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"Estudios moleculares de *Plasmodium falciparum* y *P. vivax* en la Provincia de Sucumbíos, Ecuador"

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HOJA DE APROBACIÓN DE TESIS

"Estudios moleculares de *Plasmodium falciparum* y *P. vivax* en la Provincia de Sucumbíos, Ecuador"

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Resumen:

El tratamiento de los casos de malaria no complicada causados por *Plasmodium falciparum* está basado en la administración de Sulfadoxina/Pirimetamina (S/P) combinado con Artesunato (AS), mientras que las infecciones por *P. vivax* son tratadas con Cloroquina y Primaquina. Aun cuando no existen datos a nivel molecular en la Provincia de Sucumbíos, estudios *in vivo* sugieren que la resistencia a CQ está ampliamente distribuida en el país. Los Polimorfismos de un nucleótido (SNP's) en el gen Transportador de resistencia a CQ (crt), dihidrofolato reductasa (dhfr) y dihidropteroato sintasa (dhps) confieren resistencia a CQ e hidrofolatos y su monitoreo podrían ayudar a los servicios de salud a desarrollar un adecuado sistema de vigilancia de resistencia. En este estudio hemos identificado las mutaciones más importantes en los gen es *crt, dhfr, dhps* en *P. falciparum* y *dhfr* en *P. vivax* utilizando muestras clínicas provenientes de la Provincia de Sucumbíos. Se analiza la importancia e implicaciones de estos hallazgos en el panorama epidemiológico nacional. Adicionalmente, un estudio de epidemiología molecular a pequeña escala en *P. vivax* fue realizado con el fin de identificar los genotipos de *P. vivax*.

Abstract

Treatment of no complicated malaria cases due to *P. falciparum* is based on the administration of Sulfadoxine/Pyrimethamine (S/P) and Artesunate (AS) while *P. vivax* infections are treated with Chloroquine (CQ) and Primaquine (PQ). There is no data related to molecular markers of resistance to antimalarial drugs in Ecuador, nevertheless *in vivo* studies suggest that CQ resistance is widely spread through the country. Single Nucleotyde Polymorphisms (SNP's) in the Chloroquine Resistance Transporter gene (crt), dihydrofolate reductase (dhfr) and dihydropteroate synthetase (dhps) confer resistance to CQ and antifolates. This study identifies the most important mutations in *crt, dhfr, dhps* in *P. falciparum* and *dhfr* in *P. vivax*. We analyzed the importance and implications of these findings in the epidemiological context of Ecuador. Alternatively, a small scale molecular epidemiology study was carried out in order to identify the genotypes of *P. vivax*.

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Molecular studies of *Plasmodium vivax* and *P. falciparum* in the Province of Sucumbíos, Ecuador.

Morales F, Schousboe M, León R, Espinel M and Alifrangis M.

1. ABSTRACT

BACKGROUND: Standard treatment for non-complicated *Plasmodium falciparum* infections in Ecuador is based in Sulfadoxine/Pyrimethamine (S/P) and Artesunate (AS) while *P. vivax* infections are treated with Chloroquine (CQ) and Primaquine (PQ). No molecular data is available regarding resistance to these drugs in the Province of Sucumbíos although *in vivo* studies suggest resistance to Chloroquine is widely extended in the country. It is well known that Single Nucleotide Polymorphisms (SNP's) in Chloroquine Resistance Transporter gene (crt); dihydrofolate reductase (dhfr) and dihydropteroate synthetase (dhps) confer resistance to Chloroquine and dihidrofolates, and its monitoring could help health services to an accurate surveillance of resistance. In this study we identify the most important mutations in *crt, dhfr, dhps* in *P. falciparum* and *dhfr* in *P. vivax*. Alternatively a small molecular epidemiology study was performed in order to identify the genotypes of *P. vivax* in the Province of Sucumbíos.

SUBJECTS AND METHODS: Blood filter paper samples collected in several locations of the Province of Sucumbíos through the network of laboratories of the Ministry of Health. The samples were confirmed through PCR, and SNP's were identified in *crt, dhfr, dhps* in *P. falciparum* and *dhfr* in *P. vivax*. Amplification of Merozoite Surface Protein 3α (*Pvmsp 3α)* in *P. vivax* was used for the molecular epidemiology study.

RESULTS: The genotype of 8 *P. falciparum* samples analyzed was CVMNT in the *crt* gene. *P. falciparum dhfr* haplotype was CNCNI while dhps gene showed the haplotype SAK. In *P. vivax dhfr analysis* 16 samples were analyzed. We found the haplotypes (codons 57/58/61/117): FR1TN 6,3% (n=1); FR2TN 18,8% (n=3); FR2TS 6,3% (n=1); L2R2TS 68,8% (n=11). The genotype A was identified in 18 *P. vivax* samples; four patterns of bands were visualized through gel electrophoresis.

DISCUSSION: Low number of *P. falciparum* and *P. vivax* cases makes difficult to assess the level of resistance to antimalaric drugs in the Province of Sucumbíos, nevertheless indentification of SNP's in *crt, dhfr* and *dhps* could be useful to update guidelines to treat the disease. In *P. falciparum* single mutant haplotypes associated to resistance to CQ (K76T) were identified, suggesting that the drug is still efficient to treat the disease. In *P. vivax* double and triple mutants were detected, indicating high level of S/P drug pressure. The usefulness of molecular methods to study malaria is also discussed.

Key words: *crt, dhps, dhfr*, Single Nucleotide Polymorphisms (SNP's), mutant haplotypes.

RESUMEN:

INTRODUCCIÓN: El tratamiento oficial en Ecuador para los casos de malaria no-complicados causados por *Plasmodium falciparum* está basado en la administración de Sulfadoxina/Pirimetamina (S/P) combinado con Artesunato (AS), mientras que las infecciones por *P. vivax* son tratadas con Cloroquina y Primaquina. No existen datos moleculares relacionados con los niveles de resistencia a estos medicamentos en la Provincia de Sucumbíos aunque estudios in vivo sugieren que la resistencia a CQ está ampliamente distribuida en el país. Es bien conocido que Polimorfismos de un nucleótido (SNP's) en el gen Transportador de resistencia a CQ (crt), dihidrofolato reductasa (dhfr) y dihidropteroato sintasa (dhps) confieren resistencia a CQ e hidrofolatos y que su monitoreo podrían ayudar a los servicios de salud a desarrollar un adecuado sistema de vigilancia de resistencia. En este estudio hemos identificado las mutaciones más importantes en los gentes *crt, dhfr, dhps* en *P. falciparum* y *dhfr* en *P. vivax*. Adicionalmente, un estudio de epidemiología molecular a pequeña escala en *P. vivax* fue realizado con el fin de identificar los genotipos de P. vivax en la Provincia de Sucumbíos.

PACIENTES Y METODOLOGIA: Muestras de sangre tomadas con papel filtro fueron colectadas en distintas comunidades de la Provincia de Sucumbíos a través de la red de microscopistas del Ministerio de salud Pública. Las muestras fueron confirmadas con PCR y SNP's fueron identificados en los genes *crt, dhfr y dhps* en *P. falciparum* y *dhfr* en *P. vivax.* Para realizar el estudio de epidemiologia molecular se amplifico el gen de la Proteína de la superficie del Merozíto de *P. vivax 3α* (*Pvmsp 3α).*

RESULTADOS: El genotipo de 8 muestras de *P. falciparum* analizadas fue CVMNT en el gen *crt.* El haplotipo de *dhfr* en *P. falciparum* fue CNCNI mientras que el genotipo SAK fue identificado en dhps. 16 muestras de *P. vivax* fueron analizadas, encontrándose los siguiente haplotipos: (codones 57/58/61/117): FR1TN 6,3% (n=1); FR2TN 18,8% (n=3); FR2TS 6,3% (n=1); L2R2TS 68,8% (n=11). El genotipo A fue identificado en 18 muestras de P. vivax. Cuatro patrones de bandas diferentes fueron visualizados mediante electroforesis de agarosa.

DISCUSIÓN: El reducido número de casos de *P. falciparum* y *P. vivax* hace difícil el estudio de niveles de resistencia a antimaláricos en la Provincia de Sucumbíos, sin embargo la identificación de SNP's en los genes crt, dhfr y dhps puede ser útil en la actualización de protocolos de tratamiento de la enfermedad. En *P. falciparum* mutaciones únicas asociadas con resistencia a CQ fueron identificadas (K76T), lo que sugieres que este esquema de tratamietno es aún eficiente para el tratamiento de esta

parasitosis. En P. vivax se detectaron mutantes dobles y triples lo que indica un alto nivel de presión sobre antifolatos (S/P). En este trabajo además se discute la utilidad de las herramietas moleculares en el studio de malaria.

Key words: *crt, dhps, dhfr*, Polimorfismos de un solo nucleotido (SNP's), haplotipos mutantes.

2. INTRODUCTION

Malaria is a major cause of morbidity in Ecuador, where more than 60% of the population is at risk of acquiring the disease (SNEM, 2009). In recent years the number of cases of Malaria in the country has dramatically fallen from more than 120.000 cases in 2001 to only 4.065 patients notified by the Malaria Eradication National Service (SNEM) in 2009. More than 90% of positive cases were due to *Plasmodium vivax* infections with no fatalities reported in the last two years (SNEM, 2009). The Province of Sucumbíos in Ecuador, located in the Northeast part of the country (Graphic 1) is a low transmission area where outbreaks consist of sporadic cases of malaria with an irregular seasonal pattern. Last year, from the 300 cases reported by SNEM, all were reported as *P. vivax* except for 2 that corresponded to non-complicated malaria due to *P. falciparum* (SNEM, 2009). Treatment of malaria at the Amazon region is always challenging, mainly because of the difficult access to rural communities where the majority of the cases occur and because it is difficult to measure the adherence of the treatment in such remote areas. The best treatments

for Malaria in Ecuador include a combination of Chloroquine (CQ) and Primaquine (PQ) in a 7 days period for *P. vivax (*4 extra days with Chloroquine to prevent relapses) Sulfadoxine/Pyrimethamine (S/P) and Artesunate (AS) in a 3 days scheme in case of infections with *P. falciparum.* Other drugs that have been also used in specific cases as second line treatments include Lumefantrine and Quinine.

In this complex scenario, the real impact of parasite resistance to antimalarial drugs in Ecuador is mostly unknown. Most of the studies related to resistance to antimalarial drugs have evaluated the efficacy of standard treatment of *P. falciparum* and *P. vivax* conducting clinical trials following the recommendations of the World Health Organization (WHO), nevertheless low transmission of *P. falciparum* in the Amazon basin, as well as logistic problems related to the follow-up of cases make complicated in this kind of studies. In Ecuador, in vivo studies showed that clinical resistance to CQ was widely extended in the country, and for this reason, a new strategy in the treatment of the disease based on AR + S/P has been applied since 2007 for *P. falciparum* non complicated infections, replacing the old treatment with CQ+PQ (SNEM, 2009)[1]. Data from Colombia[2] suggests that resistance to antimalarial drugs could be a significant public health issue, although very few studies have evaluated the incidence of clinical failure of Malaria cases at a molecular level, consequently, an update in the guidelines and protocols as well as an evaluation of resistance to Malaria treatment is in urgent need not only in Ecuador but also in the Amazon basin of South America. The K76T mutation in the *P. falciparum crt* gene has been extensively reported in Latin America[3] [4] [5] nevertheless no significant association between the specific haplotypes CVMNT and SVMNT and the high rate of clinical

failure of non complicated *P. falciparum* malaria treated with CQ + S/P in Ecuador and Peru[6] has been confirmed.

Across the Andean mountains in the Province of Esmeraldas (coastal region) a study of 14 samples of *P. falciparum* revealed the presence of genotypes NCSI and NCNI in the *dhfr* gene[7] (codons 51/59/108/164) nevertheless it is clear that both regions show different epidemiologic patterns of transmission, the difference lies not only in the intensity of transmission but also in geographical and sociodemographic issues. Interestingly, the Andean Mountains could also act as a natural barrier that prevents the movement of haplotypes found in the coast into the Amazon region[8].

Currently, the use of CQ + PQ is the standard treatment for *P. vivax* malaria in Ecuador, and clinical failure has not been reported to date. Resistance to CQ was notified recently in Peru and Brazil[9] [10]; nevertheless only a few data of mutations in *dhps* and *dhfr* are available. In a study carried out in French Guiana during the 2000-2005 period, Bernadas et al. found a predominance of the quintuple mutant-type 58R/117N/173L–382C/383G in the dhfr and dhps genes in spite of the fact that S/P is not the first line drug, suggesting an stable fixation of this haplotypes in *P. vivax*[11].

Considering that the monitoring of resistance to antimalarial drugs is a key tool in the control of malaria, this study addresses the status of antimalarial drug resistance in the province of Sucumbíos, Ecuador through the identification of Single Nucleotide Polymorphisms (SNP's) in *P. falciparum crt, dhps* and dhfr genes and in *P. vivax* dhrf genes. The haplotypes were identified through a simple enzyme-linked immunosorbent assay (ELISA) using sequence specific oligonucleotide probes

(SSOP's)[12] Furthermore, a small scale molecular epidemiology study was conducted to analyze the distribution of haplotypes of *P. vivax msp 3α* (Merozoite Surface Protein 3α).

3. MATERIALS AND METHODS

3.1 Study Site and sample processing

The samples were collected between 2007 and 2009 in 10 different communities in the province of Sucumbios, Ecuador. All the samples were collected in rural communities near primary or secondary rainforest, except for those taken in Lago Agrio which is the capital of the Province. The ethnic composition of this province is diverse and ranges from "colonos" to Quechua and Cofán indigenous communities.

All the samples were collected by finger prick blood in filter paper Whatman No. 3 by personnel from SNEM. DNA extraction was carried out by the chelex-100 method described elsewhere[13].

3.2 Diagnostic Nested PCR

We used two nested PCR protocols in order to detect P. *falciparum* and *P. vivax* parasites. In the outer PCR reaction that is common to both protocols, we used PLU 5 & 6 primers to amplify the 18S SSUrRNA gene type A, according to the protocol described by Snounou[14]. For the first protocol named common nested PCR, 0.5 µl of the PCR Outer product was used in the nested reaction with the primers N1+N2 in order to identify P*lasmodium* species

according to the protocol used by Rajakaruna et al.[15] The expected size of the PCR products was 352 bp. and 369 bp. for *P. vivax* and *P. falciparum* respectively. For the second protocol named species-specific nested PCR we used nested PCR specific primers for *P. falciparum* (rFAL1&2) and *P. vivax* (rVIV 1&2) yielding a PCR product of 120bp. and 205 bp. for *P. vivax* and *P. falciparum* respectively. In all cases electrophoresis was carried out using 1.5% Agarose gels.

3.3 Molecular markers of resistance .

To amplify *dhfr* and *dhps P. falciparum* genes we used a nested PCR reaction in a protocol described by Pearce[16]. For *P. falciparum crt* gen, we followed the protocol described by Djimde [17]. Both procedures were followed according to the modifications described by Alifrangis et al.[15] Briefly, the *dhfr/dhps* outer PCR mixture consisted of 10 µl of TEMPase Hot Start Master Mix (Ampliqon III, VWR-Bie Berntsen, Denmark), 5 µl of either primer set M1/M7 (dhfr) or N1/N2 (dhps) at a final concentration of 0.25 µM pr. primer, 4 µl of H_2O and 1 µl of extracted DNA. For crt gene the outer PCR mixture consisted of 10 µl of TEMPase Hot Start Master Mix (Ampliqon III, VWR-Bie Berntsen, Denmark), 4 µl of primers P1/P2 at a final concentration of 1 µM pr. primer, 0.8 µl of $MgCl_2$ 1mM ad 4.2 µl of H2O. 1 µl of the outer product PCR was used for the nested PCR. The M9/M3b, R1/R2, and TCRD1/2 primers for the *dhfr, dhps*, and *crt* nested PCRs were biotinylated at the 5' end by the supplier (MWG Biotech,

Riskov, Denmark). Reference *P. falciparum* strains 3D7, D02 and 7g8 were used as positive controls.

Only *dhfr* gene was analyzed for *P vivax* using the nested PCR protocol described previously[18]. The nested reverse primer KH-3R was biotinylated at the 5'-end by the supplier (MWG Biotech, Riskov, Denmark)[19].

3.4. *Specific Oligonucleotide Probes- Enzyme Linked Immune Assay (SSOP-ELISA)*

Single Nucleotide Polymorphisms were analyzed in *P. falciparum* genes *dhps*, *dhfr* and *crt* through SSOP-ELISA following the protocols described by Alifrangis et al [15]. Briefly, ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with Streptavidin at 4°C overnight. The plates were then washed three times in PBS-Tween20 1X by adding and removing 100µl of PBS-Tween20 to each well. After the washing steps, 2 µl of a 1:10 dilution (*dhfr* and *dhps*) or 1:5 (*crt*) of each PCR nested product previously denatured at 95°C were added to the wells and incubated overnight. Subsequently, the plates were incubated at room temperature for one hour and washed three times in washing buffer. 100 µl of a 2µM dilution of 3' digoxigenin-conjugated SSOPs in tetramethyl ammonium chloride (TMAC) (Sigma Aldrich Chemie, Seelze, Germany) were added to each well followed by 1 hour of incubation at 53°C. Upon finishing de incubation period, each plate was washed three times with washing buffer and two times with TMAC solution (10 minutes each one) at different temperatures (Table 1). Each plate was again washed three times

with washing buffer and the secondary antibody (peroxidase-conjugated anti-digoxigenin antibody 1:1000) was added and incubated for 1 hour at room temperature. After a final washing step (three times), an o-phenylenediamine solution of 1.5 mg/mL of 1,2-phenyldiamine dihydrochloride (Dako, Glostrup, Denmark) dissolved in water containing 0.015% H2O2 was added to the wells. The reaction was stopped after thirty minutes with 50 µl of 2.5M H2SO4 and read at 492 nm. Reference strains were used as positive controls. The same procedure was used to analyze *dhfr* gene for *P. vivax* samples, except that we used pre-coated streptavidin plates (Nunc, Roskilde, Denmark) , different temperatures (Table 1) on the two-round washing steps with TMAC and a different concentration of the PCR nested product (1:4). Washing temperatures, probe concentrations and sequences of the probes used for *P. falciparum* and *P. vivax* samples are listed in Table 1. Five *P. vivax dhfr* allele samples, provided by Carol Sibley (Dept. of Genome Sciences, University of Washington) were used as positive controls.

3.5 Restriction enzyme digestion of P falciparum samples

To confirm the haplotype CVMNT in *P. falciparum* samples found through SSOP-ELISA we conducted a simple digestion with ApoI (New England Biolabs, Medinova, Glostrup, Denmark) restriction enzyme. After adding the reagents, the final volume of each reaction was 24 µl (5 µl of PCR product; 0,1 µl of the restriction enzyme; 2,1 µl Buffer NEB3; 0,22 µl Bovine Serum Albumin (BSA) and 16,48 µl water).

3.6 *Genotyping of P. vivax through Restriction Fragment Length Polymorphisms of Merozoite Surface Protein 3α*

In order to genotype *P vivax*, a simple PCR reaction to amplify the *Merozoite Surface Protein 3α* (*Pvmsp 3α)* gene was conducted as described elsewhere[20] PCR products were digested with Hhal (England Biolabs, Medinova, Glostrup, Denmark) in a 22 µl mixture of the following reagents (0,05 µl restriction enzyme; 2,2 µl Buffer NEB2; 0,22 µl BSA and 15,53 µl of water). All digested products were visualized by electrophoresis on 2–2.5% agars gels.

Cloning and DNA *Sequencing analysis*

To confirm the haplotypes found through SSOP-ELISA sequencing was performed in all the *P. falciparum* and *P. vivax* samples; PCR products were sequenced on an 3130/3100-Avant™ Genetic Analyzer Capillary Arrays.

3.7 *Ethical approval*

The blood samples were collected after obtaining an informed consent from each patient. The protocol was approved by the Bioethics Committee at Universidad San Francisco de Quito.

4. RESULTS

4.1. Diagnostic PCR

Thirty six blood filter paper samples (28 *P. vivax* and 8 *P. falciparum*) were studied . The samples were randomly selected from the cases in the Province of Sucumbíos, Ecuador that were previously notified to the national system to control Malaria as positive samples examined through microscopy. From all 36 samples analyzed, 16/28 (57.14%) *P. vivax* and 6/8 (75%) *P. falciparum* were detected by microscopy, common nested PCR and species-specific nested PCR. In *P. vivax*, from the 8 remaining samples that failed to be detected by all methods, all except two (sample 1 and 2) were positive by microscopy but negative through both nested PCRs. Sample 1 and 2 were positive by microscopy and common nested PCR, interestingly however, were negative by the species-specific nested PCR method(Graphic 2). In *P. falciparum* from the two samples that failed to be detected by all methods, one (sample 36) was positive through microscopy but negative through both nested PCRs. Interestingly sample 31 was positive through microscopy and species-specific nested PCR but failed to be detected by the common nested PCR method (Table 2).

Analysis through Kappa Index showed a positive correlation between both nested PCR methodologies used (Kappa Index=0,8085; IC95%= 0,601-1). Due to the significant proportion of samples especially in *P. vivax* detected by microscopy but negative using both nested PCR's, a second round of DNA extraction was done with the same results.

4.2. P. falciparum dhfr / dhps analysis.

Codons 50/51/59/108 and 164 were analyzed in *P. falciparum dhfr* gene. 100% of the samples showed the single mutated haplotype CNCNI. Regarding *P. falciparum dhps* gen codons 436/437 and 540 were studied. The wild-type haplotype SAK was identified in all the samples. The SSOP-ELISA data was confirmed through sequence analysis (Annex 1).

4.3. P. falciparum crt analysis

The genotype of 8 samples was identified through SSOP-ELISA. Five Three different probes were used to analyze the samples: CVMNT, CVMNK, CVIET, SVIET, SVIEK and SVMNT (Table 1). Although no positive controls were available for SVIET, SVIEK and CVMNT, the Optical Density (OD's) using these probes suggested the presence of the haplotype CVMNT. In order to further verify this data PCR RFLP using ApoI digestion was performed (Graphic 3 and 4). None of the samples were digested, which supports our previous identification. To confirm this findings, the samples were subsequently DNA sequenced through standard procedures. DNA analysis corroborates our previous results and confirms the identification of the CVMNT haplotype in our samples. 3D7 strain was used as the reference strain. The alignments of the sequences are presented in Annex 1.

4.4P. vivax dhfr analysis

We used 16 samples for the analysis. Four different haplotypes were found (codons 57/58/61/117): FR1TN 6,3% (n=1); FR2TN 18,8% (n=3); FR2TS 6,3% (n=1); L2R2TS 68,8% (n=11). The results were confirmed through sequence analysis (Annex 2). No mixed haplotypes were identified.

4.5 Genotyping of P. vivax Merozoite Surface Protein 3-α

18 samples were successfully genotyped. According to the genotypes reported by Bruce[24] only one single type of Pv MSP3-α locus was found, showing the 1900 bp. band corresponding to genotype A. RFLP of the isolates showed four different restriction patterns (Table 3). The most important allele found was type 1 (53%, n=9), followed by 4 (24%, n=4); 2 (17%, n=3) and 3 (17%, n=2). No mixed infections were found.

5. DISCUSSION

5.1 Diagnostic PCR

In developing countries, microscopy continues to be the most important diagnostic method for malaria, it is widely used and in most of the cases it is the only option considering that the implementation of other more accurate methods such as PCR is expensive, requires personnel adequately trained in specific molecular techniques and requires sophisticated amplification equipment. Amplification of the 18S ribosomal genes of *P. falciparum* and *P. vivax* is nevertheless considered the gold

standard method to detect blood-stage Malaria parasites[21], in spite it has not been yet implemented in many countries where malaria is endemic, for the above mentioned reasons. PCR amplification and parasite detection through agarose gel electrophoresis of the PCR products is especially useful though to diagnose low parasitemia cases where microscopy has been shown to be less sensitive. But in general terms, it is considered that a positive malaria diagnosis using microscopy is reliable even if it is done in the field knowing that it is carried out by experienced adequately trained technicians from SNEM that most likely diagnose the disease on a daily basis on endemic areas in Ecuador. Accordingly, in our study, in the majority of samples, 22/36 (61.11%) for both parasite species, microscopic detection of Malaria was confirmed by positive common nested and species-specific nested PCRs. Nevertheless,a significant number of samples, 12 and 2 microscopically diagnosed as *P. vivax* and *P. falciparum* respectively, at least one of the two PCR techniques used showed a negative result. From the 14 samples in total that showed different microscopic and molecular results, 11/14 were negative for both PCR reactions suggesting a microscopic misdiagnosis. All laboratory reactions were conducted using the appropriate positive and negative controls and a subsequent second round of DNA extraction and amplification was conducted with the same results, eliminating the possibility of false negatives due to errors in the procedure. These results were unexpected although possible considering that personnel from SNEM often work under poor laboratory conditions often using poor maintained staining reagents and equipment. The three remaining samples (1, 2, and 31) showed more contradicting results: they were microscopically identified as *Plasmodium* parasites, positive for

only one PCR but negative for the other PCR (Table 2) suggesting that amplification of a portion of 18S SSUrRNA gene type A is neither 100% sensitive to detect *P. vivax* and/or *P falciparum* species. Other possible explanations which are unlikely but need to be further investigated is the presence of other human blood parasites that could have been microscopically misidentified as *Plasmodium* parasites by personnel from SNEM or the presence of other *Plasmodium* species in Ecuador that were detected by nested PCR procedures that intend to be species specific. Studies from neighbor countries report sporadic cases of *P. malariae* distributed mainly in the Coast of Colombia[22], although it could be also possible the transmission in the Department of Guaviare in the Amazon basin. It is also probable that at least some positive samples might correspond to imported cases knowing that population migration within a province, from other provinces or even neighbor countries is common especially in rural areas of the Amazon basin. Overall, these results suggest that in endemic areas where malaria epidemiology is not well understood nor characterized, parasite detection and identification should be based on morphology (microscopy) as well as on molecular methods (PCRs) ideally using species specific primers to all human *Plasmodium* parasite species.

5.2 P. falciparum dhfr / dhps analysis.

Interestingly, the only genotype found in this study was the single mutant CNCNI. The mutation S58N is the first to appear in the developing of resistance to Pyrimethamine[23], but alone is not able to confer resistance to this drug[24]. In South America, several studies have been carried out, identifying double and triple mutants

associated to clinical failure[25] [26]. In 2008, Bedoya analyzed 14 *P. falciparum* samples from the Province of Esmeraldas in the Northwestern coastal Ecuador through PCR dot-blot [10] and identified point mutations in codons 51/59/108/164 of the dhfr *P. falciparum* gene, two genotypes were found: NCSI (n=11) and NCNI (n=3) (unpublished data). Although the limited number of samples analyzed, it is clear that the predominant haplotype is different in Esmeraldas compared to Sucumbíos that is located to the east across the Andes. These differences could be possibly explained if we consider that the Andean mountains act as a physical barrier which separates both regions and prevent a homogenous pattern of distribution of genotypes, a phenomenon described previously in Colombia[11]. The resistance to sulphadoxine was evaluated identifying codons 436/437/540 in dhps gene. No mutant haplotypes were found in the samples analyzed.

5.3 P. falciparum crt analysis

The simple mutated haplotype CVMNT (K76T) was identified in the *P. falciparum* samples. This haplotype has been described previously in South America by Huaman et al. in Peru (2004)[27] and Patti in Asia (2007)[28]. In 2004, Plumer et al. found the CQ sensitive CVMNT genotype in samples from French Guiana, although they did not find a clear relation between clinical failure and the K76T mutation[29]. In Ecuador, clinical resistance to CQ in *P. falciparum* infections were reported in the 80's, nevertheless it was not until 2007 when the Ministry of Public Health resolved to change the therapeutic scheme to a combination of AS+S/P. Further analysisare needed to explain the poor efficiency of Chloroquine in the treatment of non-

complicated *P. falciparum* malaria. Lack of adherence to the treatment protocol in rural areas the field, where often treatment has to be done at home instead of in a health center might be a real cause of resistance to CQ. CQ is extensively commercialized in Ecuador even without prescription, which put this drug under a high pressure to develop resistance. Taking into consideration that samples were taken along a period of two years (2007-2009), the wide availability of antimalarial drugs in Ecuador and the limited number of samples analyzed, the data would suggest that the K76T mutation has remained stable in the region as an emerging pattern of resistance to CQ. Unfortunately a rapid decrease in the number of cases of *P. falciparum* recently would need to be further analyzed, probably will not allow an accurate surveillance of the resistance to this drug in the future. In contrast, dihidrofolates has remained as the first-line treatment for *P. falciparum* non complicated infections in combination with AR since 2007.

In conclusion SNP's in *P. falciparum*, dhps, dhfr and crt genes demonstrated a low level of resistance to antimalarial drugs. Further studies are needed to determine if the therapy used in the last years needs to be modified based on a more accurate surveillance of resistance to CQ and SP through molecular methods. Considering that the number of cases in Ecuador has rapidly decreased in the last years, an adequate surveillance of resistance would lead to successful treatment of the sporadic *P. falciparum* cases with the perspective of starting a pre elimination phase of the disease in the country.

5.4 P. vivax dhfr analysis

In contrast to *P. falciparum* information, only a few data on resistance to S/P in *P. vivax* is available. Most of the studies were done in Asia, where the majority of the reported cases are due to *P. vivax* infections. In our study, more than half of the samples (68,8%; n=11; IC95% 41,3- 89,0%) showed the double mutated L2R2TS haplotype, it was unexpected that we did not find the wild type FSTS (codons 57/58/61/117). This finding is interesting considering that other studies in Sri Lanka found a predominance of the wild type FSTS [21]. In South America, the S58R and S117N mutations have been commonly reported[14,30] , although there are no signs of clinical failure to S/P. Considering that Pyrimethamine is not the first line drug to treat *P. vivax* infections, it is somewhat surprising that all the genotypes were mutants (even though no triple or quadruple haplotypes were identified). S/P as well as CQ is available and extensively commercialized in Ecuador , especially in remote areas where the use of residual medication is common among the population. Alternatively cotrimoxazol and other antifolate drugs are also widely used as the first line treatment for non complicated urinary infections. Our results suggest that there is a high external pressure to develop resistance not related to S/P itself, which is most likely responsible for the high number of single and double mutants found in this study.

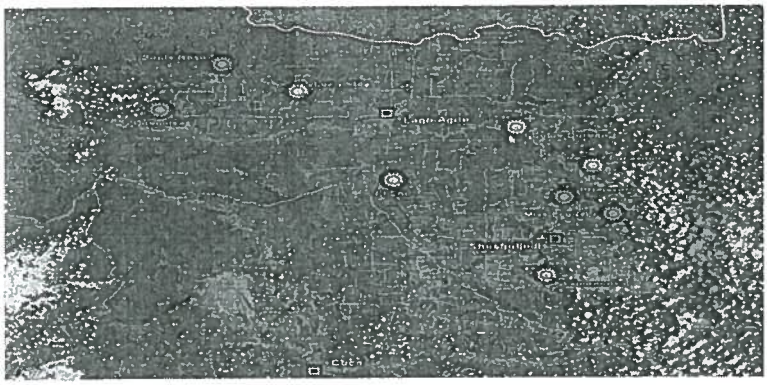
5.5P. vivax Merozoite Surface Protein 3-α analysis

Monitoring *P. vivax* infections through molecular methods is a useful tool to assess the dynamics of malaria transmission, and could give key information to implement more effective antimalarial interventions. In this study we used the MSP3-α gene to investigate the genetic diversity of 16 isolates of *P. vivax*. Four different restriction

patterns were identified, which indicate a high genetic diversity in the study area. In 2008 Cristiano et al. analyzed 56 isolates from Tierralta located in the department of Córdoba, in Northwestern Colombia and found predominance of the genotype A and 9 different restriction patterns through simple digestion with Hhal[31]. Although we analyzed a limited number of samples, we obtained a clear distribution of genotypes according to geographical localization. In 8 samples we found the predominant haplotype 1 , 6 of them coming from the city of Lago Agrio. Haplotype 4 was present only in 4 out of 5 samples from Pusino. The distribution of the genotypes in each community was statistically significant ($p<0,05$).

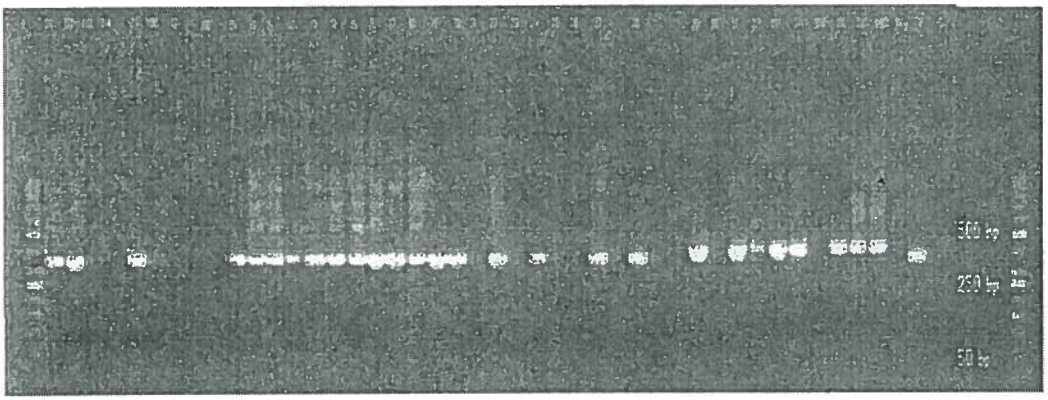
To conclude, malaria molecular studies offer a great opportunity to assess several aspects of the disease, one of the most important is the identification of molecular markers of resistance to antimalarial drugs. Considering that parasite resistance is a serious problem worldwide, close malaria surveillance and monitoring of resistance is necessary, especially in countries like Ecuador where the adherence to medication is low and prescription of antimalarial drugs is not under complete control. More clinical and molecular data is also needed in order to measure the real impact of these issues on malaria control programs.

6. GRAPHICS AND TABLES



Graphic 1. Satellital image of the area of study.

A.



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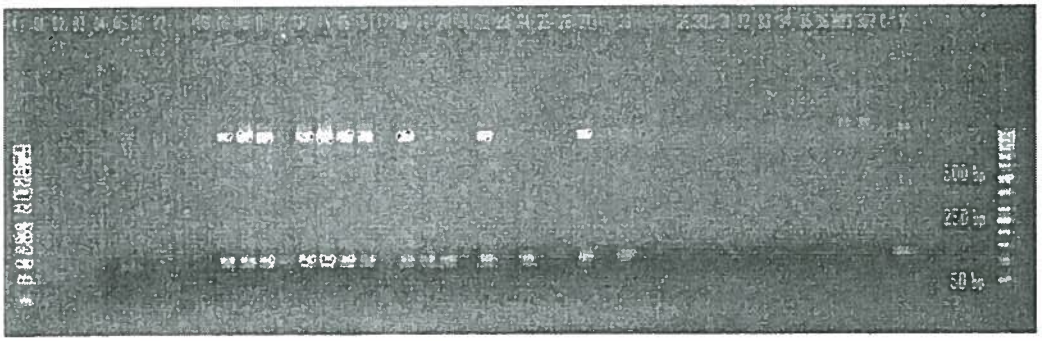
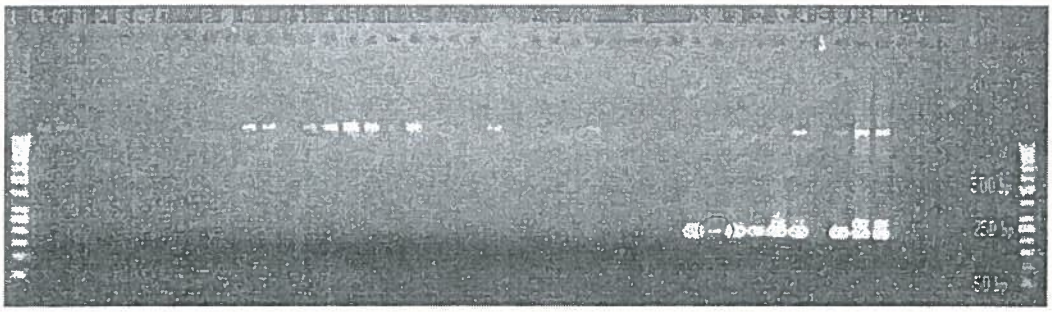
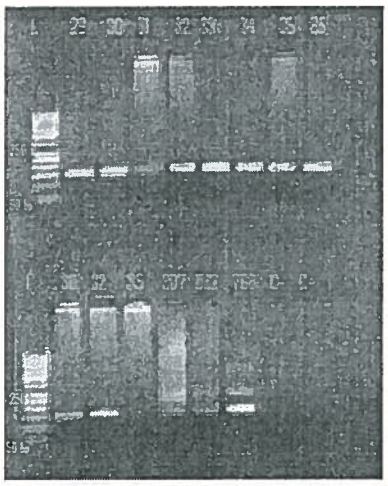
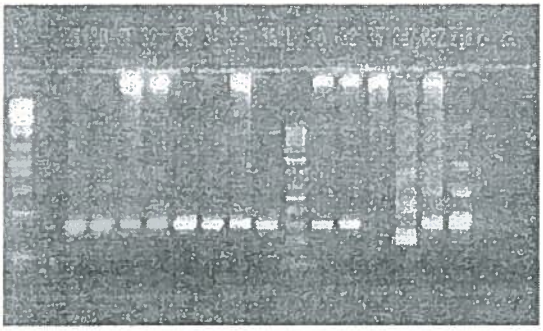




Graphic 2.
A. Common nested diagnostic PCR (Primers N1/N2) showing the 352pb product for *P. vivax* (lanes 1-28) and 369 bp *P. falciparum* (lanes 29-36). The circle shows samples 1 and 2 which were not amplified. Ladder 50 bp.
B. Specific nested diagnostic PCR showing the 120 bp *P. vivax* (lanes 1-28) specific band (primers rVIV 1&2) and *P. falciparum* (lanes 29-36) 205 bp (primers rFAL1&2). The red circle shows sample 27 which was not amplified with the common nested PCR of A. Ladder 50 bp.



Graphic. 3. *P. falciparum* crt Nested PCR showing the 145 bp band. Bottom samples correspond to a second round of DNA extractions. Ladder 50 bp.



Graphic 4. *P. falciparum* RFLP using ApoI. The non-digested 145 bp band is present in all the lanes. Ladder 50 bp.

SSOP's	Sequence	Washing Temperature	Probe concentration	Positive Controls
P. falciparum crt				
72-76 CVMNK	TA **TGT** GTA **ATG AAT AAA**A	60 C°	4 nM	3d7
72-76 CVMNT		60 C°	4 nM	-
72-76 CVIET	TA **TGT** GTA **ATT GAA ACA** A	60 C°	4 nM	Fcr3, Dd2
72-76 SVMNT	TA **AGT** GTA **ATG AAT ACA** A	60 C°	4 nM	7g8
72-76 SVIET		60 C°	4 nM	-
72-76 SVIEK		60 C°	4 nM	-
P. falciparum dhfr				
50/51CN	TGG AAA **TGT AAT** TCC CTA	58 C°	20nM	3d7, Fcr3
50/51CI	TGG AAA **TGT ATT** TCC CTA	58 C°	20nM	Dd2, 7g8
59 C	AAT ATT TTT **GTG** CAG TTA	60 C°	4 nM	3d7, Fcr3
59 R	AAT ATT TTC **GTG** CAG TTA	60 C°	4 nM	Dd2, K1
108S	A AGA ACA AGC **TGG** GAA AG	62 C°	4 nM	3d7
108T	A AGA ACA ACC **TGG** GAA AG	62 C°	4 nM	Fcr3
108N	A AGA ACA AAC **TGG** GAA AG	62 C°	4 nM	Dd2, 7g8, K1, Hb3
164I	GT TTT ATT **ATA** GGA GGT T	60 C°	4 nM	3d7, Fcr3, Dd2, 7g8
164L	GT TTT ATT **TTA** GGA GGT T	60 C°	4 nM	TN518
P. falciparum dhps				

436/437 SA	GAA TCC **TCT GCT** CCT TTT	60 C°	4 Nm	Fcr3
436/437 SG	GAA TCC **TCT GGT** CCT TTT	60 C°	4 nM	7g8, K1
436/437 FG	GAA TCC **TTT GGT** CCT TTT	60 C°	4 nM	Dd2
436/437 AG	GAA TCC **GCT GGT** CCT **T**TT	60 C°	4 nM	-
436/437 AA	GAA TCC **GCT GCT** CCT TTT	60 C°	4 nM	-
P. vivax dhfr				
57/58/61FST	AC **TTC AGC** TCG GTG **ACG** A	60 C°	4 nM	Sri Lanka samples
57/58/61FR1T	AC **TTC AGA** TCG GTG **ACG** A	60 C°	4 nM	UW19-1
57/58/61FR2**T**	AC **TTC AGG** TCG GTG **ACG** A	60 C°	4 nM	UW24C
57/58/61L1R2M	AC**CTC AGG** TCG GTG **ATG** A	62 C°	4 nM	UW16-5
57/58/61L2ST	AC **TTG AGC** TCG GTG **ACG** A	60 C°	4 nM	JM56B
57/58/61L2R1**T**	AC **TTG AGA** TCG GTG**ACG** A	64 C°	4 nM	5758117 T
57/58/61L3R2T	AC **TTA AGG** TCG GTG **ACG** A	62 C°	4 nM	ppLRTS
117S	G AGA AGC **AGC** TGG GAG AG	64 C°	4 nM	Sri Lanka samples
117N	G AGA AGC **AAC** TGG GAG AG	64 C°	4 nM	UW19-1, UW24C
117T	G AGA AGC **ACC** TGG GAG AG	64 C°	4 nM	UW16-5, JM56B, 5758117 T

Table 1. Sequences of the probes used in *P. falciparum* and *P. vivax* SSOP-ELISA

SAMPLE NUMBER	AGE	GENDER	PLASMODIUM	PARASITEMIA	PROVINCE	CANTON	PARROQUIA	COMMUNITY	COMMON NESTED PCR	SPECIE SPECIFIC NESTED PCR
1	17	F	V	+	Sucumbíos	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	+	-
2	31	F	V	+	Sucumbíos	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	+	-
3	42	F	V	< 1/2+	Sucumbíos	Lago agrio	Santa Cecilia	Comuna Guacamayos	-	-
4	16	M	V	++	Sucumbíos	Lago agrio	Jambelí	Jambelí	-	-
5	5	F	V	++	Sucumbíos	Lago agrio	Santa Cecilia	Comuna Guacamayos	+	+
6	32	M	V	< 1/2+	Sucumbíos	Lago agrio	Santa Cecilia	Comuna Guacamayos	-	-
7	4	F	V	++	Sucumbíos	Lago agrio	Santa Cecilia	Comuna Guacamayos	-	-
8	2	M	V	++	Sucumbíos	Lago agrio	Santa Cecilia	Comuna Guacamayos	-	-
9	16	F	V	++	Sucumbíos	Lago agrio	Santa Cecilia	Comuna Guacamayos	+	+
10	12	M	V	++	Sucumbíos	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	+	+
11	4	M	V	++	Sucumbíos	Lago agrio	Pacayacu	Coop. Rio Pacayacu	+	+
12	9	M	V	++	Sucumbíos	Lago agrio	El Eno	Comuna Amazonas	+	+
13	30	F	V	< 1/2+	Sucumbíos	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	+	+
14	12	M	V	++	Sucumbíos	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	+	+
15	12	F	V	++	Sucumbíos	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	+	+
16	16	F	V	++	Sucumbíos	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	+	+
17	5	F	V	+	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	+	+
18	5	F	V	++	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	+	+
19	46	M	V	+	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	+	+
20	9	M	V	+	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	+	+
21	10	M	V	+	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	-	-

22	12	M	V	++	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	+	+
23		M	V	++	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	-	-
24	2	M	V	++	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	+	+
25	6	M	V	++	Sucumbíos	Lago agrio	EL ENO	Comuna Pusino	-	-
26	59	V	V	+	Sucumbíos	Lago Agrio	Cofán Dureno	Comuna Cofán Dureno	-	-
27	61	M	V	+	Sucumbíos	Lago Agrio	Cofán Dureno	Comuna Cofán Dureno	+	+
28	n/a	n/a	V	+	Sucumbíos	Lago Agrio	Lago Agrio	Lago Agrio	-	-
29	n/a	F	F	+	Sucumbíos	Lago agrio	Vía Los Tetetes	Coop. Cristóbal Colón	+	+
30	n/a	M	F	+	Sucumbíos	Lago agrio	Comuna Pachakutik	Comuna Pachakutik	-	+
31	n/a	M	F	+	Sucumbíos	Lago agrio	Comuna Pachakutik	Comuna Pachakutik	+	+
32	n/a	F	F	+	Sucumbíos	Lago agrio	Vía Los Tetetes	Coop. Cristóbal Colón	+	+
33	n/a	F	F	+	Sucumbíos	Lago agrio	Comuna Pachakutik	Comuna Pachakutik	+	+
34	n/a	M	F	+	Sucumbíos	Lago agrio	Comuna Pachakutik	Comuna Pachakutik	+	+
35	n/a	M	F	+	Sucumbíos	Lago agrio	Comuna Pachakutik	Comuna Pachakutik	+	+
36	n/a	M	F	+	Sucumbíos	Lago agrio	Vía Los Tetetes	Coop. Cristóbal Colón	-	-

Table 2. Samples analyzed and results of the diagnostic PCR in *P. falciparum* and *P. vivax.* Parasitemia was measured through microscopy.

SAMPLE NUMBER	CANTON	PARROQUIA	COMMUNITY	TYPE OF MSP 3-α LOCUS	RESTRICTION PATTERN
1	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	A	1
2	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	A	1
4	Lago agrio	Jambelí	Jambelí	A	2
5	Lago agrio	Santa Cecilia	Comuna Guacamayos	A	3
9	Lago agrio	Santa Cecilia	Comuna Guacamayos	A	1
10	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	A	1
11	Lago agrio	Pacayacu	Coop. Rio Pacayacu	A	3
12	Lago agrio	El Eno	Comuna Amazonas	A	2
13	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	A	1
14	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	A	1
15	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	A	2
16	Lago agrio	Nueva Loja (Cabecera Cantonal)	Lago Agrio	A	1
17	Lago agrio	EL ENO	Comuna Pusino	A	4
18	Lago agrio	EL ENO	Comuna Pusino	A	4
19	Lago agrio	EL ENO	Comuna Pusino	A	1
20	Lago agrio	EL ENO	Comuna Pusino	A	4
22	Lago agrio	EL ENO	Comuna Pusino	A	4

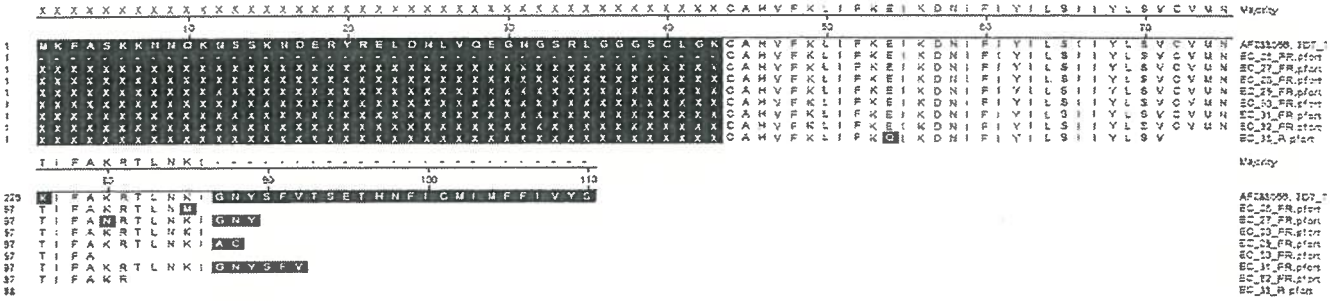
27	Lago Agrio	Cofán Dureno	Comuna Cofán Dureno	A	1

Table 3. Distribution of Restriction Patterns in each community

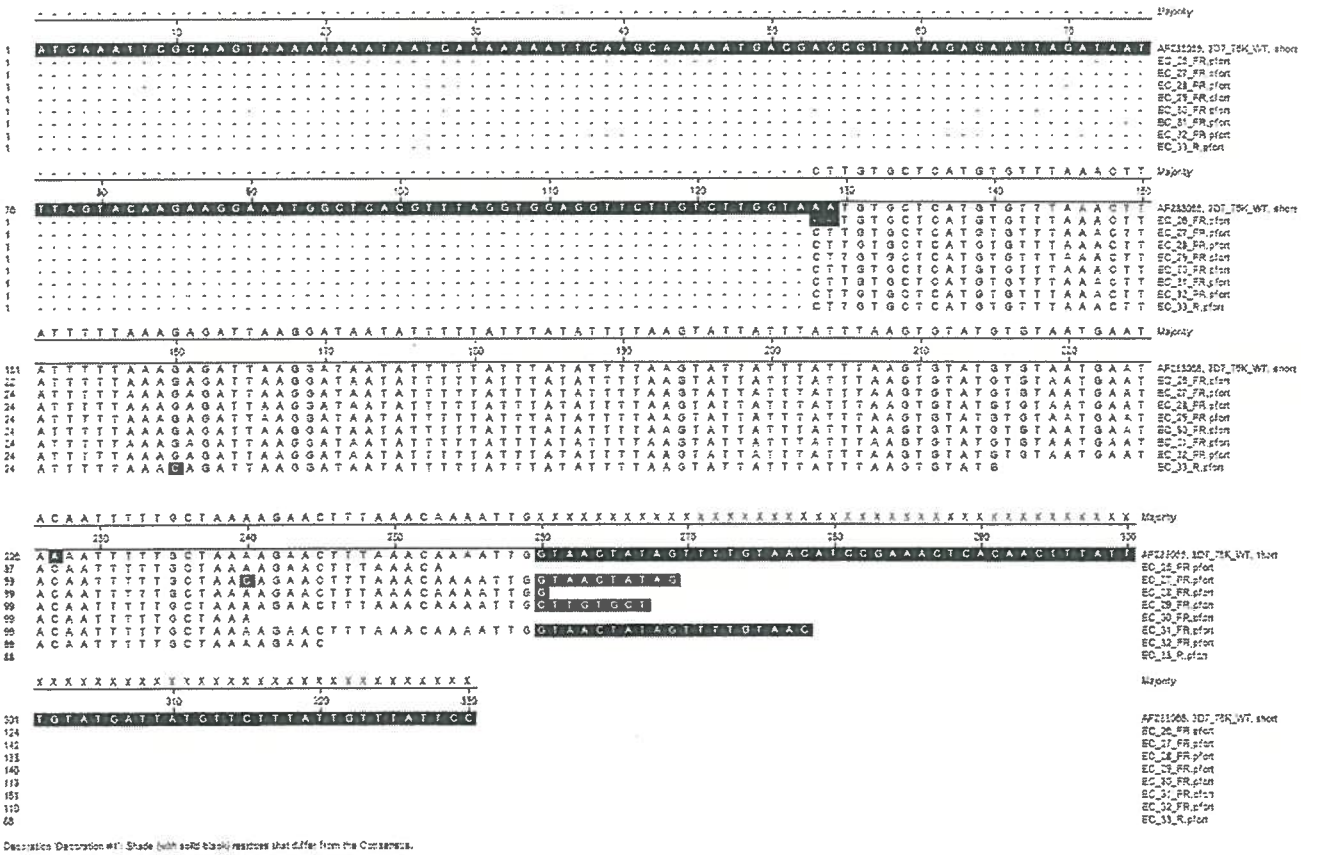
7. ANNEXES

Annex 1. *Plasmodium falciparum* sequence aligments.

Pfcrt, amino acids:

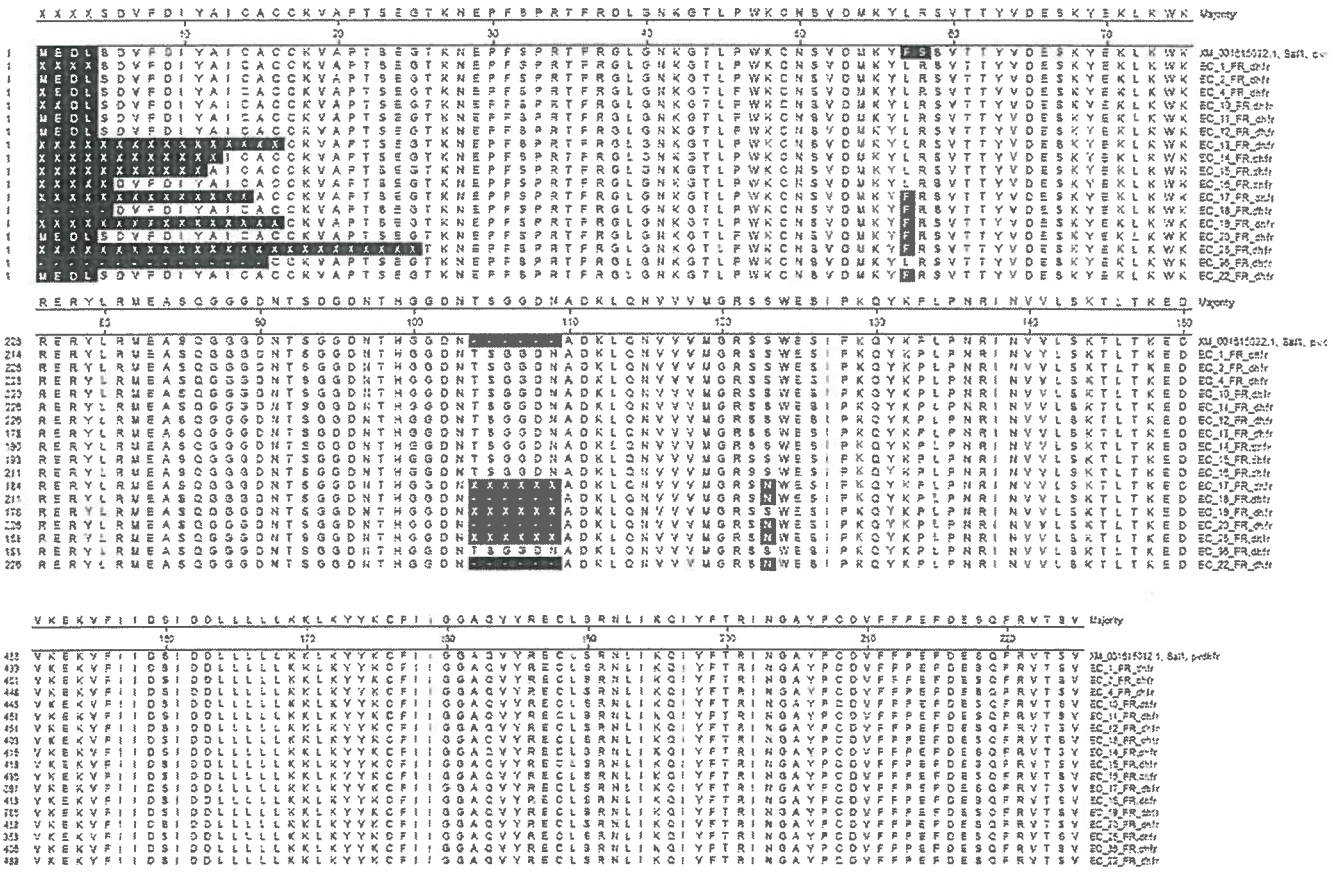


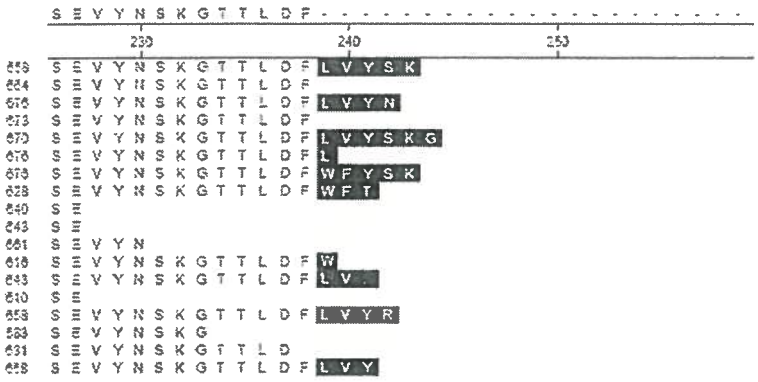
Pfcrt, nucleotides:



Annex 2. Plasmodium vivax sequence aligments

Pvdhfr, aminoacids:





Decoration 'Decoration #1': Shade (with solid black) residues that differ from the Consensus.

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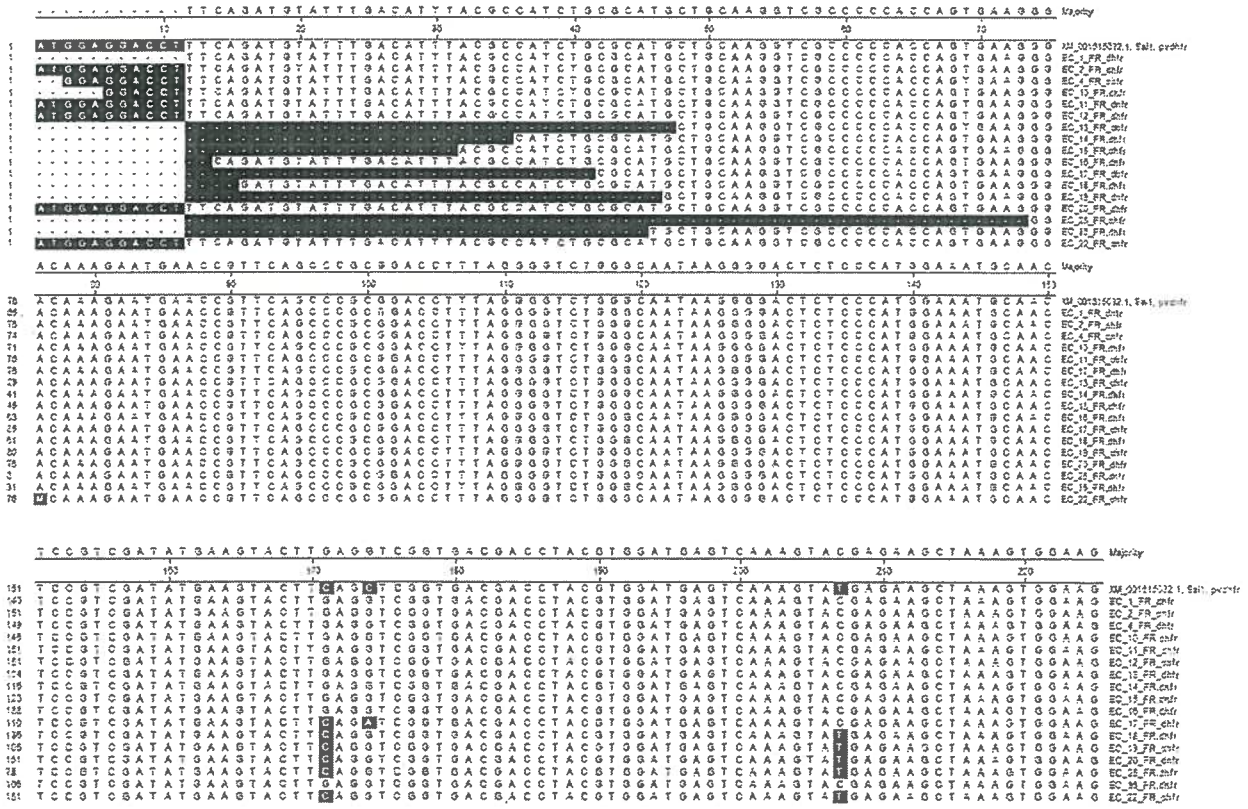

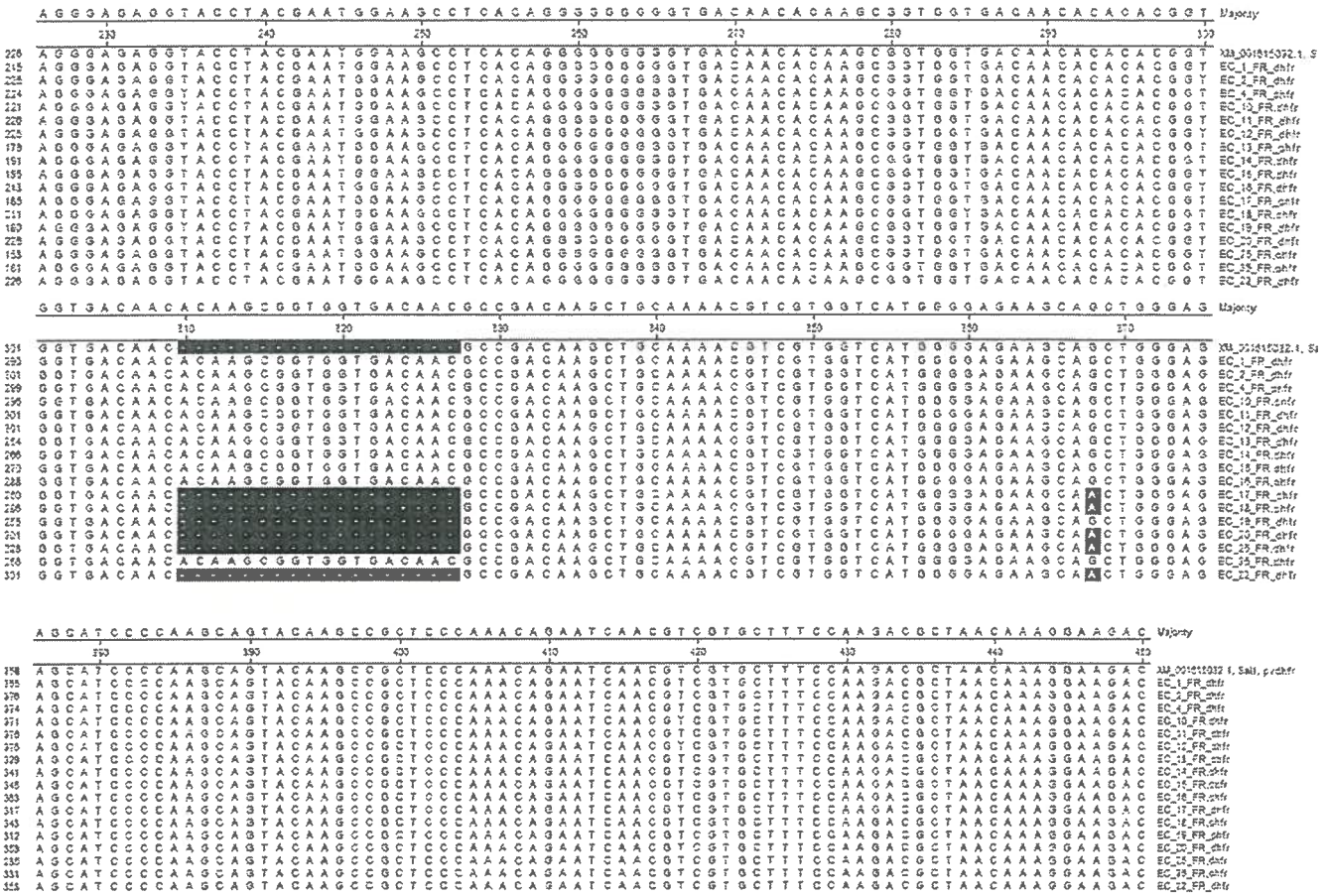

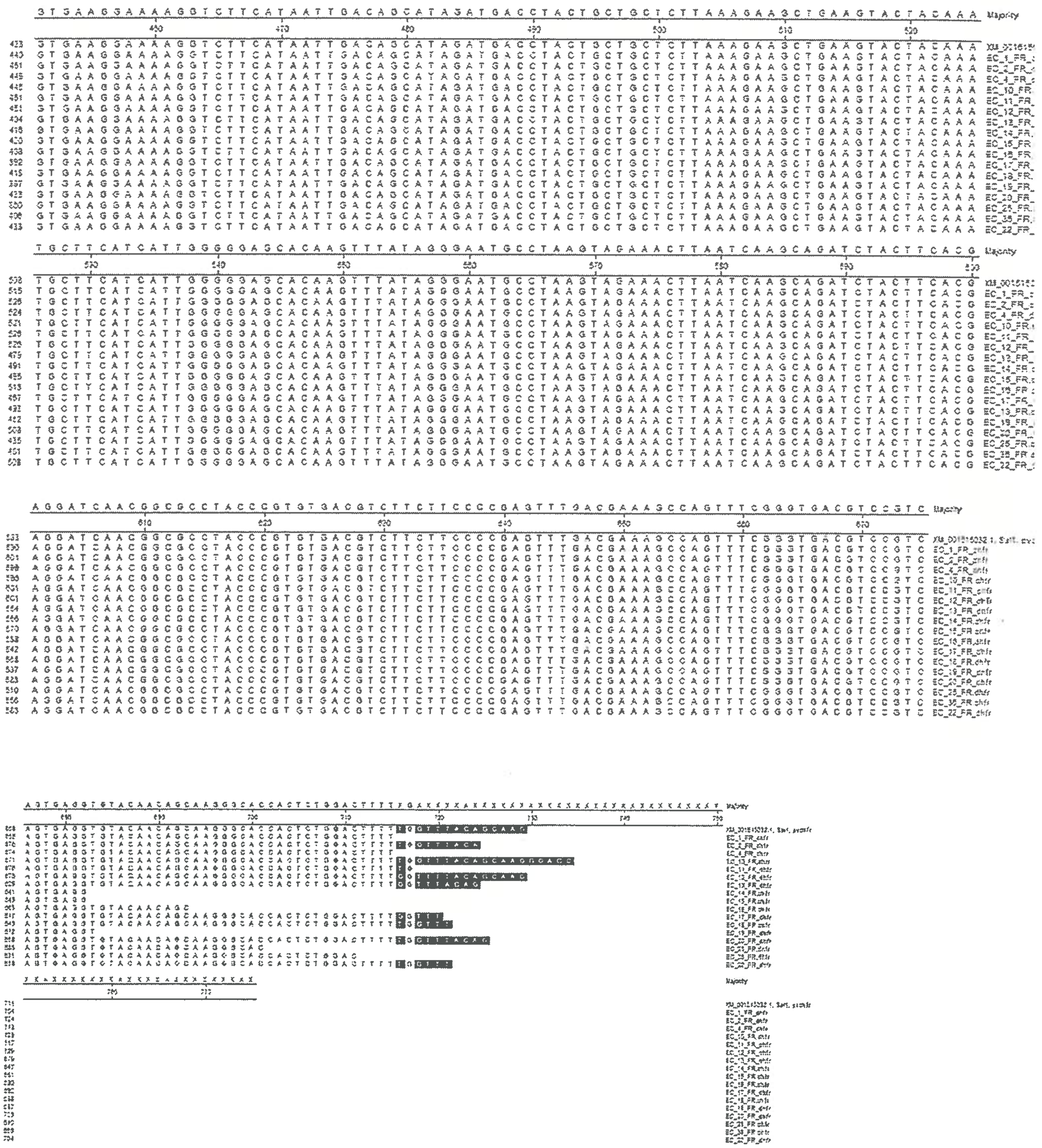

Decoration 'Decoration #1': Shade (with solid black) residues that differ from the Consensus.

8. REFERENCES

[1] Trenton K, Ruebush Ii, Marquino W, Zegarra J, Neyra D, Villaroel R, Avila JC, Diaz C, and Beltra E. Practical Aspects of in vivo antimalarial drug efficacy testing in the Americas. Am. J. Trop. Med. Hyg., 68(4), 2003, pp. 391–397

[2] Galindo J, Cristiano F, Knudson A, Nicholls R, Guerra AP. Point mutations in dihydrofolate reductase and dihydropteroate synthase genes of Plasmodium falciparum from three endemic malaria regions in Colombia. Biomedica 2010, 30:56-64.

[3] Gonzalez IJ, Varela RE, Murillo C, Ferro BE, Salas J, Giraldo LE, *et al.* Polymorphisms in cg2 and pfcrt genes and resistance to chloroquine and other antimalarials *in vitro* in *Plasmodium falciparum* isolates from Colombia. Trans R Soc Trop Med Hyg 2003, 97:318-324.

[4] Eliana Restrepo, E. Carmona-Fonseca J., Maestre A. *Plasmodium falciparum*: high frequency of pfcrt point mutations and emergence of new mutant haplotypes in Colombia. Biomedica. 2008, 28:523-530.

[5] Huaman M, Roncal N, Nakazawa S, Ton That Ai Long, Gerena L, Garcia C, Solari L, Magill A and Kanbara H. Polymorphism of the *Plasmodium falciparum* multidrug resistance and chloroquine resistance transporter genes and in vitro susceptibility to aminoquinolines in isolates from the Peruvian Amazon. Am. J. Trop. Med. Hyg., 2004, 70:461–466.

[6] Durand S, Marquiño W, Cabezas C, Utz G, Fiestas V, Cairo J, Puray M, Lucas C, Salas C, Gutierrez S. Unusual pattern of *Plasmodium falciparum* drug resistance in the northwestern Peruvian Amazon region Am. J. Trop. Med. Hyg. 2007, 76 614–618.

[7]Bedoya, C. Utilizacion de técnicas moleculares y radio-isotópicas para lel fortalecimietno de la vigilancia de la malaria en la región andina. Project RLA/6/055. 2005.

[8] Corredor V, Murillo C, Echeverry DF, Benavides J, Pearce RJ, Roper C, Guerra AP, Osorio L. Origin and dissemination across Colombian Andes mountain range of Plasmodium falciparum Sulfadoxine-Pyrimethamine resistance. Antimicrob Agents Chemother 2010, 54:3121-3125.

[9] Ruebush TK II, Zegarra J, Cairo EM, Andersen M, Green DR, Pillai W, et al. Chloroquine-resistant Plasmodium vivax malaria in Peru. Am J Trop Med Hyg 2003, 69:548–552.

[10] Alecrim MGC Estudo clínico, resistência e polimorfismo parasitário na malária pelo Plasmodium vivax, Manaus – AM. PhD thesis. Brasília: Universidade de Brasília; 2000.

[11] Barnadas C, Musset L, Legrand E, Tichit M, Briolant S, Fusai T, Rogier C, Bouchier C, Picot S, and Ménard D. High Prevalence and fixation of Plasmodium vivax dhfr/dhps mutations related to Sulfadoxine/Pyrimethamine resistance in French Guiana. Am. J. Trop. Med. Hyg 2009 81:19-22.

[12] Alifrangis M, Enosse S, Pearce R, Drakeley C, Roper C, Khalil IF, Nkya WM, Ronn AM, Theander TG, Bygbjerg IC: A simple highthroughput method to detect Plasmodium falciparum single nucleotide polymorphisms in the dihydrofolate reductase dihydropteroate synthase, and P. falciparum Chloroquine resistance transporter genes using polymerase chain reaction and enzyme linked immunosorbent assay-based technology. Am J Trop Med Hyg 2005, 72:155-162.

[13] Wooden J, Kyes S, Sibley C. PCR and Strain Identification in *Plasmodium falciparum*. Parasitol Today 1993, 9: 303–305.

[14] Snounou GS, Viriyakosol XP, Zhu W, Jarra L, Pinheiro , do Rosario, VThaithong VS, Brown KN: High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 1993, 61:315-320.

[15] Rupika H, Alifrangis M, Priyanie P, Konradesen F. Pre-elimination stage of malaria in Sri Lanka: assessing the level of hidden parasites in the population. Malar J. 2010, 20:9:25.

[16] Pearce R, Drakeley C, Chandramohan D, Mosha F, and Roper C. Molecular determination of point mutation haplotypes in the dihydrofolate reductase and dihydropteroate synthase of Plasmodium falciparum in three districts of northern Tanzania. Antimicrobial Agents and Chemotherapy 2003, 47:1347–1354

[17] Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, Coulibaly D, 2001. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 344: 257–263.

[18] de Pecoulas P, Tahar R, Yi P, Thai KH, Basco LK: Genetic variation of the dihydrofolate reductase gene in Plasmodium vivax in Snoul, Northeastern Cambodia. Acta Trop 2004, 92:1-6.

[19] Schousboe M, Rajakaruna R, Salanti A, Hapuarachchi H, Galappaththy G, Bygbjerg C, Amerasinghe P, Konradsen F and Michael Alifrangis. Island-wide diversity in single nucleotide polymorphisms of the Plasmodium vivax dihydrofolate reductase and dihydropteroate synthetase genes in Sri Lanka. Malaria Journal 2007, 6:28

[21] Mixson-Hayden T, Lucchi N, and Udhayakumar V. Evaluation of three PCR-based diagnostic assays for detecting mixed Plasmodium infection BMC Res Notes 2010; 3: 88.

[22] Vergara J, Hurtado S, Alvarez V, Arevalo M, Socrates H. Caracterización de la transmisión de *Plasmodium malariae* en cuatro regiones colombianas endémicas de malaria. Biomédica 2001, 21:53-61

[23] Cortese J, Caraballo A, Contreras C, Plowe C. Origin and dissemination of *Plasmodium falciparum* drug-resistance mutations in South America. J Infect Dis 2002, 186:999-1006.

[24] Plowe C, Cortese J, Djimde A, Nwanyanwu O, Watkins W, Winstanley P, et al. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J Infect Dis. 1997, 176:1590-1596.

[25] Bacon D, Tang D, Salas C, Roncal N, Lucas C, Gerena L, Tapia L, Llanos-Cuentas A, Garcia C, Solari L, Kyle D, Magill A Effects of point mutations in Plasmodium falciparum dihydrofolate reductase and dihydropterate synthase genes on clinical outcomes and in vitro susceptibility to sulfadoxine and Pyrimethamine. PLoS One 2009 26;4 6762.

[26] Contreras C, Cortese J, Caraballo A, Plowe C. Genetics of drug-resistant Plasmodium falciparum malaria in the Venezuelan state of Bolivar. Am J Trop Med Hyg. 2002 67:400-405.

[27] . Huaman M, Roncal N, Nakazawa S, Long T, Gerena L, Garcia C, Solari L, Magill AJ, Kanbara H. Polymorphism of the Plasmodium falciparum multidrug resistance and chloroquine resistance transporter genes and in vitro susceptibility to aminoquinolines in isolates from the Peruvian Amazon. Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan. Am J Trop Med Hyg 2004 70:461-466.

[28] Pati S, Mishra S, Mohanty S, Mohapatra D, Sahu PK, Priyadarshi N, Kumar S, Sharma SK, Tyagi P, Chitnis C, Das B. Pfcrt haplotypes and in vivo chloroquine response in Sundergarh district, Orissa, India. Trans R Soc Trop Med Hyg 2007, 101650-654.

[29] Plummer B, Pinto Pereira L, Carrington C. Pfcrt and pfmdr1 alleles associated with chloroquine resistance in *Plasmodium falciparum* from Guyana, South America. Mem Inst Oswaldo Cruz. 2004, 99:389-392.

[30] Hawkins V, Auliff A, Kumr Prajapati S, Rungsihirunrat K, Hapuarachchi H, Maestre A, O'Neil M, Cheng Q, Joshi H, Na- Bangchang K and Sibley C. Multiple origins of resistance-conferring mutations in Plasmodium vivax dihydrofolate reductase. Malaria Journal 2008, 7:72.

[31] Cristiano F, Pérez M, Nicholls R, Guerra A. Polymorphism in the Plasmodium vivax msp 3α gene in field samples from Tierralta, Colombia. Memórias do Instituto Oswaldo Cruz Mem. Inst. Oswaldo Cruz 2008, 103: