# Molecular variation of *Plasmodium vivax* dehydrofolate reductase in Mexico and Nicaragua contrasts with that occurring in South America

Lilia González-Cerón, MSc, D en C,[1] Mario H Rodríguez, MSc, PhD,[2] Alberto Montoya, MV, M en C,[3] Frida Santillán-Valenzuela, QFB,[1] Josselin C Corzo-Gómez, QFB, M en C.[1,4]

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## Abstract

**Objective.** To research mutations associated to pyrimethamine resistance in dihydrofolate reductase (*pvdhfr*) of *Plasmodium vivax* from Mexico and Nicaragua and compare it to that reported in the rest of America. **Materials and methods.** Genomic DNA was obtained from *P. vivax*-infected blood samples. A *pvdhfr* gene fragment was amplified and sequenced. The identified gene variations were compared to those observed in other affected sites of America. **Results.** No mutations in *pvdhfr* were detected in *P. vivax* from Mexico and Nicaragua. One synonymous change and variation in the repeat domain was detected in Nicaraguan parasites. In South America, a high frequency of variant residues 58R and 117N associated to pyrimethamine resistance was reported. **Conclusions.** The lack of polymorphisms associated with pyrimethamine resistance suggests that drug-resistant *P. vivax* has not penetrated Mesoamerica, nor have local parasites been under selective pressure. These data contribute to establish the basis for the epidemiological surveillance of drug resistance.

Keywords: *Plasmodium vivax; pvdhfr*; polymorphism; pyrimethamine; Mexico; Nicaragua

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## Resumen

**Objetivo.** Determinar mutaciones en la dihydrofolato reductasa de *P. vivax* (*Pvdhfr*) en parásitos de México y Nicaragua, y comparar con lo reportado en América. **Material y métodos**. Del ADN de sangres infectadas con *P. vivax* de pacientes, el gen *pvdhfr* se amplificó y secuenció, y se contrastócon lo observado en América. **Resultados**. No se detectaron mutaciones asociadas con la resistencia debida a pirimetamina. Los parásitos de Nicaragua tuvieron una mutación sinónima y variación en la región repetida. Se reportaron frecuentes mutaciones asociadas con la resistencia a la pirimetamina en Sudamérica. **Conclusiones**. La ausencia de polimorfismos en *Pvdhfr* sugiere que no se han seleccionado ni introducido parásitos resistentes en la zona de estudio, lo que resulta muy útil para la vigilancia epidemiológica.

Palabras clave: *Plasmodium vivax*; *pvdhfr*; variación molecular; pirimetamina; México; Nicaragua

(1) Centro Regional de Investigación en Salud Pública, Instituto Nacional de Salud Pública. Tapachula, Chiapas, México.
(2) Centro de Investigación en Enfermedades Infecciosas, Instituto Nacional de Salud Pública. Cuernavaca, Morelos, México.
(3) Departamento de Parasitología, Centro Nacional de Diagnóstico y Referencia, Ministerio de Salud. Managua, Nicaragua.
(4) Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional. Ciudad de México, México.

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Corresponding author: Dra. Lilia González-Cerón. 4 avenida Norte y 19 Calle Poniente. 30740, Tapachula, Chiapas, México.
email: lgonzal@insp.mx

The overall progressive trend in the control of malaria worldwide halted in 2016. The estimated global incidence rate decreased from 76 to 63 cases (18%) per 1 000 people at risk; yet, although the estimated 216 million malaria cases that occurred in 2016 represent a decline compared to 237 million cases in 2010, this figure is higher than that (211 million) estimated for 2015.[1]

In Latin America, where *Plasmodium vivax* causes 60-70% of malaria cases, the incidence rate declined 67% between 2000 and 2014, i.e. from 1 181 000 cases in 2010 to 389 390 cases in 2014;[1,2] however, substantial increases occurred between 2015 and 2016. Venezuela contributed 34.4% of the cases in the region (240 000 cases, which amount to an increase of 76% in the country) and, together with Peru and Brazil, accumulated 65% of the cases in the region. In Nicaragua, fewer than 1 000 cases were reported between 2008 to 2011, but over 10 times more cases (10 846) were reported in 2017.[3] On the other hand, in Argentina, Ecuador, El Salvador, Mexico and Paraguay, substantial declines in the incidence of malaria have encouraged initiatives to accelerate its elimination. In Mexico, only 715 *P. vivax* cases were reported in 2017.[4]

A decrease in funding, a growing insecticide resistance of mosquito vectors, and an increasing resistance of malaria parasites to drugs are the main difficulties that hinder progress in the control and elimination of malaria.[1] In South America, chloroquine (CQ) and pyrimethamine were used in massive drug administration campaigns during the global malaria eradication initiative,[5,6] and resistance of *Plasmodium falciparum* strains to both drugs was detected since the early 1960s.[7,8] In the 1970s, pyrimethamine reformulated with sulfadoxine (SP) was re-introduced to fight CQ-resistant *P. falciparum* strains throughout the malaria-infested areas of the planet.[9-11] In South America, *P. falciparum* resistant to SP was detected shortly after its introduction,[12,13] and by the 1990s SP was replaced by other antimalarials, such as mefloquine, and tetracycline.[14] Currently, the standard treatment for *P. falciparum* is artemisinin-based combination therapy (ACT). ACTs recommended by the WHO include artemether-lumefantrine (Coartem), artesunate-mefloquine, artesunate-amodiaquine and artesunate-sulfadoxine/pyrimethamine.[14,15]

SP inhibits malaria parasites growth through sequential inhibition of folate biosynthesis. Pyrimethamine binding to dihydrofolate reductase (DHFR) and sulphadoxine binding to dihydropteroate synthase (DHPS) inhibit these metabolic enzymes.[15,16] In *P. falciparum*, mutations at DHFR residues C50R, N51I, C59R, S108N/T and I164L are associated to pyrimethamine resistance.[11,17,18] SP alone is not recommended for treating *P. vivax* infections, because this parasite rapidly develops resistance.[19,20] However, as *P. vivax* has coexisted with *P. falciparum* in most affected areas outside Africa,[21,22] in patients with mixed infections, SP treatment to *P. falciparum* can unintentionally expose *P. vivax* to SP selective pressure.[10,23] This explains why *P. vivax* expressing *dhfr* mutations associated to pyrimethamine resistance have been detected in most malaria areas under present or past SP treatment.[24]

*Pvdhfr* residues (F57, S58, T61, S117 and I173) correspond topologically to those that confer pyrimethamine resistance in *P. falciparum*.[18,25] *In vitro* studies in which a yeast system expressing different *Pvdhfr* haplotypes was utilized showed that residues 58R+117N might reduce sensitivity to pyrimethamine, but the accumulation of other mutations (e.g. 57L, 61M, 117T and 173F) may increase the risk of resistance to this drug.[26,27] In South America, *Pvdhfr* mutations at codons S58R and I117N were reported.[28-35] Resistance has also been documented in areas not exposed to SP, probably due to the introduction of resistant parasites through human migrations.[36,37] Mexico and Central America have not officially used SP to treat malaria,[15,38,39] and the molecular susceptibility status of local *P. vivax* to SP is unknown.

Mexico has brought malaria to pre-elimination status, and Central America struggles to advance control toward its elimination; both need evaluation of the pertinence of new treatment schemes. Molecular approaches would be helpful to establish surveillance strategies and map the dispersion of drug resistance strains. In the present study, *Pvdhfr* polymorphism was researched in parasites from southern Mexico and Nicaragua, and the results were compared to published studies in other affected sites of Latin America.

## Materials and methods

*Plasmodium vivax*-infected blood samples were obtained from symptomatic patients seeking malaria diagnosis in Mexico and Nicaragua.[40-42] The patients' personal information was encrypted and only their municipality of origin was used for data interpretation. The Ethics Committee of the National Institute of Public Health of Mexico and the Ethics Committee of the National Center for Diagnosis and Reference (CNDR) of the Ministry of Health of Nicaragua approved the study.

*P. vivax samples.* From 2008 to 2010, 85 *P. vivax*-infected blood samples were obtained at the Regional Research Center for Public Health (CRISP-INSP), from patients living in southern Chiapas (SCH), the Mexican southernmost region bordering with Guatemala.[40-42] In

Nicaragua, the sentinel laboratory network established by the Health Ministry collected 100 blood samples. Seventy samples were from patients living in the North Caribbean Coast Autonomous Region (RACCN) in 2011-2012. Another 18 and 12 samples were obtained during an outbreak in 2012 and in 2006-2007[40] from the North Pacific Coast (NPC),[42] respectively. Blood samples collected in filter paper (Whatman # 2) were dried and kept in the dark at 4°C until used.

*Molecular analysis.* Genomic DNA was extracted from blood samples using the QIAmp DNA blood Minikit protocol and reagents, according to the manufacturer's instructions (Qiagen, Valencia, CA USA). The oligonucleotides forward 5'-GACATTTACGCCATCTGCG -3' (nt 25-44) and reverse 5'-CGTTGATCCTCGTGAAG-TAGATC-3' (nt 570-592) were used to amplify a *Pvdhfr* gene fragment comprising codons 57, 58, 61, 117 and 173. The PCR reactions were prepared as follows: 10$\mu$L of 5× PCR buffer, 4$\mu$L of $MgCl_2$ (25mM), 2.5$\mu$L of dNTPs (1.25mM), 1.9 $\mu$L of each primer (10$\mu$M), 0.5$\mu$L of Go Taq DNA polymerase 5u/$\mu$L (Promega, Madison, WI, USA) and 2-4 $\mu$L of extracted DNA for a final volume of 50 $\mu$L. The PCR reaction conditions were as follows: 3 min at 94 °C followed by 35 cycles: 40s at 94 °C, 40s at 54 °C, and 1 min at 72 °C; afterwards, there was a final extension of 72 °C for 5 min in a MyCycler thermal cycler (BioRad, Hercules, CA, USA). The PCR products of the correct molecular size were purified using a MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA). The purified products were Sanger-sequenced, using the forward and reverse primers, at the High Throughput Genomics Unit (University of Washington, Seattle, WA, USA; http://www.htseq.org/). The consensus sequences were submitted to the NCBI GenBank [KX180639-KX180789]. *Pvdhfr* mutations and haplotypes were identified, and their frequency calculated for each study site. The Salvador I strain (Sal I) orthologous sequence XM_001615032 was used as reference.[43] This strain is sensitive to pyrimethamine *in vitro*.[12]

*Comparison of PvDHFR mutations among sites in America.* *Pvdhfr* mutations and haplotypes detected in this study were compared to sequences reported in the literature on affected areas of Central and South America (NCBI, Google Scholar). Information referring to sample collection date, municipality/region and country, number of isolates, DNA sequences, synonymous and non-synonymous mutations (if available), prevalence of nucleotide/residue variants, haplotypes and their frequency were analyzed.

# Results

A *dhfr* gene fragment comprising codons 16 - 198 was obtained from 151 parasites; 73 samples, from southern Mexico; 54, from RACCN, and 24 from NPC in Nicaragua. All Mexican sequence isolates and 55 (70.5%) from Nicaragua (51 from RACCN and four from NPC) were identical to that of Sal I (sensitive strain) (tables I and II). Only three parasites (5.5%) from RACCN had a synonymous mutation at codon Y69 (tat→tac). These parasites were obtained from blood samples of patients living in the Rosita, Bonanza and Waspam municipalities, between December 2011 and March 2012. Twenty samples from NPC (83.3%) had mutation at codon Y69 and 18 extra nucleotides coding for the TSGGDN domain, between codons 103 and 104, in 60% of the parasites collected in 2006 (n = 6) and 100% of the parasites collected in 2012 (n = 14) (table I).

Information concerning *Pvdhfr* gene polymorphism was obtained from French Guiana,[35] Colombia,[28-32] Peru,[28] Brazil,[28,33,34] Honduras[44] and Mexico[28] (tables I-III). In those previous studies, *P. vivax*-infected blood samples were collected between 2001 and 2013. A substitution at residue P322L in one of 19 isolates was reported in Southern Mexico. The synonymous mutation Y67 was reported in Honduras (22%) and, with a high frequency, in Colombia and French Guiana (82.9 and 85.6%, respectively). Haplotypes expressing 58R and 117N were reported in Colombia, French Guiana, Brazil and Peru (table I and II). Although variation at residues 57L and 173L was not very frequent in South America, it was reported at 173L in Brazil, Peru, French Guiana and Amazon Colombia. The amino acid change 57L was reported only in one isolate in Amazon Colombia and two isolates in Honduras (table I and II). Residues 58N/K were reported in Northern Peru and Amazon Colombia, respectively. Other mutations were private to certain geographic sites (table I). A tandem repeat sequence observed in the Sal I strain was also observed in samples collected in this study in Mexico and, less frequently, in the NPC of Nicaragua; it was also reported, at different high frequencies, in Honduras, French Guyana and Colombia (table III). A variation of these tandem repeat sequence detected in this study in NPC of Nicaragua was previously observed in Honduras. Other tandem sequence variations were reported for Colombian parasites (table III).

# Discussion

Mutations associated to pyrimethamine (PM) resistance were not detected in *P. vivax* dehydrofolate reductase

from affected patients living in southern Mexico and Nicaragua. Accordingly, no mutations in *dhfr* were observed in *P. falciparum* in Honduras[44] or Nicaragua.[45] Interestingly, the variant repeat sequence found in NPC Nicaragua and reported in parasites from Honduras,[44] might not be present in the RACCN of Nicaragua. The synonymous mutation at codon Y69 observed in parasites of Nicaragua, and in those of Honduras,[44] Colombia[32] and French Guiana,[35] seem to be widely distributed: it was reported in parasites of South Korea[46] and Thailand.[47]

On the other hand, those observations contrast with records from previous studies carried out in South America. Mutations at *pvdhfr* conferring pyrimethamine resistance, such as those found in residues 58R/N and 117N and haplotypes having both substitutions, have been very frequently detected in Colombia,[28-32] Peru,[28] French Guiana[35] and Brazil.[28,33,34] However, the distribution of parasites carrying other important mutations varies among regions of the same country. For instance, mutation 173L was reported at various frequencies in different locations of South America, the highest frequency (17%) occurring in *P. vivax* in the Amazon region of Colombia,[32] but was absent in samples collected in different regions of the same country.[28-31] Other mutations were more restricted to certain sites, although few DNA sequences were available. In Panama, *P. falciparum* expressing residues 51I-108N at DHFR was detected in malarious regions next to the Colombia border,[48] indicating that *P. vivax* might had been also exposed to pyrimethamine in these regions.

Mutations 58R and 117N were reported to be common in malarious areas of Asia and Indonesia;[27,30,49-55] the 57L substitution, rare in South America, was more frequent in South and Southeast Asia, and Oceania,[29,30] and the double mutation 57L /58R reported in Honduras[44] has been observed in Sri Lanka.[29] Interestingly, the variant residue 173L seems to be exclusive of South

**Table I**
**MOLECULAR VARIATION IN *PLASMODIUM VIVAX DHFR* IN LATIN AMERICA, 2001-2013**

| | | | *Amino acid substitutions and their frequency, %* | | | | | |
|---|---|---|---|---|---|---|---|---|
| # residue: | | | 57 | 58* | 117 | 173 | *Other mutations: S, synonymous NS, nonsynonymous (%)* | *References* |
| Sal I strain: | | | F (ttc) | S (agc) | S (agc) | I (att) | | |
| Amino acid substitution: | | | *L (ttg)* | *R /N /K* | *N (aac)* | *L (ctt )* | | |
| *Samples origin* | *Period* | *N* | | | | | | |
| Nicaragua (all areas) | 2011-2012 | 78 | - | - | - | - | S:Y69 (25.6) | This study |
| Mexico (Southern) | 2008-2010 | 73 | - | - | - | - | - | This study |
| Mexico (Southern) | 2001-2008 | 19 | - | - | - | - | NS: P322L (5.2) | 28‡ |
| Honduras (all areas) | 2004-2009 | 59 | 3.4 | 3.4 | - | - | S:Y69 (22), L39 (3), S85 (5) | 44 |
| Colombia | 2005 | 9 | - | 100 | 100 | - | nucleotide changes not given | 29 |
| Colombia (all areas) | 2001-2004 | 53 | - | 98.1 | 100 | - | nucleotide changes not given | 30§ |
| Colombia (Northern) | 2011-2013 | 7 | - | 57/42.8/ | 100 | - | 58R cgc, aga | 31 |
| Colombia (Pacific Coast) | 2012-2013 | 24-31 | - | 73.3/4.1/ | 100 | - | SN:A15V (3.2), H99N (41)/R (44). 58R [cgc, agg/aga] | 28‡ |
| Colombia (Amazon) | Not indicated | 41 | 2 | 83/-/15 | 98 | 17 | S:Y69 (82.9), V19 (12.2). NS:A15V (12.2), N50I (2.4), G175E (5). 57L[ttg], 58 R[agg, aga], K[aag] | 32‡ |
| Peru (North) | 2008-2013 | 21-37 | - | 95.4/22.8/ | 100 | 2.7 | 58R [agg, aga], 58N [aac] | 28‡ |
| Brazil (Manaus) | 2007-2008 | 19-14 | - | 79-64 | 84-93 | 5-36 | Amino acid changes not given[1] | 33,34 |
| Brazil (Acre) | 2011 | 2-4 | - | 100 | 100 | 50 | - | 28‡ |
| French Guiana | 2001-2005 | 90 | - | 98.9 | 100 | 34.4 | S:Y69 (85.6), L19 (3.3). NS:A15V (6.7), S116G (3.5). 58R [agg,aga, cgt] | 35‡ |

* Multiple codons.
‡ Assuming the most common amino acid change.
§ Genomic or genetic data available.
Single letter amino acid code: N, asparagine; F, phenylalanine; S, serine; I, isoleucine; G, glycine; L, leucine; R, arginine; T, threonine; E, glutamic acid; K, Lysine; Y, tyrosine; P, proline; V, valine; H, histidine; A, alanine.

**Table II**
**Differences in *P. vivax* DHFR haplotypes among parasites of Latin America**

| *Geographic origin/strain* | *N* | *Residues/haplotype* | *%* | *References* |
|---|---|---|---|---|
| Sal I* strain (1980), Nicaragua strain (1980s), Panama strain (1966) | | 50-57-58-116-117-173-175/<br>NFSSSIG | | 29, 43 |
| Mexico (Southern) | 73 | NFSSSIG | 100 | This study |
| Nicaragua (all areas) | 78 | NFSSSIG | 100 | This study |
| Honduras | 59 | 57-58-117-173/<br>FSSI<br>LRSI | <br>96.6<br>3.4 | 44 |
| Colombia | 9 | 57-58-61-117-173/<br>FRTN | <br>100 | 29 |
| Colombia (Northern) | 6 | FRSNI | 100 | 31 |
| Colombia (all areas) | 53 | 57-58-117/<br>FRN<br>FRS | <br>94.3<br>5.7 | 30 |
| Colombia (Amazon) | 41 | 50-57-58-117-173-175/<br>NLRSIG<br>IFSNIG<br>NFRNIG<br>NFRNIE<br>NFRNLG (triple mutant)<br>NFKNIG | <br>2<br>2<br>59<br>5<br>17<br>15 | 32 |
| Belem strain/Brazil (1980) | | NFRSNI | - | 47 |
| French Guiana | 90 | 57-58-116-117-173/<br>FSSNI<br>FRSNI<br>FRSNL<br>FRSNI<br>FRGNI<br>FRSNL (triple mutant) | <br>1.2<br>4.7<br>1.2<br>30.2<br>3.5<br>59.3 | 35 |

* Susceptiblle residues.
N: number of isolates.
Amino acids are indicated by one letter code: N, asparagine; F, phenylalanine; S, serine; I, isoleucine; G, glycine; L, leucine; R, arginine; T, threonine; E, glutamic acid. Variant residues are underlined.

**Table III**
**Limited tandem repeat types of the *Pvdhfr* in isolates from Latin America**

| *DHFR: tandem repeat arrangement:* | *RACCN** | *NPC** | *SCH** | *HOND* | *FG* | *COL* |
|---|---|---|---|---|---|---|
| | *Number of isolates:* | | | | | |
| | 54 | 24 | 73 | 54 | 90 | 48 |
| | | | % | | | |
| GGDNTS‡ GGDNTH GGDNAD | 100 | 17 | 100 | 78 | 100 | 75 |
| GGDNTS GGDNTH GGDNTS GGDNAD | 0 | 83 | 0 | 22 | 0 | 0 |
| GGDNTS GGDNAD | 0 | 0 | 0 | 0 | 0 | 16.7 |
| GGDNTS GGDNTH GGDNTH GGDNAD | 0 | 0 | 0 | 0 | 0 | 2.1 |

* This study.
‡ Sal I strain sequence (NBCI, Gen Bank: XM_001615032): amino acids 89-106.
Place /date of sample collection: RACCN, North Atlantic Autonomous Region/2011-2012; NPC, North Pacific Coast/2006, 2012; SCH, Southern Chiapas Mexico/2008-2010; HOND, Honduras/2004-2009 (Ref. 44); FG, French Guiana/2001-2005 (Ref. 35); COL, Colombia/2001-2004 (Ref. 30). Amino acids are indicated by one letter code. G, glycine; D, aspartic acid; N, asparagine; T, threonine; H, histidine; A, alanine; S, serine.

America, while a different substitution (173F) was reported in South Korea, Myanmar,[49] India[27] and Vanuatu.[55] Furthermore, other amino acid substitutions conferring pyrimethamine resistance were unseen in Latin America, e.g. those present at residues 61 and 117. The variant T61M is frequently linked to S117T; both seem to have arisen in Thailand, Indonesia and Oceania.[49]

In South America, the emergence of pyrimethamine resistance arose under the pressure of SP implemented in the 1980s-1990s to treat *P. falciparum*.[56-59] *Pfdhfr* mutations associated to pyrimethamine resistance were documented shortly after at different sites and at various frequencies.[48,56,57,59] At that time, mixed *P. falciparum* and *P. vivax* infections were common in areas where SP were used, and resistant *P. vivax* resulted from bystander exposure.

In Mexico and in most of Central America, only chloroquine and primaquine have been used to treat malaria since the 1950s,[15] while pyrimethamine was used in some malarious areas of South America and Panama,[48,59] explaining the absence of *Pvdhfr* resistance-associated mutations in our samples and their variable distribution in the southern countries. Nevertheless, a consequence of past and current human migrations may be the introduction of resistant parasites into areas with no resistance. Our results suggest a lack of exposure of *P. vivax* to pyrimethamine and the non-introduction of parasites with *Pvdhfr* mutations conferring resistance to this drug in Mesoamerica. The pattern of mutations in *Pvdhfr* at a local and global level would help establish molecular surveillance and monitoring of the dispersion of drug resistant strains through human migration. Following the WHO's recommendations to implement ACT (including artesunate-sulfadoxine/pyrimethamine)[15] and choosing the best treatment requires, among other things, information on the susceptibility status of *P. vivax* in the region, an accurate diagnosis of the medical condition of the patient, and availability of anti-malarial medications.

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*Declaración de conflicto de intereses.* Los autores declararon no tener conflicto de intereses.

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