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Genotypes and Phenotypes of Resistance in Ecuadorian *Plasmodium falciparum*

Tesis previa a la obtención del título de Magister en Biología de las Enfermedades Infecciosas

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Certifico que la Tesis de Maestría en Biología de las Enfermedades Infecciosas de la Lcda. Gabriela Paola Valenzuela Sánchez ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Fabián Sáenz Ph.D.
Director de Tesis
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A mis padres, mis hermanos y mi esposo

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TABLA DE CONTENIDOS

ABBREVIATIONS LIST

Abbreviation	Meaning
ACT	Artemisinin in combination with other antimalarial
ART	Artemisinine
ATM	Arthemeter
CQ	Chloroquine
DHA	Dihydroartemisinin
HF	Halofantrine
iRBC	Infected red blood cell
MQ	Mefloquine
LUMF	Lumefantrine
Pfcrt	*P. falciparum* chloroquine resistance transporter
Pfdhfr	*P. falciparum* dihydrofolate reductase

ABBREVIATIONS LIST

Abbreviation	Meaning
Pfdhps	*P. falciparum* dihydropteroate synthetase
Pfmdr1	*P. falciparum* multidrug resistance 1
QN	Quinine
RBC	Red blood cell

FIGURE LIST

TABLE LIST

1 RESUMEN

Introducción: La malaria continúa siendo endémica en la costa y Amazonia de Ecuador. Determinar la situación actual de resistencia de *P. falciparum* a medicamentos en el país contribuirá con la eliminación de la enfermedad. En este estudio *P. falciparum* ecuatorianos fueron analizados para determinar su fegotipo y genotipo de resistencia.

Métodos: Se realizaron análisis moleculares para determinar mutaciones en los genes *pfcrt*, *pfdhfr*, *pfdhps*, *pfmdr1* y *k13*. El número de copias del gen *Pfmdr1* se determinó a través de PCR en tiempo real. El transporte de Fluo 4 fue usado para determinar la actividad transportadora de la proteína PFMDR1. Ensayos *in vitro* se usaron para determinar la susceptibilidad de los parásitos a cloroquina, quinina, lumefantrina, mefloquina, dihydroartemisinina y artemeter.

Resultados: La mayoría de muestras presentaron el genotipo CVMN**T** en el gen *pfcrt* (72-26), mutaciones (NED**F** S**D**FD) en el gen *pfmdr1* y los genotipos salvajes en los genes *pfdhfr*, *pfdhps* y *k13*. La cepa ESM-2013 presentó resistencia *in vitro* a la cloroquina, pero susceptibilidad a quinina, lumefantrina, mefloquina, dihydroartermisinina y artemeter. Además, ESM-2013 presentó inhibición en el transporte de Fluo 4 AM, desde el citosol hacia la vacuola digestiva. Todas las muestras analizadas presentaron solo una copia del gen *Pfmdr1*.

Conclusión: Este estudio nos indica que los parásitos ecuatorianos presentan el genotipo y fenotipo de resistencia a cloroquina, pero de sensibilidad a sulfadoxina – pirimetamina, artemeter – lumefantrina, quinina, mefloquina y dihydroartemisinina. Los resultados de este estudio sugieren que el actual tratamiento está siendo efectivo. Este estudio pretende clarificar la situación

actual de resistencia a los antimaláricos en Ecuador y de esta manera contribuir con la eliminación de malaria causada por *P. falciparum*.

Palabras claves: *Plasmodium falciparum*, malaria, Ecuador, resistencia, genotipos, fenotipos.

2 ABSTRACT

Background: Malaria continues to be endemic in the coast and amazon regions of Ecuador. Clarifying the current situation of *P. falciparum* resistance in the country will support malaria elimination efforts. In this study, Ecuadorian *P. falciparum* were analyzed to determine their drug resistance genotypes and phenotypes.

Methods: Molecular analyses were performed in searching of mutations in resistance (*pfcrt*, *pfdhfr*, *pfdhps*, *pfmdr1* and *k13*) markers. *Pfmdr1* copy number was determined by qPCR. Fluo-4 transport by live cell imaging was used to characterize the PFMDR1 transporter activity. Chloroquine, quinine, lumefantrine, mefloquine, dihydroartemisinine and artemeter sensitivities were measured by *in vitro* assays.

Results: The majority of samples from this study presented the CVMN**T** genotype for *pfcrt* (72-26), NED**F** S**D**FD mutations in *pfmdr1* and wild type genotypes for *pfdhfr*, *pfdhps* and *k13*. The Ecuadorian *P. falciparum* strain ESM-2013 showed *in vitro* resistance to chloroquine, but sensitivity to quinine, lumefantrine, mefloquine, dihydroartemisinine and artemeter. In addition, ESM-2013 inhibited the transport of Fluo-4 AM from cytosol into the digestive vacuole. All the samples analyzed showed one copy of *Pfmdr1*.

Conclusion: This study indicates that Ecuadorian parasites presented the genotype and phenotype for chloroquine resistance, however, they are sensitive to sulfadoxine-pirimetamine, arthermeter-lumefantrine, quinine, mefloquine, and dihydroartemisinin. The results of this study suggest that the current treatment employed in the country is effective. Our results help clarifying the situation of

antimalarial resistance in Ecuador and inform the *Plasmodium falciparum* elimination campaigns in the country.

Keywords: *Plasmodium falciparum*, malaria, Ecuador, resistance, genotypes, phenotypes

REVISTA

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TÍTULO

Genotypes and Phenotypes of Resistance in Ecuadorian *Plasmodium falciparum*

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Genotypes and Phenotypes of Resistance in Ecuadorian *Plasmodium falciparum*

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3 Background

According to the World Health Organization (WHO), 132 million people were at risk of malaria infection in 2015 in the Americas. There was an estimated 31% decrease from 2010 to 2015 in malaria incidence in this region, as well as a 37% decrease in malaria related mortality. Nevertheless, approximately 450,000 cases were reported in 2015 in the region [1], 30% of them in Venezuela, Colombia reported around 48,000 cases and Peru reported 66,000 cases in the same year [1], 30% of which were caused by *Plasmodium falciparum.* Malaria continues to be endemic in the coast and amazon areas of Ecuador. The country reported 558 cases in 2015 [3] and 916 cases in 2016 and *P. falciparum* was responsible for 31 % of cases in the country in the same year [4].

Current treatment for *P. falciparum* infection in Americas is based on ACT (artemisinin in combination with another antimalarial) [2]. The treatment in Ecuador used for uncomplicated malaria cases caused by *P. falciparum* is arthemeter – lumefantrine + primaquine and for *P. vivax* the treatment used is chloroquine + primaquine [3]. *P. falciparum* has developed resistance to almost all available antimalarials and an adequate knowledge of the antimalarial drug effectiveness is necessary, especially in low transmission areas where malaria elimination is underway, since the inflow of resistant parasites can generate unexpected outbreaks.

Chloroquine (CQ) resistance by *P. falciparum* was reported in 1960 in the Thailand – Cambodia border and almost at the same time in Colombia and Venezuela, before spreading to the rest of the world [5]. Drug resistance in South

America spread almost at the same time as in South East Asia. Currently, CQ resistance is present in all of South America [5] and the molecular marker K76**T** is considered fixed in this region [6]. The haplotype CVMN**T** (positions 72-76) has been reported in Colombia and Peru. Also, the haplotypes CVM**ET** and CV**EIT** have been reported in Colombia and Venezuela and **S**VMN**T** has been reported in the amazon of Brazil and Peru [7].

During the 70s sulfadoxine and pirimetamine were introduced in combination as a treatment against *P. falciparum* in South America, however, shortly after their use, resistance to these drugs was reported [7]. Colombian, Brazilian and Peruvian isolates showed mutations in *Pfdhps* mainly in positions 437, 540 and 581. The mutation A437**G** was present in Colombia, the mutations A437**G** and K540**E** were present in Peru [7, 8, 9]. In addition, Venezuela and Bolivia reported the mutation K540**E** in ninety percent of the samples [7, 8]. *Pfdhfr* mutations C50**R**, I165**L** and S108**N/T** are frequent around South America [8, 9]. All of these mutations are associated with sulfadoxine and pyrimetamine resistance [10].

Pfmdr1 codifies for a permeability glycoprotein (MDR1) that is a transporter protein, it is part of adenosine triphosphate–binding cassette transporters. Mutations in *Pfmdr1* (*Plasmodium falciparum* multidrug resistance gene) are associated with multidrug resistance, reduced susceptibility to mefloquine (MQ), halofantrine (HF), quinine (QN) and possibly to lumefantrine (LUMF) [11, 12]. The mutations N86Y and Y184F are more common in Asia and Africa, in contrast in South America the mutations S1034C, N1042D and D1246Y are the most common ones [12].

Several studies associate *Pfmdr1* copy number modification to MQ resistance and quinine (QN) and CQ susceptibility [13, 14, 15]. Recent research suggested that an increase in *Pfmdr1* copy number was related to artemisinin resistance [14, 13]. In South America, there are reports of modifications in *Pfmdr1* copy number, specifically in samples from the Pacific region, Atlantic region and southeast of Colombia. In this area, there was an increase of two to five copies in 30 % of the samples [14]. Nevertheless, Peru reported single copy number for *Pfmdr1* [16].

Resistance to artemisinine (ART) in *P. falciparum* has been reported in five Asian countries: China, Viet-Nam, Cambodia, Thailand and Myanmar. The current treatment to control *P. falciparum* infections is based on ART derivatives combined with a partner antimalarial (MQ, LUMF, primaquine) [17]. *Kelch 13* (K13) propeller mutations have been associated with ART resistance, these mutations can be used as molecular markers to monitor the possible emergence of resistance to ART [3, 18]. ACT treatment continues to be effective in South America. New studies in Brazilian, Peruvian and Colombian isolates show a lack of *k13* mutations previously associated with artemisinin resistance [5, 19, 20].

In addition to the study of genetic variability, drug resistance phenotypes can be characterized by *in vitro* assays. In particular, Colombia reported low *in vitro* susceptibility to CQ and amodiaquine (AQ) in almost 90% of the samples analyzed showing IC_{50} > 100nM [14, 21]. Furthermore, all samples presented high susceptibility to dihydroartemisinin (DHA), LUMF and arthemeter (ATM) [14,

21]. Brazilian samples from the Amazon region also presented resistance to CQ and AQ with an increase in IC_{50} to these drugs [22]. *In vitro* assays have been limited in South American samples because the culture adaptation of field parasites to laboratory conditions can take a long time and parasites present intrinsic culture capabilities [23].

Studies in Ecuador in 2002, reported mutations in samples collected in Esmeraldas. The parasites presented CVMN**T** genotype for *Pfcrt*, and at least one mutation (position 108**N**) and double (positions 108**N**, 164**L**) or triple (51I, 108N, 164L) mutations in *Pfdhfr* [24]. In 2013, wild type genotypes for *Pfdhfr* and *Pfdhps* were reported and genotypes CVMN**T** and CVM**ET** were present in *Pfcrt*, and the mutations Y184F and N1042D in *Pfmdr1* in an outbreak occurred in Esmeraldas city [25]. These genotypes showed that Ecuadorian strains had resistance to chloroquine and were mostly sensible to sulfadoxine and primimetamine [25]. This genotype was shared with Ecu 1110, an isolate from 1990 of the same area. Also, Ecu1110 presents an *in vitro* phenotype of resistance to CQ (IC_{50} > 90 nM) [26].

In this study, *in vitro* assays were used to determine the drug susceptibility phenotypes. In addition, drug resistance genotypes were analyzed in five genes of Ecuadorian *P. falciparum* isolates. The aim of this study was to understand the current situation of antimalarial resistance in Ecuador, in order to support malaria elimination efforts in the country.

4 Materials and Methods

Ethics statement

The samples used in this study were obtained from the malaria control and elimination program and approved by the Ethical Review Committee of Pontificia Universidad Católica del Ecuador (CBE-016-2013 and CEISH-163-2016). Written informed consent was provided by study participants and/or their legal guardians.

Study Site and Sample Collection

Sixty-nine samples were analyzed in this study, sixty-two of them (89.9%) were collected in Esmeraldas province, all the samples collected in this province came from Esmeraldas and San Lorenzo counties, where the incidence of *P. falciparum* is the highest in Ecuador. Esmeraldas is located in northwest Ecuador and borders with Colombia. Four samples were collected in Carchi province, located in the north of Ecuador, east of Esmeraldas province. Three samples were collected in Sucumbíos, a province located in the north Amazon of Ecuador, east of Carchi province (Fig 1).

All samples were collected between 2013 and 2015 by SNEM (National Service for Control of Diseases Transmitted by Arthropod Vectors); 85% of them as whole blood and 15% in filter paper and kept at 4 ˚C.

DNA Extraction and Confirmation of infection

DNA from 69 samples was isolated from filter paper using QIamp DNA mini-spin kit (QIAGEN, Valencia, CA, USA), and from whole blood using Axyprep body fluid viral DNA/RNA Miniprep (AXYGEN, Union City, CA, USA). Confirmation of *P.*

falciparum infection was performed by microscopy and nested-PCR using of the 18S ribosomal RNA gene [27]

Drug resistance markers

P. falciparum 3D7, W2, D6, CAM6 and C2B isolates were used as controls and Ecuadorian *P. falciparum* were analyzed to identify drug resistance associated mutations in *Pfcrt* (positions 72-76), *Pfmdr-1* (positions 134,184,1034,1042,1226 and 1246), *Pfdhfr* (positions 51,59,108 and 164), *Pfdhps* (positions 436,437,540,581 and 613), and *K13* (positions 476, 493, 539, 543 and 580) using conditions and primers reported previously in other studies [9, 27, 28, 29] (Table 1).

All PCR amplicons were visualized in 2% agarose gels and purified with 5 µl of Illustra ExoProStar (GE Healthcare, Piscataway NJ, USA) at 37°C for 25 minutes and 20 minutes at 80°C. After visual confirmation of amplification in the agarose gel, all amplicons were submitted to MACROGEN, South Korea for capillary sequencing.

Molecular Analysis of Drug Resistance Markers

All samples were sequenced for specific drug resistance associated SNPs. The sequencing results were analyzed and aligned using Geneious software version 10 (Biomatters, Inc, Newark, NJ, USA). The sequences were compared to controls (3D7, W2, D6, CAM6, C2B) to establish which samples sequences present mutations. The sequence alignments were analyzed, and SNP

differences were established to determine the mutation frequencies for each gene. All mutations frequencies were analyzed using Microsoft Excel 16.9.

Pfmdr1 Copy number

Pfmdr1 copy number was established by real time PCR, using TaqMan probes for *pfmdr1* (target gene) and the housekeeping gene *Seryl-t-rna-synthetase* was used as a control.

The primers used for quantifying copy number were: *PF_F:* 5TTAAGTTTTACTCTAAAAGAAGGGAAAACATA,PF_*R:* 5′TCTCC TTCGGTTGGATCATAAAG, seryl_F:5′GATTTATTAAGAAAAATAGGTGGAGC TA, seryl_R:5′TATAGCATTATGTAATAAGAAACCTGC, and the Taqman probes were: *PF_FAM:* 5′FAMCATTTGTGGGAGAATCAGGTTGTGGGAAAT_TAMRA, seryl_probe:5′VICAAGGTATACAAGTAGCAGGTCATCGTGGTT_TAMRA [31].

Each 20 µl of reaction mix contained 15 ng DNA, 300 nM primers, 250 nM TaqMan probes and 10 ul of TaqMan Fast Advance Mix, Applied Biosystems (Austin, TX, USA). Triplicates for each sample were analyzed with the following amplification protocol initial activation: 94 ˚C at for 3 min, followed by 40 cycles of: 94 ˚C for 1 min, 60°C for 1 min, and 72 ˚C for 30 sec. Fluorescence was recorded after each elongation step. Real-time PCR was carried out in StepOne™ Real-Time PCR System (Applied Biosystems). Copy numbers were calculated relative to 3D7, which is known to have only one *pfmdr1* gene copy and the seryl gene is expressed during all of the parasite cycle. The copy number of *pfmdr1* was calculated using a comparative threshold method, with the formula

$\Delta\Delta Ct = (Ct_{mdr1} - Ct_{seryl})_{Sample} - (Ct_{mdr1} - Ct_{seryl})_{3D7}$, $2^{-(\Delta\Delta Ct)}$ where Ct is the threshold cycle for each gene [17, 31].

Phenotype characterization

Parasites

The following standard strains were used as controls for *in vitro* susceptibility assays: 3D7 (CQ sensitive) and W2 (CQ and SP resistant). The *P. falciparum* isolate ESM-2013 was obtained from a patient in Esmeraldas city in 2013and subsequently adapted to laboratory conditions by Dr. Fabián Sáenz.

Parasite culture conditions

Parasites were cultured in human O+ erythrocytes, following previously reported methods [33], with modifications. RPMI 1640, supplemented with 25 mM of HEPES, 8,9 mM of Sodium Bicarbonate and human plasma O+ was employed. All parasites were cultured in red blood cells at 4% of hematocrit with complete medium at 37 ˚C and mixed gas (5% O_2, 5% CO_2 and 90% of N_2).

Chemicals

In vitro sensitivity assays were performed with the following drugs: chloroquine disphosphate (CQ), dihydroartemisinin (DHA), quinine sulfate (QN), mefloquine hydrochloride (MQ), lumefantrine (LUMF) and Artemether (ATM). The drugs were gently provided by Dr. Dennis Kyle, University of South Florida

Drug sensitivity assay

The sensitivity of the parasites using *in vitro* assays with antimalarial drugs was measured by microscopy to establish the IC_{50}s. Drugs stock solutions were prepared in dimethyl sulfoxide (DMSO) or water, at an initial concentration of 1mg/ml. The experiments were set up in 96-well plates with 2-fold dilutions of each drug across the plate in a total volume of 150 µl and at a final red blood cell concentration of 1.5% (vol/vol). The experiment was started at an initial parasitemia of 0.5% (80% rings) synchronous parasite-infected red blood cells (PRBC). The plates were incubated for 72 h at 37°C in an atmosphere of 5% CO2, 5% O2, and 90% N2.

A light microscope was used to look for the presence of schizonts in a thick smear to establish parasite growth. Parasitemias were determined by counting 1000 red blood cells (RBCs) and the number of infected cells (iRBCs) using the equation (No. iRBCs*100/No. RBCs). All *in vitro* assays were performed in duplicate with at least two replicates.

Data analysis

Thin smears were stained with 20% GIEMSA at for 20 minutes. The data was analyzed using Excel 16.9 software to make comparisons between parasitemias of each well vs drug concentration. Parasitic IC_{50} curves were obtained using non-linear regression and IC_{50}s were calculated by using the equation of the curve.

Live cell imaging

Parasite cultures of standard laboratory strain Dd2 and Ecuadorian *P. falciparum* ESM-2013 were used for this experiment. iRBC were washed twice with Ringer's solution and then were loaded with 5 µM of Fluo-4 AM (Life Technologies, Burlington, ON, Canada) in Ringer's solution for 45 min at 37°C and transferred to a confocal microscope chamber. Parasites were kept at 37°C during microscopy. A series of images per parasite was taken using a Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany) using the objective (63x/1.20 W Korr M27) and a 488 nm laser. The ratio was calculated by intensity of fluorescence using the Zen software (Carl Zeiss) [34].

5 Results

Genotyping of drug resistance markers

In this study, 62 samples were analyzed for mutations in five genes: *Pfcrt* (positions 72-76), *Pfdhps* (positions 436, 437, 540, 581, 613), *Pfdrfr* (positions 50,51,59, 108, 164), *Pfmdr1* (positions 86, 130, 144, 184, 1034, 1042, 1226, 1246) and *K13* (positions 476, 493, 539, 543, 580). Thirty-two samples were collected in Esmeraldas county, 23 samples in San Lorenzo county, in total 55 samples were collected in Esmeraldas province, 4 samples collected in Carchi province and 3 in Sucumbíos province, F50 is a sample collected in Orellana province and Ecu 1110 (Esmeraldas 1990) was included in the analysis.

The samples collected in Esmeraldas county presented two mutated haplotypes of *Pfcrt*: 97% of the samples presented the CVMN**T** haplotype with a simple mutation in the position 76 (K76T) and 3% of the samples showed the CVM**ET**

haplotype with double mutations at positions 75 and 76 (N75**E**). In San Lorenzo county, the same haplotypes were found but in different frequencies, 78.3% of the samples presented the haplotype CVMN**T** and 21.3% showed CVM**ET**. All of the samples collected in Carchi showed the CVMN**T** haplotype**.** This haplotype occurs in 33.3% of Sucumbíos samples, while, 66.7% presented the CVM**ET** haplotype (Fig.2a). Only, the sample F50 collected in Orellana presented the haplotype **S**VMN**T** with double mutations at positions 72 and 76 (C72**S**). The Ecu1110 isolate, collected in Esmeraldas in 1990, also presented the CVMN**T** haplotype for *Pfcrt*. In conclusion, 100% of the samples analyzed showed at least one mutation in the 72-76 positions of *Pfcrt.* All of the genotypes found are associated to CQ resistance (Table 2).

The *Pfdhfr* gene presented the wild genotype CNCSI in the majority of samples (97%) in Esmeraldas county. In San Lorenzo county, 82.6%, 66.7% in Sucumbíos and 100% in Carchi. The CNC**N**I haplotype with a simple mutation in the position S108N was found in 13.04% of San Lorenzo samples and the C**I**C**N**I haplotype (double mutant at positions N51I and S108N in 4.36% of San Lorenzo samples and one of Sucumbíos samples (Fig 2b). The double mutation (C**I**C**N**I) was also found in F50 (Orellana). In contrast, Ecu 1110 presented the wild genotype CNCSI. San Lorenzo county shows higher diversity of haplotypes for the *Pfdhfr* gene (Table 2), although, the wild type genotype was the most frequent in the population.

The wild type genotype (SAKAA) of *Pfdhps* was present in the highest frequency in all samples analyzed: 97% for Esmeraldas and 100% for San Lorenzo, Carchi

and Sucumbíos (Fig 2c). One sample from Esmeraldas county presented a synonymous mutation in the position 540, the sample F50 from Sucumbíos had the mutation K540**E,** showing the haplotype SA**E**AA (Table 2).

Mutations in the gene *K13* have been related to artemisinin resistance. This gene was analyzed in five different positions. All the samples studied in Esmeraldas, San Lorenzo, Carchi and Sucumbíos presented the wild type haplotype CRYG (Fig 2d).

Pfmdr1 presented two main mutations: Y184F and N1042D in the majority of samples analyzed in this study. In fact, the NED**F**S**D**FD haplotype was present in 97% of Esmeraldas county samples, 78.3% of San Lorenzo county samples and 100% of Carchi and Sucumbíos samples (Fig 2e). Surprisingly, the F50 sample and the Ecu 1110 presented the same genotype. In contrast, just 3% of Esmeraldas county (F31 sample) presented only the mutation 1042 (NEDYS**D**FD) and 21.7% of San Lorenzo county samples presented only one mutation in the position 184 (NED**F**SNFD). The mutations 184 and 1042 were frequent in all the samples (Table 2).

Copy number of *Pfmdr1*

Pfmdr1 copy number was determined by qPCR. The housekeeping gene *Seryl* was used as an internal control. 3D7 and Dd2 strains were used as copy number controls, 3D7 showed one copy and Dd2 two copies of *Pfmdr1* gene. All results were calculated with relative quantification (RQ) by the calculation of ΔΔCt, using the Ct generated by *Seryl* and *Pfmdr1for* each sample. Sixty-two samples were

analyzed, 75% (45/62) of the samples were amplified by qPCR. One-hundred percent of the samples collected in Esmeraldas county, San Lorenzo county, Carchi and Sucumbíos presented only one copy of *Pfmdr1* (Fig 3).

Live cell imaging

Ecuadorian *P. falciparum* was analyzed for eight different amino acid positions and copy number variations of *Pfmdr1*. These parasites presented two main mutations: Y184**F** and N1042**D** and only one copy of the gene. Fluo 4 AM is a fluorescence solution that attaches to Ca_2+ free and it is transported by PFMDR1 from the cytosol to the digestive vacuole. Using Fluo 4 AM can help investigate the role of the PFMDR1 transporter. The mutation N1042D has been related to blocking the transport of Fluo 4 AM from the cytosol to the digestive vacuole [34]. The parasite ESM-2013, an isolate that presented the mutation N1042**D**, was cultured with Fluo 4 AM and visualized by confocal microscopy to establish the transport of the dye. Dd2 strain was used as control that allows the transport of Fluo 4 AM to its digestive vacuole. The results show that ESM-2013 inhibits the transport of Fluo 4 AM to its digestive vacuole in the trophozoites and in the gametocytes (Fig 4). The ratio of fluorescence intensity (DV/cytosol) of Dd2 was 6.9, in contrast ESM-2013 presented ratio of 0.50.

***In vitro* Drug Sensitivity Characterization**

In vitro assays were used to test the sensitivity of Ecuadorian *P. falciparum* to CQ, MQ, QN, ATM, DHA and LUMF. ESM-2013, was exposed to antimalarial drugs to establish its IC_{50}. This parasite also presented the genotype associated with resistance to CQ (CVMN**T**). The parasite ESM-2013 showed an IC_{50} of 93.71

nM (Table 3) (Fig 5). Furthermore, ESM-2013 presented considerable lower IC_{50} for MQ, QN, LUMF, ATM and DHA. ESM-2013 presented 4.94 nM IC_{50} for MQ, 7.63 nM for QN and 2.60 nM for DHA indicating a phenotype of sensitivity for those antimalarial drugs. The current treatment for *P. falciparum* infections in Ecuador is based on ATM and LUMF, this treatment was tested in ESM-2013 isolate and this parasite showed an IC_{50} of 3.59 nM for LUMF and 1.14 nM for ATM (Table 3).

6 Discussion

Ecuador has been very successful in reducing the number of malaria cases. It is estimated that a 99% prevalence reduction has taken place since 2000 [35]. In this context, understanding the drug resistance genotypes and phenotypes of Ecuadorian *Plasmodium* isolates constitutes crucial information for supporting the National Malaria Program to achieve elimination of the disease in the country.

Ninety percent of the samples analyzed in this study were collected in Esmeraldas province (Esmeraldas and San Lorenzo counties), where most *P. falciparum* cases concentrate. In 2016, this province reported 125 *P. falciparum* cases; roughly 45% of the total of *P. falciparum* cases in the country [4].

This study determined the mutations associated with five different genes involved in CQ, SP, MQ, QN and ART resistance present in Ecuador. The results showed that Ecuadorian parasites presented the CQ resistance haplotypes CVMN**T**, CVM**ET** and **S**VMN**T** in *Pfcrt* (72-76)**.** The genotype CVMN**T** was found in Esmeraldas, San Lorenzo, Carchi and Sucumbíos while the CVM**ET** genotype

was found in San Lorenzo and Sucumbíos, the SVMNT genotype was found in one sample from Orellana. CVMN**T** and CVM**ET** genotypes have previously been reported Colombia [7]. CVMN**T** was reported in the Pacific coast of Peru and the CVM**ET** genotype was reported in the Amazon of Peru [9] The genotype **S**VMN**T** has been found in the Peruvian [9] and Brazilian Amazon [8].

The CVMN**T** haplotype had been previously reported in a parasite isolate obtained in Esmeraldas in the 1990 [26] Additionally, this genotype was reported in Ecuador during an outbreak occurred in 2013, a parasite from the 1990s. This suggests that this haplotype has been circulating in Ecuador for decades and is still maintained in the country. Griffing and collaborators suggest that parasites carrying this haplotype, were circulating in Colombia, then they were transmitted to Ecuador and later entered Peru [9].

The resistance to CQ is considered fixed in South America since CVMNT, CVMET and **S**VMN**T** are common genotypes in the region [7, 9]. However, in Ecuador, the national treatment regime was changed from CQ in 2004 to artesunate + sulfadoxine-pirimethamine [36] and; more recently, to ATM-LUMF [25]. *P. falciparum* parasites circulating in the region continue to have the K76**T** genotype of *Pfcrt*. This could be related to a continuous drug pressure in the population, because, the treatment to control infections caused by *P. vivax* is CQ.

The wild type genotype CNCSI for *Pfdhfr* was found in all studied locations in Ecuador, in Peru and Colombia as well as in the Ecu 1110 isolate [26]. The genotype with a simple mutation (CNC**N**I) was present in San Lorenzo county.

These genotypes have previously been reported in Ecuador [25, 24], Colombia [7] and Peru [9]. The resistance genotype C**I**C**N**I was found in this study in San Lorenzo county and Orellana. In addition, Peru, Colombia and Brazil reported this genotype [9, 7].

Pfdhfr was more diverse in San Lorenzo than in the other Ecuadorian locations. San Lorenzo is located close to the Colombian border suggesting that the migration of parasites from Colombia to Ecuador could be related with the distribution of these mutations. In fact, have been frequently reported in Colombia double and triple mutations in *Pfdhfr* and resistance to SP [7].

The wild type genotype SAKAA for *Pfdhps* presented the highest frequency in all studied locations and in Ecu 1110 [26]. Only the sample F50 from Orellana presented mutation in this position (SA**E**AA) this mutation is common in Brazil, [24]Venezuela, Bolivia and Peru [7, 9]. The wild type genotype was previously found in samples from Esmeraldas in studies performed in 2002 and 2013. Mutations in this gene have not been reported previously [24,25].

Despite of the presence of mutations in *Pfdhfr* and *Pfdhps* in samples from Ecuador, the high prevalence of wild type genotypes, suggests sensitivity to SP in the country. In 2002 90% of samples collected in Esmeraldas presented at least one mutation (position 108N). In addition, double (positions 108N, 164L) and triple (51I, 108N, 164L) mutations were reported [24]. The decrease in the frequency of parasites carrying *Pfdhfr* mutations in Esmeraldas province could

be related to the change in treatment from artesunate + sulfadoxine-pirimetamine to ATM-LUMF which relieved the parasites from drug pressure.

Pfmdr1 codes for a transmembrane P-glycoprotein in the digestive vacuole (DV) of the parasites involved in transport of substrates from the cytosol to the DV. This protein belongs to the adenosine triphosphate-binding cassette transporter family [11]. Two factors have been associated with alteration of function in PFMDR1: mutations present in *Pfmdr1* and copy number increase [34]. The mutations N86**Y** and Y184**F** are more common in Asia and Africa, in contrast, in South America the mutations S1034**C**, N1042**D** and D1246**Y** are found to be more common [12]. All of these mutations are associated with multidrug resistance [37].

In Ecuador, the mutations 184**F** and 1042**D** were frequently found and were present in the majority of samples from an outbreak that occurred in Esmeraldas in 2013 [25]. These double mutants were found in this study in samples from Esmeraldas, San Lorenzo, Carchi and Sucumbíos. Furthermore, this genotype was found in Orellana and previously reported in Ecu 1110 [26]. The *Pfmdr1* polymorphisms (86, 1034, 1042 and 1246) have been associated with resistance to QN, MQ, DHA and HF [12, 38]. The 184F mutation has not been associated with any specific drug resistance and the mutation 1042D has been linked to MQ and QN resistance [34]. However, Ecuadorian parasites do not present resistance to MQ and QN, suggesting that these drugs can be considered as an alternative to the current treatment in the future.

The increase in *Pfmdr1* copy number has been associated with of *P. falciparum* resistance to MQ, QN and ART [13, 14, 15]. All Ecuadorian *P. falciparum* parasites in this study showed one copy of this gene, suggesting that these parasites are sensitive to MQ. Efficacy *in vivo* studies of artesunate and MQ combination showed that these drugs were an effective treatment in Ecuador in 2000 [39]. In South America, there are reports of modifications in copy number in samples from the Pacific region, Atlantic region and southeast of Colombia (2009-2012), where 32% of the isolates had a *Pfmdr1* copy number increase of two to five copies [14]. Nevertheless, Peru reported single copy number for *Pfmdr1* in 2009 in the Amazon region [16].

The mutation 1042**D** and the increase in copy number have been associated with MQ resistance [34]. MQ forms hydrogen bonds with the residue 1042 of PFMDR1 and the change of N (asparagine) to D (acid aspartic) in this position could result in the inhibition of MQ passage through the DV membrane [40]. In order to test this hypothesis live cell imaging using Fluo 4 AM was performed.

Fluo 4 AM is a fluorescence marker that attaches to free Ca^{+} and can be used as a marker for the transport of substrates from the cytosol to the DV. Previous studies have described Fluo 4 AM as a marker to establish PFMDR1 transporter activity [34]. The parasites that present N1042 (wild type genotype) accumulate Fluo 4 AM in the DV, showing that there is no inhibition of transport in Fluo-4, in contrast the parasites with 1042**D** (mutated) inhibit the transport of Fluo 4 AM and accumulate in the cytosol of the parasites [31].

The mutation N1042**D** was found in most Ecuadorian *P. falciparum* tested in this study. The Fluo 4 AM assay was then performed in the isolate ESM-2013 from Esmeraldas to confirm that this mutation inhibited the transport of this marker. The ratio of fluorescence intensity (DV/cytosol) of Dd2 (N1042) was higher in the DV than in ESM-2013 (which presented less concentration in the DV but higher concentration in the cytosol). Despite the fact that Ecuadorian *P. falciparum* presented the mutation 1042**D** and inhibited the transport of Fluo 4 AM to the DV, it did not present resistance to MQ, suggesting that the resistance to the drug can be related with the synergy between polymorphisms in *Pfmdr1* and the increase in copy number this drug. Nevertheless, the results showed that PFMDR1 of ESM-2013 is not completely functional, because there was inhibition of Fluo 4 AM transport. These results are not directly related with the current treatment for *P. falciparum* in Ecuador but should be considered in case a treatment change is planned.

Resistance to ART by *P. falciparum* has been reported in Southeast Asian countries particularly in the Grand Mekon area (China, Viet Nam, Cambodia, Thailand and Myanmar). ART resistance has been associated to mutations in *k13* [41, 18, 20, 17]. One hundred percent of Ecuadorian samples presented the wild type CRYGI (positions 476, 493, 539, 543 and 580) genotype for this gene. Similarly, recent studies in Brazilian, Peruvian and Colombian isolates have shown a lack of *k13* mutations and the ACT treatment appears to be effective in South America [41]. The mutation C580**Y** (associated with ART resistance in South East Asia) was found in 5% of *P. falciparum* in Guyana, even though ART showed 100% efficacy [19]. Although no ATM resistance widely reported

mutations occur in the studied samples, the spread of other mutations related to ART resistance cannot be ruled out.

Drug resistance phenotypes can also be characterized by *in vitro* assays. In this study, *in vitro* assays were used to associate drug susceptibility phenotypes to drug resistant genotypes. *In vitro* studies are used to monitor the drug susceptibility of *P. falciparum* and help guide the drug policy in each country [42]. These studies can give a better idea of how the interaction between the parasite and the drug occur and can help establish parasite sensitivity.

Ecuadorian parasites were cultured and exposed to common antimalarial drugs to establish their susceptibility to antimalarials. The ESM-2013 parasite showed an IC_{50} of 93.7 nM related with CQ resistance. The *in vitro* resistance to CQ in Ecuadorian parasites has been reported previously in Ecu1110. It presented an IC_{50} >90.9 nM [26] in comparison with 3D7 that showed an IC_{50} < 10 nM. Other *in vitro* studies have shown resistance to CQ in Colombia, where 90% of parasites analyzed presented IC_{50} > 100nM [21]. ESM-2013 showed *in vitro* sensitivity to QN, MQ, DHA, LUMF and ATM; similarly Colombian samples had *in vitro* sensitivity to DHA, LUMF and ATM [14].

The *in vitro* assays confirmed a CQ resistance phenotype in Ecuadorian *P. falciparum* having the mutation K76T. The resistance to this drug has been present since 1980s in Ecuador and was also reported in Ecu 1110 (1990), suggesting that the resistance to CQ is fixed in Ecuadorian *P. falciparum*.

The current treatment for *P. falciparum* in Ecuador is ATM-LUMF, the results of this study suggest that this treatment continues to be effective in the country, as in the rest of Latin America where there is no reported resistance to ACT treatments [41]. It is important to note that even though Ecuadorian parasites have CQ resistance genotype and phenotype and present mutations in *Pfdhfr* and *Pfmdr1*, they have the same resistance profile as Ecu 1110 an isolate collected in 1990 [25]. These results suggest that the mutations in drug resistance genes have been maintained for several years in spite of a lack of selective pressure. This could be explained by fixation of drug resistance mutations and the ability of the parasites to be present as asymptomatic reservoirs.

Some of the limitations of this study include the difficulties of culture adaptation of field samples to laboratory conditions and the need for a large number of samples from the Amazon region to clarify the current situation of resistance in Ecuador. This study showed the importance of combining molecular and *in vitro* studies as surveillance tools to contribute with malaria elimination in the country by the early determination of resistance to the current treatment used for *P. falciparum* infections.

7 Declarations

Ethics approval

The study protocol was approved by the Ethical Review Committees of Pontificia Universidad Católica del Ecuador.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests

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Author's contributions

FES conceived and designed the study; GV and FES wrote the manuscript; GV and PR performed the experiments; GV, FES and PR analyzed the data. All authors approved the manuscript.

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9 FIGURES

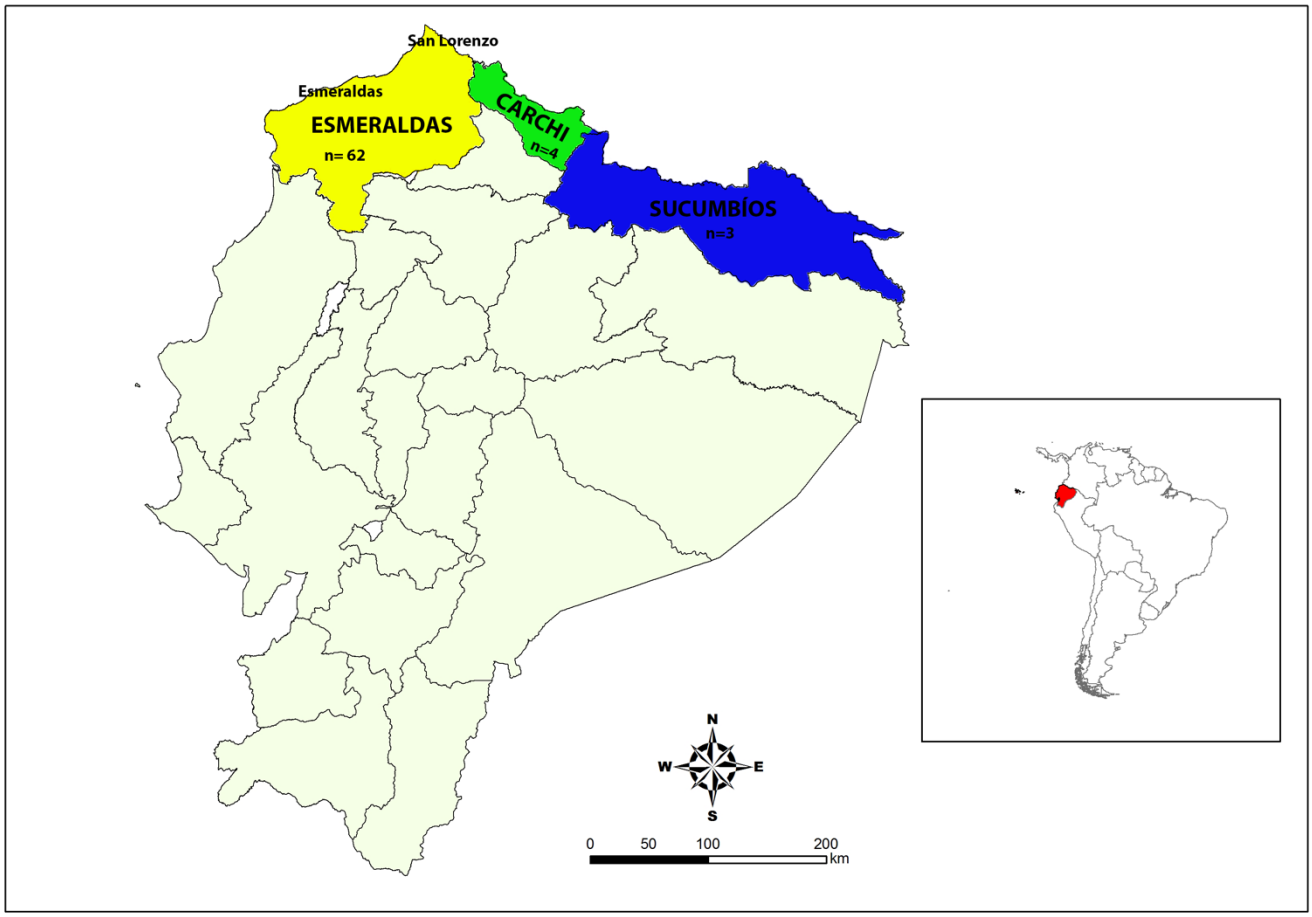

Figure 1. Study Site. All samples were collected in the three provinces of the North of Ecuador (Esmeraldas n=62 samples, Carchi n=4 samples and Sucumbíos n=3 samples).

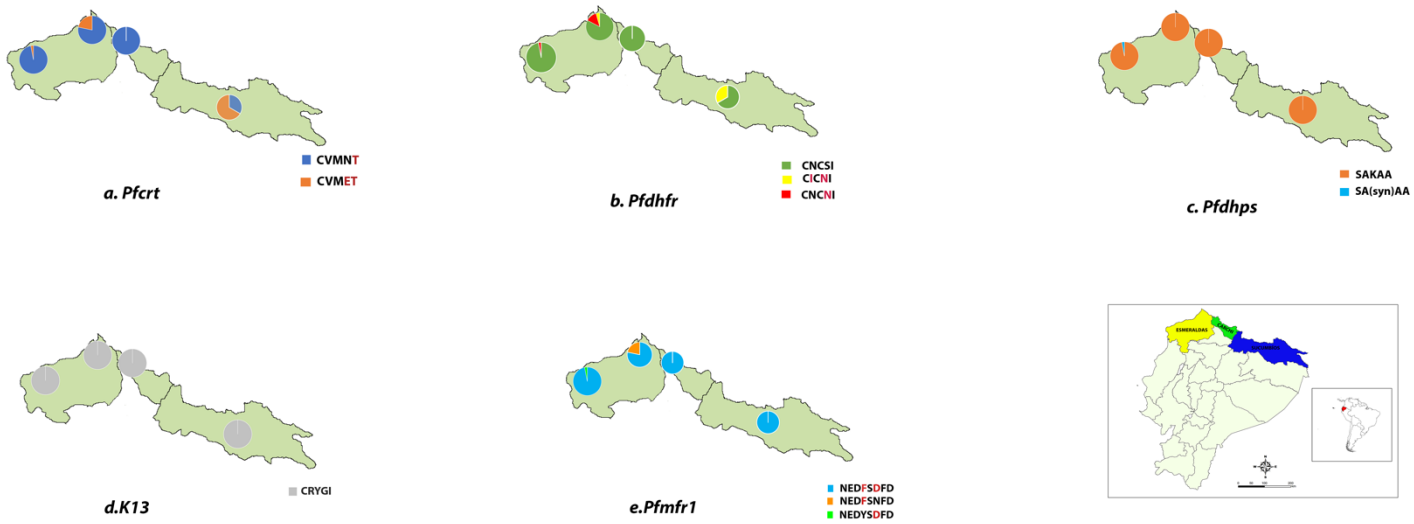

Figure 2. Drug resistance haplotypes distribution and frequency in Ecuadorian *P. falciparum* in the study area. a. Distribution of *Pfcrt* haplotypes b. Distribution of *Pfdhfr* haplotypes c. Distribution of *Pfdhps* haplotypes. d. Distribution of *k13* haplotypes. e. Distribution of *Pfmdr1* haplotypes.

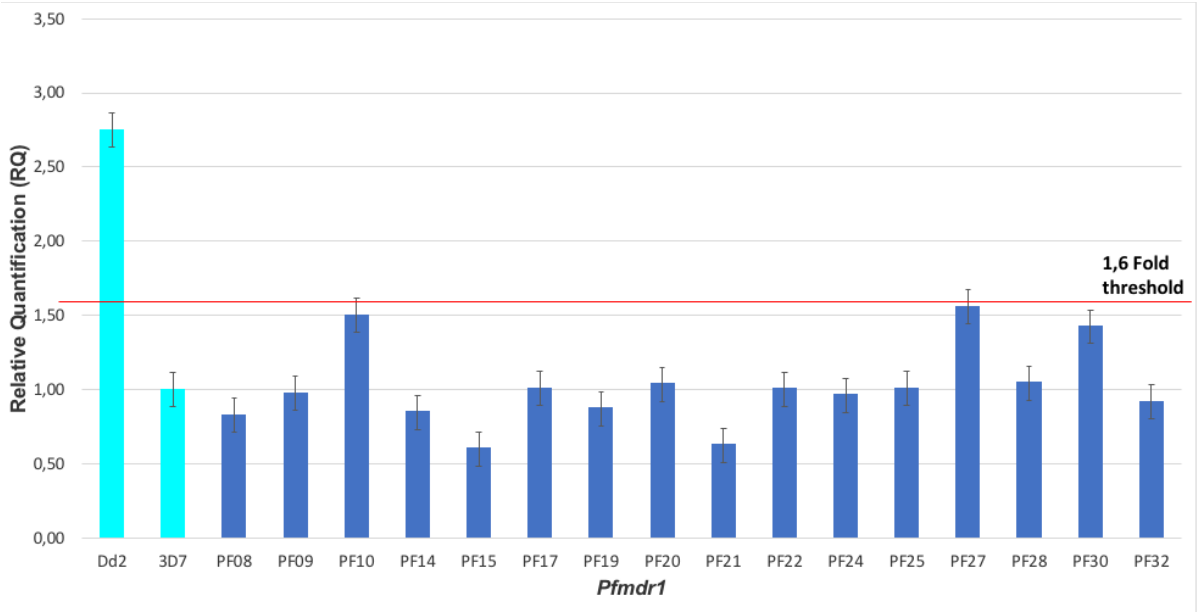

Figure 3. *Pfmdr1* copy number. Dd2 presents two copies and 3D7 one copy. All samples analyzed presented one copy of *Pfmdr1*. Copy number > 1,6 was defined as a duplication of *pfmdr1* [32].

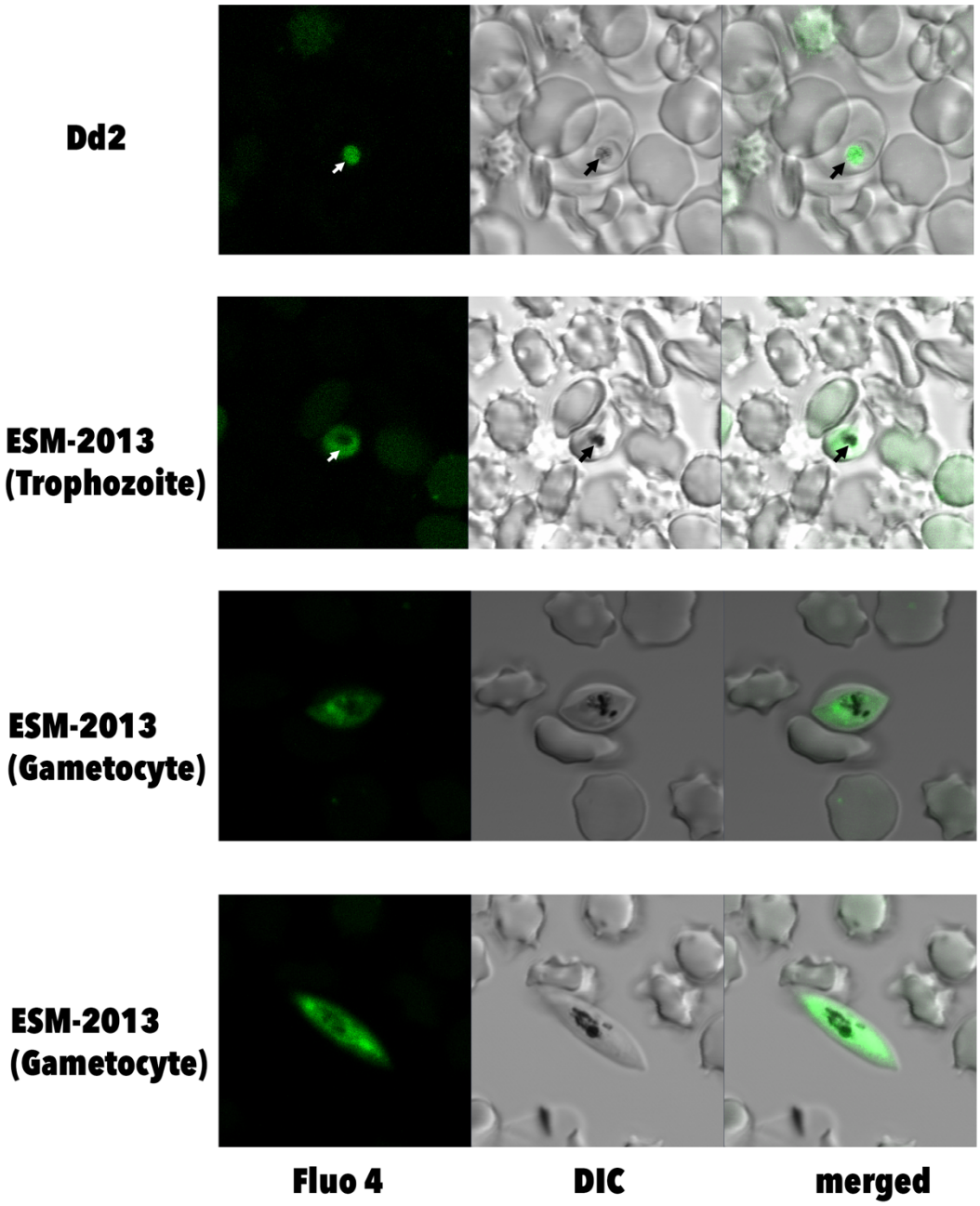

Figure 4. Fluo 4 AM fluorescence in *P. falciparum* parasites. Parasites were incubated with 5 μM Fluo 4. Dd2 accumulates Fluo 4 AM in its digestive vacuole and ESM-2013 inhibits the transport of Fluo 4 AM to its digestive vacuole in trophozoites and gametocytes. Arrow: digestive vacuole.

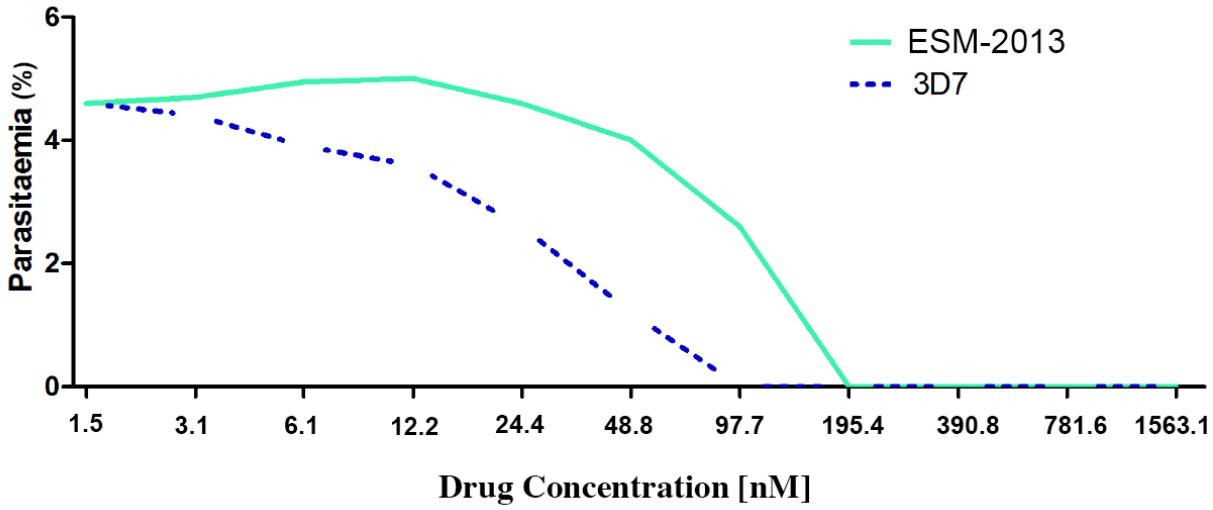

Figure 5. Inhibitory concentration 50 (IC_{50}) curves for ESM-2013 and 3D7 for CQ. The parasitemia and drug concentrations were compared to determine the IC_{50} of each strain for CQ. 3D7 presented an IC_{50} of 8.36 nM and ESM-2013 showed an IC_{50} of 93.71 nM.

10 TABLES

Table 1. Primers and conditions used for amplification of drug resistance markers

Gene	Primer Sequence	Amplification Conditions	Amplicon Size (bp)	Reference
Pfcrt (72-76)	5' AGCAAAAATGACGAGCGTTATAG 3'	94/10 min; (35 cycles of 94/30 sec; 59/30 sec; 72/30 sec); 72/10 min	559	Griffing et al., 2010
	5' ATTGGTAGGTGGAATAGATTCTC 3'			
	5' TTTTTCCCTTGTCGACCTTAAC 3'	94/10 min (30 cycles of 94/30 sec; 56/30 sec; 72/30 sec); 72/10 min	264	
	5'AGGAATAAACAATAAAGAACATAATCATAC 3'			
Pfdhfr (50, 51, 59, 108 and 164)	5' TCCTTTTTATGATGGAACAAG 3'	94/5 min; (35 cycles of 95/30 sec; 50/30 sec; 68/1 min); 68/5 min	633	Alam et al., 2011
	5' AGTATATACATCGCTAACAGA 3'			
	5' TTTATGATGGAACAAGTCTGC 3'	94/5 min (30 cycles of 95/30 sec; 52/30 sec; 68/1 min); 68/5 min	616	
	5' ACTCATTTTCATTTATTTCTGG 3'			
Pfdhps (436, 437, 540, 581 y 613)	5'AACCTAAACGTGCTGTTCAA 3'	95/5 min (35 cycles of 95/30 sec; 50/30 sec; 68/1 min) 68/5 min	711	Vinayak et al., 2010
	5' AATTGTGTGATTTGTCCACAA 3'			
	5' ATGATAAATGAAGGTGCTAG 3'	95/5 min (30 cycles of 95/30 sec; 52/30 sec; 68/1 min); 68/5 min	528	
	5' TCATTTTGTTGTTCATCATGT 3'			
	5' CCATTCTTTTTGAAATAATTGTAAT 3'			
Pfmdr-1 (86, 130, 144 y 184)	5' CCGTTTAAATGTTTACCTGCAC 3'	94/10 min (35 cycles of 94/1 min; 57/1 min; 72/1 min); 72/10 min	1014	Vinayak et al., 2010
	5' TGGGGTATTGATTCGTTGCAC 3'			
	5' CCGTTTAAATGTTTACCTGCAC 3'	94/5 min (30 cycles of 94/30 sec; 55/30 sec, 72/30 sec); 72/10 min	746	
	5' TGGGGTATTGATTCGTTGCAC 3'			
Pfmdr-1 (1034, 1042, 1226 y 1246)	5' GCATTTAGTTCAGATGATGAAATG 3'	94/5 min (35 cycles of 94/30 sec; 56/30 sec; 68/1 min); 68/10 min	1016	Vinayak et al., 2010
	5' CCATATGGTCCAACATTTGTATC 3'			
	5' TATGCATACTGTTATTAATTATGG 3'	94/5 min (25 cycles of 94/30 sec; 55/30 sec; 68/1 min); 68/10 min	910	
	5' TTCGATAAATTCATCTATAGCAG 3'			
K 13 propeller	5' GCAAATAGTATCTCGAAT 3'	94/5 min (35 cycles of 94/30 sec; 46/1 min; 68/2 min); 68/10 min	2126	Talundzic et al., 2015
	5' CTGGGAACTAATAAAGAT 3'			
	5' GATAAACAAGGAAGAATATTCT 3'	94/5 min (30 ciycles of 94/30 sec; 54/30 sec; 68/1 min) 68/10 min	748	
	5' CGGAATCTAATATGTTATGTTCA 3'			

Table 2. Drug resistance markers haplotypes by locations

	Genes				
Location	***Pfcrt***	***Pfdhfr***	***Pfdhps***	***Pfmdr1***	***K13***
Esmeraldas	CVMN**T**	CNCSI	SAKAA	NED**F**S**D**FD	CRYGI
	CVM**ET**	CNC**N**I	SA(Syn)AA	NEDYS**D**FD	
San Lorenzo	CVMN**T**	CNCSI	SAKAA	NED**F**S**D**FD	CRYGI
	CVM**ET**	CNC**N**I		NED**F**SNFD	
		C**I**C**N**I			
Carchi	CVMN**T**	CNCSI	SAKAA	NED**F**S**D**FD	CRYGI
		C**I**C**N**I			
Sucumbíos	CVMN**T**	CNCSI	SAKAA	NED**F**S**D**FD	CRYGI
	CVM**ET**				
F50 (Orellana)	**S**VMN**T**	C**I**C**N**I	SA**E**AA	NED**F**S**D**FD	CRYGI
Ecu1110 (1990)	CVMN**T**	CNCSI	SAKAA	NED**F**S**D**FD	

Table 3. IC_{50} Values of *P. falciparum* ESM-2013 and 3D7

Drug	ESM-2013 IC_{50} [nM]	3D7 IC_{50} [nM]
CQ	93.71 ± 33.07	8.36 ± 5.6
MQ	4.94 ± 0.6	19.3 ± 2.15
QN	7.63 ± 1.08	23 ± 1.8
LUMF	3.59 ± 0.4	7.46 ± 2.6
ATM	1.14 ± 0.7	3.1 ± 0.8
DHA	2.6 ± 1.4	2.4 ± 1.3

11 SUBMISSION GUIDELINES

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