Clonal population expansion in an outbreak of *Plasmodium falciparum* on the northwest coast of Ecuador

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Abstract

**Background:** Determining the source of malaria outbreaks in Ecuador and identifying remaining transmission foci will help in malaria elimination efforts. In this study, the genetic signatures of *Plasmodium falciparum* isolates, obtained from an outbreak that occurred in northwest Ecuador from 2012 to 2013, were characterized.

**Methods:** Molecular investigation of the outbreak was performed using neutral microsatellites, drug resistance markers and *pfhrp2* and *pfhrp3* genotyping.

**Results:** A majority of parasite isolates (31/32) from this outbreak were of a single clonal type that matched a clonal lineage previously described on the northern coast of Peru and a historical isolate from Ecuador. All but one isolate carried a chloroquine-resistant *pfcrt* genotype and sulfadoxine- and pyrimethamine-sensitive *pfhdps* and *pfhdfr* genotypes. *Pfmdr1* mutations were identified in codons 184 and 1042. In addition, most samples (97%) showed presence of *pfhrp2* gene.

**Conclusions:** This study indicates that parasites from a single clonal lineage largely contributed to this outbreak and this lineage was found to be genetically related to a lineage previously reported in the Peruvian coast and historical Ecuadorian parasites.

**Keywords:** *Plasmodium falciparum*, Malaria, Ecuador, Outbreak, Microsatellite markers

Background

Some countries in Latin America have achieved and surpassed the goals set by the World Health Assembly for reducing malaria cases (reduction of malaria burden by 75% between 2000 and 2015) [1]. In particular, Ecuador has decreased the number of malaria cases from more than 100,000 (approximately 13.6 cases per 1000 individuals) in 2000 to 378 cases in 2013 (approximately 0.05 cases per 1000 individuals), thus placing it in the pre-elimination phase [1–3]. Nevertheless, outbreaks of both falciparum and vivax malaria still occur in both the Amazon and Coastal regions of Ecuador.

Molecular tools are valuable in providing insight about the potential source of malaria parasites in outbreak investigations [4–8]. Neutral microsatellite markers, single nucleotide polymorphism (SNP) based molecular barcodes and genomics tools have been used to characterize *Plasmodium falciparum* parasites in South America [5, 9–17]. These studies have suggested that *P. falciparum* parasites in this region have undergone population bottlenecks and in many parts of Peru and Colombia there are clonal population of parasites that are highly prevalent in different geographical regions.

One molecular investigation of *P. falciparum* isolates from Peru, using seven neutral microsatellite markers, revealed that the parasite isolates collected between 1999 and 2000 (during peak malaria expansion after the malaria elimination era) comprised five distinct clonal lineages named A, B, C, D, and E [12]. In the coastal
In order to understand the source of this outbreak parasite population the following questions were answered: (a) are these parasite populations genetically similar to parasite populations in Ecuador, Peru (southern border) and Colombia (northern border)? (b) is there any evidence to suggest these parasites may have origins outside this region (Andean)? and (c) did these outbreak parasites belong to a single clonal lineage or to multiple lineages?

Methods

Ethics statement
The parasite samples used in this study were obtained from the malaria surveillance protocol approved by the Ethical Review Committee of Pontificia Universidad Católica del Ecuador. Written informed consent was provided by study participants and/or their legal guardians.

Study site
Esmeraldas city is the largest city in Esmeraldas Province in northwest Ecuador. All samples used in this study were collected in Esmeraldas city during a malaria outbreak that occurred between November 2012 and November 2013.

Samples
The malaria outbreak occurred between November 2012 and November 2013. Samples used in this study were collected from patients at 11 time points in 2013 between epidemiological weeks 5 and 45, with a higher
concentration of samples collected in weeks 21–23 (Fig. 2). Differences in sample collection patterns were due to logistical challenges, including lack of informed consent, lack of sample collection and delayed training of microscopists. A total of 32 blood samples collected from patients who were initially reported to be microscopically positive for *P. falciparum* infection, and from whom informed consent was obtained, were available for this molecular investigation. The blood samples were collected by finger prick or by drawing peripheral whole blood and spotted on 3MM Whatman filter paper. All the samples were collected in Esmeraldas city between January and November 2013 and most (28 samples, 87.5 %) came from the neighbourhoods in the southern part of the city where the majority of cases were reported (Fig. 3).

Reference isolates
In this study, laboratory adapted *P. falciparum* isolates 3D7, Dd2 and HB3 were used as controls for genotyping. In addition, Ecu 1110, a *P. falciparum* culture-adapted strain that was originally isolated in Ecuador in 1990 (provided by Dr Tom Wellems, National Institutes of Health, Bethesda, USA) was included as a reference strain from Ecuador.

DNA extraction and confirmation of infection
DNA was isolated from filter paper or whole blood using the QIAamp DNA mini-spin kit (QIAGEN, Valencia, CA, USA) or Chelex-100 [19]. *Plasmodium falciparum* infection status was further confirmed by two different molecular methods: nested PCR using primers for 18S ribosomal RNA [20], and photo-induced electron transfer (PET) real-time PCR using specific fluorogenic primers for *Plasmodium* (genus) and *falciparum* (species) [21].

Detection of the *pfhrp2* and *pfhrp3* genes by PCR
Nested PCR amplifications of *pfhrp2* and *pfhrp3* genes were performed using reaction conditions and primers reported previously [15, 22]. In vitro cultured isolate Dd2 was used as a negative control for *pfhrp2* amplification experiments while HB3 served as a negative control for *pfhrp3* amplification because the laboratory isolates lacked the respective genes. The resulting PCR amplicons were visualized on a 2 % agarose gel. If a visible band of the appropriate size was observed, the isolate was scored as positive for the presence of the respective gene and that result was recorded as final. If no amplification occurred, PCR amplification was repeated to confirm this observation. If no amplification occurred during the second amplification attempt, thus showing concordance with the original PCR result, no further testing was done and the outcome was reported as negative. However, if there was discordance between the first and second result, the PCR was repeated again and the two matching results out of three were scored as the final result.

Microsatellite typing
Genomic DNA was used for microsatellite characterization. Samples were genotyped for seven neutral microsatellite loci spanning six chromosomes that had been used in previous studies (*TA1*, chromosome 6; *Polya*, ch. 4; *PfPK2*, ch. 12; *TA109*, ch. 6; and 2490, ch. 10; *C2M34*, ch. 2; *C3M69*, ch. 3) [23–25]. Genomic DNA was amplified by PCR using previously described methods [12, 15, 23–25] and fluorescently labelled (HEX and FAM) PCR products were separated by capillary electrophoresis on

![Fig. 2](image-url) Number of samples collected during the outbreak. The curve shows the total number of cases reported in each epidemiological week during 2013. The bars correspond to the number of samples collected in different epidemiological weeks that were used in this study. The samples available for molecular study were collected only at selected time points as indicated.
an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems Foster City, CA, USA). Alleles were then sized and scored using GeneMapper v3.7 (Applied Biosystems Foster City, CA, USA) and binned to the nearest two or three base pairs.

Drug resistance markers
Sanger sequencing was performed on all samples to identify drug resistance-associated mutations in *pf*cr*t*, *pf*dhfr, *pf*dhps, and *pf*mdr1 genes [26]. Mutations in the codon positions 72–76 of *pf*cr*t*, 51, 59, 108, and 164 of *pf*dhfr,
436, 437, 540, 581, and 613 of pfmdr1 and 84, 134, 184, 1034, 1042, 1226 and 1246 of pfmdr1 were identified.

Network analysis
A median-joining network diagram was generated in Network 4.6.1.3 [27] using the seven neutral microsatellites previously described to examine the genetic relationships among the 32 samples collected from the outbreak, in addition to Ecu 1110, the Peruvian historical isolates published previously [12] as well as five Colombian isolates published recently [14].

Statistical analysis
Analysis of molecular variance (AMOVA) was used to partition variation within the study parasite population and between the population of Ecuador and falciparum populations of western Peru. In addition, heterozygosity (He) and pair-wise fixation indices (Fst) were calculated. Arlequin 3.5.1.2 software (CMGP, Swiss Institute of Bioinformatics, Lausanne, Switzerland) was used for these analyses [28]. Structure v2.3.4 software [29, 30] was used to assign outbreak samples to previously known populations and Structure Harvester [31] was used to define the number of expected populations.

Results
During the P. falciparum malaria outbreak in Esmeraldas, 151 cases of P. falciparum were identified (Fig. 1). Eighty per cent of the cases were located in two parishes in the south part of Esmeraldas. Men accounted for 57 % of the cases. Fifty per cent of the total number of cases were reported in teenagers and young adults (11–25 years old), while 13 % were in children 10 years old or younger, 22 % were in adults 25–40 years old and 15 % were in adults over 40 years old. Most cases were reported between February and April 2013, which corresponds to the rainy season on the Ecuadorian coast (Fig. 1). Thirty-two of these samples were available for this molecular investigation and were confirmed positive by nested PCR and real time PET-PCR.

Genetic characterization using microsatellite markers
Among the 32 samples tested, 31 showed similar microsatellite profile for all seven microsatellites, as indicated in Table 1. There were some minor variations in four samples. For instance, one locus (TA1) could not be amplified in sample F37 but the rest of the alleles were nearly identical to parasites of the E clonal lineage. Three of the samples (F3, F9 and F26) had extra alleles in locus C3M69 (122 bp) in addition to the shared genotype. With the exception of these minor variations, 31/32 isolates shared a very similar genetic profile corresponding to the E clonal lineage of Peru. One sample (F31) had a distinct genotype from all others, differing at four of seven loci. This isolate was similar to clonal lineage D in at least six of the seven loci tested (Table 1; Additional file 1).

As expected, heterozygosity in the Esmeraldas outbreak samples was very low (He = 0.0625) for most loci, consistent with a majority of isolates being clonal. Pair-wise Fst was performed using the data from seven neutral microsatellite markers and compared with the five clonal lineages previously reported in Peru and four lineages reported in Colombia to determine their genetic relatedness. As shown in Table 2, the Fst value between Peru’s E clonal lineage and the Esmeraldas outbreak population was very close to zero, showing their close genetic relationship (P = 0.505). The pair-wise Fst with the other Peruvian clonal lineages and Colombian lineages were significantly higher (P = 0.00) (Table 2). To confirm the relatedness between the outbreak samples and clonal lineages E and D, a median joining network analysis was performed. The network analysis indicates that most of the samples are linked only to the coastal and Western Amazon parasites of Peru (clonal lineage E) and to the clone Ecu 1110. In addition, five samples from Valle del Cauca in Colombia [14] were closely related to clonal lineage E and most outbreak samples. The exception was sample F31 which appears to be more closely related to clonal lineage D (Fig. 4).

Drug resistance markers
All the samples obtained from the outbreak were tested by sequencing for single nucleotide polymorphisms (SNPs) in the anti-malarial drug resistance genes pfcrt, pfmdr1, pfldhfr, pfldhps, and pfmdr1 (Table 3). All 31 samples that were related to the E clonal lineage carried the pfcrt genotype CVMNT (codon 72–76) while the singular D clonal lineage-related isolate, F31, had the CVMET genotype. The E clonal lineage-related 31 samples were wild type for all SNPs tested in the pfldhfr and pfldhps genes.

| Table 1 | Microsatellite alleles amplified in isolates collected during the outbreak |
|---------|--------------------------------------------------|
| Clonal type | Number of samples | TA1 Ch.6 | Poly-α Ch.4 | PIPK2 Ch.12 | TA109 Ch.6 | 2490 Ch. 10 | C2M34 Ch. 2 | C3M69 Ch 3 |
| E | 31 | 171 | 147 | 174 | 160 | 72 | 226 | 140 |
| D | 1 | 171 | 174 | 174 | 160 | 81 | 232 | 122 |

* The clonal types of these parasites were comparable to previously described P. falciparum populations in Peru as demonstrated in Additional file 1.
Sample F31 (D clonal lineage) on the other hand, had the S108 N mutation in \textit{pf}dhfr and a synonymous mutation in codon 540 of \textit{pf}dhps. The Y184F mutation was found in the \textit{pf}mdr1 gene of all 31 E clonal lineage parasites while the lone D clonal lineage parasite was wild type at this codon. In addition, all 32 samples had the mutation N1042D in \textit{pf}mdr1. No mutations were found in any of these samples in codons 86, 130, 144, 1034, 1226, and 1246 of \textit{pf}mdr1 (Table 3; Additional file 2).

\section*{Pfhrp2 and Pfhrp3 genotyping}

The samples from this outbreak were genotyped for the presence of \textit{pf}hrp2 and \textit{pf}hrp3 genes. The results indicated that both genes were intact in all 31 E clonal lineage-related parasite isolates. In contrast, both \textit{pf}hrp2 and \textit{pf}hrp3 were deleted in the D clonal lineage isolate F31 (Table 3).

\section*{Discussion}

As Ecuador progresses towards the pre-elimination phase of the malaria control programme, it is important to thoroughly investigate any malaria occurrences in the country. In this context, understanding the origins of a population of parasites during an outbreak will help the national programme to develop strategies for prevention of future outbreaks. The current molecular epidemiologic investigation has determined that, with the exception of one parasite isolate, the isolates from the 2012–2013 outbreak that were tested carried a nearly identical genotypic profile which is related to the \textit{P. falciparum} E clonal lineage parasites circulating on the north coast of Peru in 1999–2000. Interestingly, a...
single historical isolate collected in 1990 from the coast of Ecuador was also found to be similar to the E clonal lineage highlighting the historical connection between Peru and Ecuador in sharing a genetically similar population of parasites. These isolates also shared a similar drug resistance marker profile in \(pfcrt\), \(pfdhfr\), \(pfdhps\), and \(pfmdr1\) and had intact \(pfhrp2\) and \(pfhrp3\) genes. Interestingly, a single isolate collected in epidemiological week 38 was found to be distinct from the rest of the outbreak isolates in its genetic profile and was related to another clonal lineage (D) that was reported previously in Peru (Fig. 5).

Overall, these findings suggest that the main source of this outbreak might be parasites belonging to the E clonal lineage. However, not all samples from this outbreak were available due to logistical challenges and the unwillingness of some participants to provide consent. Since not all outbreak samples were available for molecular analysis, it was not possible to identify the genetic profile of all the parasite isolates, which would have helped to unambiguously describe the origin of the entire outbreak parasite population. Nevertheless, this finding clearly illustrates the utility of molecular epidemiological investigations in determining the origin(s) of outbreak parasite populations in future investigations as Ecuador moves towards malaria elimination.

The high clonality of \(P. falciparum\) observed in this low-transmission area is consistent with reports from Peru [12, 13, 32], Colombia [10, 14, 21] and Panama [5]. Using the same set of microsatellite markers used in this study, recent \(P. falciparum\) outbreaks in the north coastal region and Cusco area of Peru, were determined to have originated in a region of the Peruvian Amazon [7, 8]. A molecular epidemiological study conducted previously in the north coast of Peru determined that E clonal lineage parasites were circulating in the 1990s [12]. Importantly, \(P. falciparum\) transmission was eliminated in the north coast of Peru from 2005 until the malaria outbreak of 2010–2012, during which a clonal population of parasites belonging to \(B_{V1}\) lineage from Peruvian Amazon, were introduced to the region [8]. These findings demonstrate the utility of molecular tools for epidemiological investigations.

Since the genetic profile of \(P. falciparum\) parasites that were circulating in Ecuador is not known, it is difficult to clearly attribute whether the current major outbreak population

| Table 3 Drug resistance markers haplotypes and \(pfhrp2\) and \(pfhrp3\) presence |
|----------------------------------------|----------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Number of samples | MS clonal type | \(Pfcrt\) | \(Pfdhfr\) | \(Pfdhps\) | \(Pfmdr1\) | \(Pfhrp2\) | \(Pfhrp3\) |
| Esmeraldas 2013       | 31             | E         | CVMNT    | CNCSI     | SAKAA     | NEDF      | SD        | F         | S         | D         | FD        | 1         | +         | +         |
| E P. falciparum lineage 1999\(^a\) | 42             | E         | CVMNT    | CNCSI     | SAKAA     | NEDF      | SNF       | NEDY SD    | F         | S         | D         | FD Y NEDY SD | +         | +         |
| Ecu 1110 1990\(^b\)  | 1              | E         | CVMNT    | CNCSI     | SAKAA     | NEDF      | SD        | F         | S         | D         | FD        | +         | +         |

\(^a\) Data as reported in [12]
\(^b\) Data as reported in [34]
expanded from a residual, vestigial population of parasites that may still be circulating in Ecuador. The finding that a single historical isolate from Ecuador collected about two decades ago genetically resembled the major outbreak population and that sporadic cases have been occurring in the area (Fig. 1) is consistent with the hypothesis that E clonal lineage-related P. falciparum strains were present in Ecuador throughout this time period. Based on this finding, one could argue for the possibility of a potential local expansion of residual reservoirs of parasites with E clonal genetic signatures in this outbreak due to some ecological changes. However, further genetic evidence using parasite isolates collected in recent times from this region is needed to support such a hypothesis.

An alternative explanation for the source of this outbreak, based on epidemiological investigations, suggests that the migration of human cases between Colombia and Ecuador may be the cause of this outbreak (Ministry of Public Health of Ecuador, pers. comm.). However, there is no strong evidence to support this hypothesis as explained below. In a recent study, five parasite isolates closely related to the E clonal lineage were detected in sample collections from 2008 and 2009 obtained from Valle de Cauca Department, Colombia. However, three of these parasites differed from this outbreak samples in the chromosomal 10 locus 2490 and two differed in the chromosomal 6 locus TA109. While mostly CVMNK and CVMNT genotypes of pfcrtp were reported in Ecuador [33], Colombian parasites have been reported to be CVMET genotype [34]. Since there was no availability of parasite specimens from the index case(s), it was not possible to link the genetic data obtained with travel history information.

The F31 sample donor, who reported travel outside of the Esmeraldas area and worked in the Amazon region of Ecuador (Ministry of Public Health, pers. comm.), had parasites belonging to the D clonal lineage. Interestingly, this parasite isolate carried pfcrtp genotype of CVMET that is characteristic of Colombian parasites, as documented in previous studies [34, 35]. Based on the travel history and pfcrtp genotype, one can hypothesize that this traveler may have acquired the D lineage parasites from Colombia or the Ecuador Amazon region; however, information about the genetic profile of P. falciparum parasites circulating in the Ecuadorian Amazon is lacking and so in order to validate the hypothesis, P. falciparum genotype data will need to be collected from the region.

Ecuador changed its P. falciparum anti-malarial treatment policy in 2006, switching from chloroquine (CQ) to artesunate-sulfadoxine-pyrimethamine (AS-SP) and recently to artemether-lumefantrine (AL) [33, 36]. It is important to note that even though the P. falciparum parasites from the outbreak have a CQ-resistant haplotype (CVMNT and CVMET), they carried wild type dhfr and dhps genotypes associated with SP sensitivity. Mutations in pfmdr1 were restricted to only two codons as opposed to mutations in three or four codons of this gene in parasite isolates from the Amazon region of South America [13, 37]. Remarkably, the most common haplotype found in the Esmeraldas outbreak had the exact same drug resistance gene profile as Ecu 1110 [34, 35, 38], an isolate collected in Ecuador in 1990. These results suggest that if these outbreak parasites are from the local residual transmission foci then they have maintained the historical CQ-resistant haplotype CVMNT in the population. In addition, the profile of wild type pfhrp2 and pfhrp3 alleles including the limited mutation pattern in pfmdr1 in the E clonal outbreak parasites indicates that this is an ancestral lineage that may predate SP introduction.

The clonal population from the Esmeraldas outbreak does not have deletions of pfhrp2 or pfhrp3, which would imply that these parasites can be detected by PFHRP2-based rapid diagnostic tests (RDTs). However, the D clonal lineage-related F31 sample had deleted both pfhrp2 and pfhrp3. This finding highlights the need for vigilance when PFHRP2-based RDTs are used for control programmes in South America since pfhrp2 deletion has been documented in parts of South America [14–16, 18].

Conclusions
Taken together, this data suggest that the P. falciparum isolates from the Esmeraldas outbreak are the result of a clonal expansion of parasites either circulating at very low levels in Ecuador or re-invading Ecuador from border countries. Some of the limitations of this study include the lack of genetic and travel information from the index case(s), lack of population-level genotypic data for P. falciparum parasites in Ecuador and the availability of only a sub-set of samples from the outbreak for analysis. Nevertheless, this study highlights the importance of molecular studies to investigate the origin and relatedness of Plasmodium parasite populations in outbreak investigations. Further genetic characterization of historical malaria parasites from Ecuador will help in determining if low-level transmission of bottle-necked, vestigial populations of historical Ecuadorian parasites still contribute to local malaria transmission. The genetic characterizations will also help in future outbreak investigations to differentiate residual bottle-necked populations from newly introduced parasites from other regions as efforts are underway to eliminate malaria from this region.

Additional files

Additional file 1. Microsatellite alleles found in the study samples. Two numbers indicate two alleles detected and DNW indicates no allele amplification at that locus.

Additional file 2. Drug resistance haplotypes and pfhrp2 and pfhrp3 presence in the study samples.
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Competing interests

The authors declare that they have no competing interests.

Disclaimers

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

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References

1. WHO. World malaria report. World Health Organization. 2013.
2. SIVEMAE 2 S, Ministerio de Salud Pública Reposte de casos de malaria en Ecuador. Guayaquil, Ecuador 2013.
3. PAHO. Report on the Situation of Malaria in the Americas 2013.
4. Patel JC, Taylor SM, Julião PC, Parobek CM, Jankó M, Gonzalez LD, et al. Genetic evidence of importation of drug-resistant Plasmodium falciparum to Guatemala from the Democratic Republic of the Congo. Emerg Infect Dis. 2014;20:932–40.
5. Obaldia N 3rd, Baro NK, Calzada JE, Santamaria AM, Daniels R, Wong M, et al. Clonal outbreak of Plasmodium falciparum infection in Eastern Panama. J Infect Dis. 2015;211:1087–96.
6. Ared AP, Snounou G, Pinto J, Sousa CA, Modiano D, Ribeiro H, et al. A clonal Plasmodium falciparum population in an isolated outbreak of malaria in the Republic of Cabo Verde. Parasitology. 1999;118:347–55.
7. Okoth SA, Chener SM, Arrospide N, Gutierrez S, Cabezas C, Matta JA, et al. Molecular investigation into a malaria outbreak in Cusco, Peru. Plasmodium falciparum BV1 lineage is linked to a second outbreak in recent times. Am J Trop Med Hyg. 2015 [Epub ahead of print].
8. Baldeyron GC, Okoth SA, Arrospide N, Gonzalez RV, Sanchez JF, Macedo S, et al. Molecular Epidemiology of Plasmodium falciparum Malaria Outbreak, Tumbes, Peru. 2010–2012. Emerg Infect Dis. 2015;21:797–803.
9. Hernandez DC, Guerra AP, Cucunuba ZM, Nicholls RS, Barrera SM. Haplotypes associated with resistance to sulfadoxine-pyrimethamine in Plasmodium falciparum in two malaria endemic locations in Colombia. Infect Genet Evol. 2013;18:183–90.
10. Echeverry DF, Nair S, Osono L, Menon S, Murillo C, Anderson TJ. Long term persistence of clonal malaria parasite Plasmodium falciparum lineages in the Colombian Pacific region. BMC Genet. 2013;14:2.
11. Arango EM, Samuel R, Aguadel OM, Carmona-Fonseca J, Maestre A, Yanow SK. Genotype comparison of Plasmodium vivax and Plasmodium falciparum clones from pregnant and non-pregnant populations in North-west Colombia. Malar J. 2012;11:392.
12. Grifﬁng SM, Moxson-Hayden T, Sridaran S, Alam MT, McCollum AM, Cabezas C, et al. South American Plasmodium falciparum after the malaria eradication era: clonal population expansion and survival of the fittest hybrids. PLoS One. 2011;6:e23486.
13. Bacon DJ, McCollum AM, Grifﬁng SM, Salas C, Soberon V, Santolalla M, et al. Dynamics of malaria drug resistance patterns in the Amazon basin region following changes in Peruvian national treatment policy for uncomplicated malaria. Antimicrob Agents Chemother. 2009;53:2042–51.
14. Murillo Solano C, Akinji Okoth S, Abdallah JF, Pava Z, Dorado E, Incardona S, et al. Deletion of Plasmodium falciparum histidine-rich protein 2 (pfhrp2) and histidine-rich protein 3 (pfhrp3) genes in Colombian parasites. PLoS One. 2015;10:e0131576.
15. Akinji S, Hayden T, Gamboa D, Torres K, Bendezu J, Abdallah JF, et al. Multiple genetic origins of histidine-rich protein 2 gene deletion in Plasmodium falciparum parasites from Peru. Sci Rep. 2013;3:2797.
16. Akinji Okoth S, Abdallah JF, Cerón N, Adhin MR, Chandrasobe J, Krishnaall K, et al. Variation in Plasmodium falciparum histidine-rich protein 2 (Pfhrp2) and Plasmodium falciparum histidine-rich protein 3 (Pfhrp3) gene deletions in Guayauna and Suriname. PLoS One. 2015;10:e0126805.
17. Grifﬁng SM, Viana GM, Moxson-Hayden T, Sridaran S, Alam MT, de Oliveira AM, et al. Historical shifts in Brazilian P falciparum population structure and drug resistance alleles. PLoS One. 2013;8:e58984.
18. Gamboa D, Ho MF, Bendezu J, Torres K, Chiodini PL, Barnwell JW, et al. A large proportion of P falciparum isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: implications for malaria rapid diagnostic tests. PLoS One. 2010;5:e8091.
19. Kain KC, Lanar DE. Determination of genetic variation within Plasmodium falciparum by using enzymatically amplified DNA from ﬁlter paper disks impregnated with whole blood. J Clin Microbiol. 1991;29:1171–4.
20. Snounou G. Detection and identiﬁcation of the four malaria parasite species infecting humans by PCR ampliﬁcation. Methods Mol Biol. 1996;50:263–91.
21. Lucchi NW, Narayanjan J, Karell MA, Xayavong M, Kariuki S, Dalisija AJ, et al. Molecular diagnosis of malaria by photo-induced electron transfer ﬂuorescent primers. PET-PCR. PLoS One. 2013;8:e56677.
22. Abdallah JF, Okoth S, Fontecha GA, Torres R, Banegas EI, Matute M, et al. Prevalence of pfhrp2 and pfhrp3 gene deletions in Puerto Lempira, Honduras. Malar J. 2015;14:19.
23. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum. Mol Biol Evol. 2000;17:1467–82.
24. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP. Twelve microsatellite markers for characterization of Plasmodium falciparum from ﬁnger-prick blood samples. Parasitology. 1999;119:113–25.
25. McCollum AM, Mueller K, Villages L, Udithayakumar V, Escalante AA. Common origin and fixation of Plasmodium falciparum dhfr and dhps mutations associated with sulfadoxine-pyrimethamine resistance in a low-transmission area in South America. Antimicrob Agents Chemother. 2007;51:2085–91.
26. Alam MT, de Souza DK, Vincaya S, Grifﬁng SM, Poe AC, Duah NO, et al. Selective sweeps and genetic lineages of Plasmodium falciparum drug-resistant alleles in Ghana. J Infect Dis. 2011;203:220–7.
27. Bandelt HJ, Forster P, Rohl A. Median-joining networks for inferring intraspeciﬁc phylogenies. Mol Biol Evol. 1999;16:37–48.
28. Excoﬀier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online. 2005;1:47–50.
29. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155:945–55.
30. Hubisz MJ, Falush D, Stephens M, Pritchard JK. Inference weak population structure with the assistance of sample group information. Mol Ecol Resour. 2009;9:1322–32.
31. Earl DA, von Holt BM. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour. 2012;4:359–61.

32. Branch OH, Sutton PL, Barnes C, Castro JC, Hussin J, Awadalla P, et al. Plasmodium falciparum genetic diversity maintained and amplified over 5 years of a low transmission endemic in the Peruvian Amazon. Mol Biol Evol. 2011;28:1973–86.

33. Arrospide N, Hijar-Guerra G, de Mora D, Díaz-Cortéz CE, Veloz-Pérez R, Gutiérrez S, et al. Alelos mutantes asociados a la resistencia a cloroquina y sulfadoxina-pirimetamina en Plasmodium falciparum de las fronteras Ecuador-Perú y Ecuador-Colombia. Rev Peru Med Exp Salud Publica. 2014;31:282–7.

34. Sa JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, et al. Geographic patterns of Plasmodium falciparum drug resistance distinguished by differential responses to amodiaquine and chloroquine. Proc Natl Acad Sci USA. 2009;106:18883–9.

35. Cortese JF, Caraballo A, Contreras CE, Plowe CV. Origin and dissemination of Plasmodium falciparum drug-resistance mutations in South America. J Infect Dis. 2002;186:999–1006.

36. Jiménez M, Valdez C, Marmion J, Barillas E. Situación de la gestión del suministro de medicamentos para el tratamiento de la malaria en los países que comparten la cuenca Amazónica y Centroamérica. Strengthening Pharmaceutical Systems, Management Sciences for Health. 2011. pp. 21.

37. Griffing S, Syphard L, Sridaran S, McCollum AM, Mixson-Hayden T, Vinayak S, et al. Pfmdr1 amplification and fixation of Pfcrt chloroquine resistance alleles in Plasmodium falciparum in Venezuela. Antimicrob Agents Chemother. 2010;54:1572–9.

38. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the P falciparum digestive vacuole transmembrane protein Pfcrt and evidence for their role in chloroquine resistance. Mol Cell. 2000;6:861–71.