusco. In the top panel, the numbers indicate the amino acid codons while the letter symbols represent the amino acid changes at those particular codons within each respective gene. In the bottom panel, the top row lists the names of the microsatellite loci while the number listed below each locus corresponds to the size (in bases) of the polymerase chain reaction (PCR)-amplified fragment. The antimalarial drug resistance marker and microsatellite allele profiles of the isolates studied were identical to those of the B_{V1} lineage parasites identified in the Tumbes outbreak³ and in Loreto Department. *Pfdhfr* = dihydrofolate reductase; *Pfprt* = chloroquine resistance transporter; *Pfdhps* = dihydropteroate synthase; *Pfmdr1* = multidrug-resistant gene, where *pf* denotes *P. falciparum*. (C) Timeline of the malaria outbreak in Cusco. The two road construction workers from Loreto who presented with malaria in Cusco in September 2013 are the likely index cases to the subsequent outbreak that occurred in November 2013. CQ = chloroquine; DIRESA = Direccin Regional de Salud (Regional Health Directorate); PQ = primaquine.

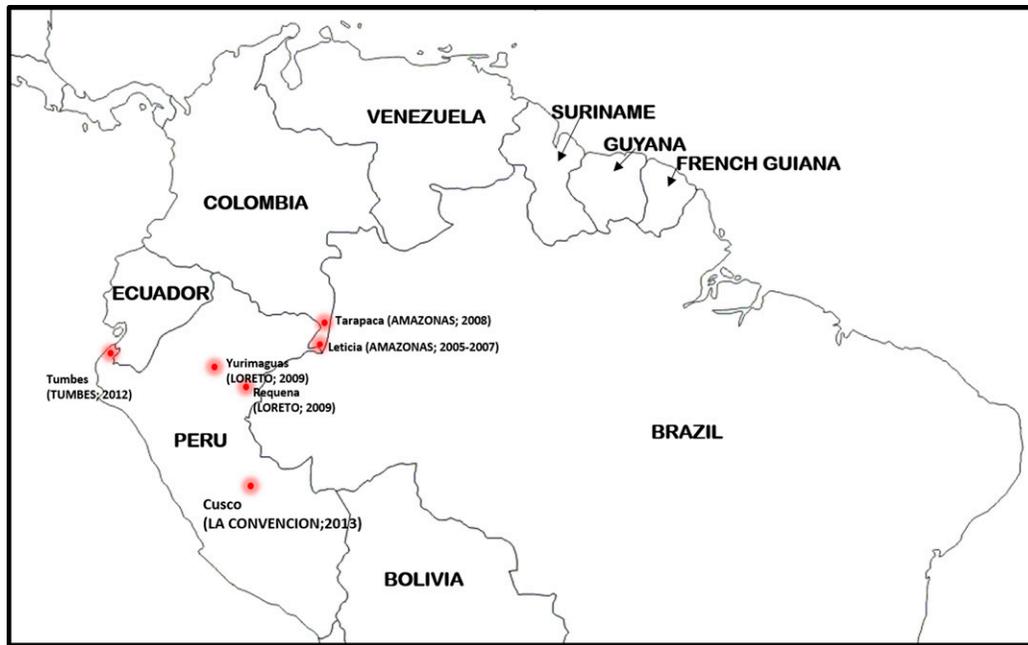


FIGURE 2. Sites in South America where B_{V1} lineage parasites (based on neutral microsatellite allele information) have been identified to date. The departments (or states) of each respective country are indicated in uppercase letters. The year(s) of collection of the specimens containing B_{V1} lineage parasites is shown in parentheses.

Pfhrp3. Overall, the genetic background of these isolates was identical to that of the B_{V1} lineage that caused the *P. falciparum* outbreak in Tumbes in 2012 (Figure 1). This finding suggests that the B_{V1} lineage is the most likely source of the falciparum malaria outbreak in Cusco. This is the second malaria outbreak in Peru that involved the same *P. falciparum* lineage.

It is important to point out that the B_{V1} lineage derived from the original “B clonal lineage” found in Peru since 1999.⁵ The highly clonal dynamic observed in this *P. falciparum* population is the result of decreased outcrossing and genetic drift primarily resulting from inbreeding and asexual replication.⁷ In general, the low *P. falciparum* genetic diversity found in South America relates to the low transmission intensity in this region. With fewer parasite types, there are limited opportunities for outcrossing during meiosis in the mosquito, which results in highly related or even clonal parasites.^{8,9} Moreover, since the *P. falciparum* population underwent strong drug selection, expansions of few drug-resistant parasite lineages is expected.¹⁰

Epidemiological data suggest that the Cusco *P. falciparum* outbreak was the result of human migration from Loreto Department when a road construction company set up a camp in Rosalina to build a road to Kiteni, Echarate District.² In September 2013, prior to the outbreak, two road workers who were originally from Iquitos were diagnosed with severe malaria and, because of their critical condition, were subsequently transferred to another city for medical examination. It is suspected that these may have been the index cases to the ensuing outbreak.² Unfortunately, specimens from these suspected index cases were not available for molecular analysis to confirm the proposed link. Nevertheless, even with the limited number of samples analyzed, this molecular investigation corroborated the epidemiological data suggesting that the

P. falciparum B_{V1} lineage from Loreto is the most likely source of the Cusco outbreak.

In addition to Peru, B_{V1} lineage parasites have been detected in isolates collected in Amazonas Department, Colombia, from as far back as 2005¹¹ (Figure 2). *Anopheles darlingi* and *Anopheles oswaldoi* are the main malaria species in Amazonas Department.¹² The discovery of B_{V1} lineage parasites in other parts of the Amazon region of South America presents a major challenge for regional malaria control and elimination due to the persistence and wide distribution of this *P. falciparum* lineage, which seems highly adaptive to different transmission settings and can escape detection by PfHRP2-based rapid diagnostic tests because of *Pfhrp2*/*Pfhrp3* deletions.

Finally, this study highlights the need for local health posts to perform high-quality malaria diagnosis to detect *P. falciparum* introductions, particularly in areas where *P. vivax* transmission is known or thought to occur exclusively, because *P. falciparum* lineages could possibly establish themselves in the long term. In addition, although there have been some reports on the use and interpretation of *Plasmodium* molecular genetics tools for outbreak investigations,^{3,7} this methodology should be standardized for routine use to identify not only the *Plasmodium* species but the source(s) of a malaria outbreak and the prevalence of long-lasting drug-resistant *P. falciparum* strains.

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