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)

González-Cerón L, Rodríguez MH, Montoya A, Santillán-Valenzuela F, Corzo-Gómez JC. Molecular variation of *Plasmodium vivax* dehydrofolate reductase in Mexico and Nicaragua contrasts with that occurring in South America. Salud Publica Mex. 2020:62:364-371.
https://doi.org/10.21149/10129

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

González-Cerón L, Rodríguez MH, Montoya A, Santillán-Valenzuela F, Corzo-Gómez JC. Variación molecular de la dehidrofolato reductasa de *Plasmodium vivax* en México y Nicaragua contrasta con la que ocurre en Sudamérica. Salud Publica Mex. 2020:62:364-371.
https://doi.org/10.21149/10129

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 amplifico 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.

Received on: October 18, 2017 • **Accepted on:** April 11, 2018
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'-CGTTGATCCTCGTGAAGTAGATC-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: 10µL of 5× PCR buffer, 4µL of $MgCl_2$ (25mM), 2.5µL of dNTPs (1.25mM), 1.9 µL of each primer (10µM), 0.5µL of Go Taq DNA polymerase 5u/µL (Promega, Madison, WI, USA) and 2-4 µL of extracted DNA for a final volume of 50 µL. 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/ NFSSSIG		29, 43
Mexico (Southern)	73	NFSSSIG	100	This study
Nicaragua (all areas)	78	NFSSSIG	100	This study
Honduras	59	57-58-117-173/ FSSI LRSI	 96.6 3.4	44
Colombia	9	57-58-61-117-173/ FRTN	100	29
Colombia (Northern)	6	FRSNI	100	31
Colombia (all areas)	53	57-58-117/ FRN FRS	 94.3 5.7	30
Colombia (Amazon)	41	50-57-58-117-173-175/ NLRSIG IFSNIG NFRNIG NFRNIE NFRNLG (triple mutant) NFKNIG	 2 2 59 5 17 15	32
Belem strain/Brazil (1980)		NFRSNI	-	47
French Guiana	90	57-58-116-117-173/ FSSNI FRSNI FRSNL FRSNI FRGNI FRSNL (triple mutant)	 1.2 4.7 1.2 30.2 3.5 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.

Acknowledgements

We wish to express our gratitude to Conacyt-Mexico, project CB-2009-01-131247; the AMI/RAVEDRA-OPS/OMS-Nicaragua project, and the Global Fund Malaria Component-Nicaragua project.

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

References

1. World Health Organization. Malaria Report 2017. Geneva: WHO, 2017.
2. Organización Panamericana de la Salud. Informe de la situación de Paludismo en las Américas, 2014. Washington DC: OPS, 2017.
3. Pan American Health Organization. Epidemiological Update. Increase of malaria in the Americas, 2018. Washington DC: OPS, 2018.
4. Secretaría de Salud. Boletín Epidemiológico Sistema Nacional de Vigilancia Epidemiológica Sistema Único de Información. Mexico City: Sinave, 2017.
5. Gabaldon A, Berti AL. The first large area in the tropical zone to report malaria eradication: North-Central Venezuela. Am J Trop Med Hyg. 1954;3:793-807. https://doi.org/10.4269/ajtmh.1954.3.793
6. Pinotti M. New method of malaria prevention: combination of an antimalarial drug with table salt used daily in food. Rev Bras Malariol Doencas Trop. 1954;6(1):5-12.
7. Maberti S. The development of resistance to pyrimethamine. Presentation of 15 cases studied in Trujillo, Venezuela. Arch Venez Med Trop Parasitol Med. 1960;3:239-59.
8. Moore DV, Lanier JE. Observations on two *Plasmodium falciparum* infections with an abnormal response to chloroquine. Am J Trop Med Hyg. 1961;10:5-9. https://doi.org/10.4269/ajtmh.1961.10.5
9. Wernsdorfer WH, Kouznetsov RL. Drug-resistant malaria--occurrence, control, and surveillance. Bull World Health Organ. 1980;58(3):341-52.
10. White NJ. Antimalarial drug resistance. J Clin Invest. 2004;113:1084-92. https://doi.org/10.1172/JCI200421682
11. Contreras CE, Cortese JF, Caraballo A, Plowe CV. Genetics of drug-resistant *Plasmodium falciparum* malaria in the Venezuelan state of Bolivar. Am J Trop Med Hyg. 2002;67:400-5. https://doi.org/10.4269/ajtmh.2002.67.400
12. Godoy GA, Volcan GS, Guevara R, Medrano C, Castro J, Texeira A. Venezuelan strains of *Plasmodium falciparum* resistant to sulfa and pyrimethamine as demonstrated by in vitro test. Rev Latinoam Microbiol. 1977;19:229-31.
13. Alecrim WD, Dourado H, Alecrim M das G, Passos LF, Wanssa E, Albuquerque B [In vivo resistance of *Plasmodium falciparum* to the combination of sulfadoxine and pyrimethamine, at RIII level, in Amazonas, Brazil]. Rev Inst Med Trop Sao Paulo. 1982;24(suppl 6):52-3.
14. World Health Organization. Antimalarial durg combination therapy: report of a WHO technical consultation. Geneva: WHO/RBM, 2001.
15. World Health Organization. Guidelines for the treatment of malaria 2015. 3rd ed. Geneva: WHO, 2015.
16. Yuthavong Y, Kamchonwongpaisan S, Leartsakulpanich U, Chitnumsub P. Folate metabolism as a source of molecular targets for antimalarials. Future Microbiol. 2006;1:113-25. https://doi.org/10.2217/17460913.1.1.113
17. Picot S, Olliaro P, de Monbrison F, Bienvenu AL, Price RN, Ringwald P. A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in falciparum malaria. Malar J. 2009;8:89. https://doi.org/10.1186/1475-2875-8-89
18. Alam MT, Bora H, Bharti PK, Saifi MA, Das MK, Dev V, *et al.* Similar trends of pyrimethamine resistance-associated mutations in *Plasmodium vivax* and *P. falciparum*. Antimicrob Agents Chemother. 2007;51:857-63. https://doi.org/10.1128/AAC.01200-06
19. Young MD, Burgess RW. Pyrimethamine resistance in *Plasmodium vivax* malaria. Bull World Health Organ. 1959;20(1):27-36.
20. Imwong M, Pukrittakayamee S, Looareesuwan S, Pasvol G, Poirreiz J, White NJ, *et al.* Association of genetic mutations in *Plasmodium vivax* dhfr with resistance to sulfadoxine-pyrimethamine: geographical and clinical correlates. Antimicrob Agents Chemother. 2001;45:3122-7. https://doi.org/10.1128/AAC.45.11.3122-3127.2001

21. World Health Organization. World Malaria Report 2015. Geneva: WHO, 2015.
22. Haghdoost AA, Alexander N. Systematic review and meta-analysis of the interaction between *Plasmodium falciparum* and *Plasmodium vivax* in humans. J Vector Borne Dis. 2007;44(1):33-43.
23. Stepniewska K, White NJ. Pharmacokinetic determinants of the window of selection for antimalarial drug resistance. Antimicrob Agents Chemother. 2008;52:1589-96. https://doi.org/10.1128/AAC.00903-07
24. Hawkins VN, Joshi H, Rungsihirunrat K, Na-Bangchang K, Sibley CH. Antifolates can have a role in the treatment of *Plasmodium vivax*. Trends Parasitol. 2007;23:213-22. https://doi.org/10.1016/j.pt.2007.03.002
25. de Pecoulas PE, Tahar R, Ouatas T, Mazabraud A, Basco LK. Sequence variations in the *Plasmodium vivax* dihydrofolate reductase-thymidylate synthase gene and their relationship with pyrimethamine resistance. Mol Biochem Parasitol. 1998;92:265-73. https://doi.org/10.1016/S0166-6851(97)00247-8
26. Hastings MD, Porter KM, Maguire JD, Susanti I, Kania W, Bangs MJ, *et al.* Dihydrofolate reductase mutations in *Plasmodium vivax* from Indonesia and therapeutic response to sulfadoxine plus pyrimethamine. J Infect Dis. 2004;189:744-50. https://doi.org/10.1086/381397
27. Hastings MD, Maguire JD, Bangs MJ, Zimmerman PA, Reeder JC, Baird JK, *et al.* Novel *Plasmodium vivax* dhfr alleles from the Indonesian Archipelago and Papua New Guinea: association with pyrimethamine resistance determined by a *Saccharomyces cerevisiae* expression system. Antimicrob Agents Chemother. 2005;49:733-40. https://doi.org/10.1128/AAC.49.2.733-740.2005
28. Hupalo DN, Luo Z, Melnikov A, Sutton PL, Rogov P, Escalante A, *et al.* Population genomics studies identify signatures of global dispersal and drug resistance in *Plasmodium vivax*. Nat Genet. 2016;48:953-8. https://doi.org/10.1038/ng.3588
29. Hawkins VN, Auliff A, Prajapati SK, Rungsihirunrat K, Hapuarachchi HC, Maestre A, *et al.* Multiple origins of resistance-conferring mutations in *Plasmodium vivax* dihydrofolate reductase. Malar J. 2008;7:72. https://doi.org/10.1186/1475-2875-7-72
30. Saralamba N, Nakeesathit S, Mayxay M, Newton PN, Osorio L, Kim JR, *et al*. Geographic distribution of amino acid mutations in DHFR and DHPS in *Plasmodium vivax* isolates from Lao PDR, India and Colombia. Malar J. 2016;15:484. https://doi.org/10.1186/s12936-016-1543-8
31. Winter DJ, Pacheco MA, Vallejo AF, Schwartz RS, Arevalo-Herrera M, Herrera S, *et al.* Whole genome sequencing of field isolates reveals extensive genetic diversity in *Plasmodium vivax* from Colombia. PLoS Negl Trop Dis. 2015;9:e0004252. https://doi.org/10.1371/journal.pntd.0004252
32. Cubides JR, Camargo-Ayala PA, Nino CH, Garzon-Ospina D, Ortega-Ortegon A, Ospina-Cantillo E, *et al.* Simultaneous detection of *Plasmodium vivax* dhfr, dhps, mdr1 and crt-o resistance-associated mutations in the Colombian Amazonian region. Malar J. 2018;17:130. https://doi.org/0.1186/s12936-018-2286-5
33. Chehuan YF, Costa MR, Costa JS, Alecrim MG, Nogueira F, Silveira H, *et al.* In vitro chloroquine resistance for *Plasmodium vivax* isolates from the Western Brazilian Amazon. Malar J. 2013;12:226. https://doi.org/10.1186/1475-2875-12-226
34. Marques MM, Costa MR, Santana Filho FS, Vieira JL, Nascimento MT, Brasil LW, *et al. Plasmodium vivax* Chloroquine resistance and anemia in the Western Brazilian Amazon. Antimicrob Agents Chemother. 2014;58:342-7. https://doi.org/10.1128/AAC.02279-12
35. Barnadas C, Musset L, Legrand E, Tichit M, Briolant S, Fusai T, *et al.* 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. https://doi.org/10.4269/ajtmh.2009.81.19
36. Mace KE, Arguin PM, Tan KR. Malaria surveillance — United States, 2015. MMWR Surveill Summ. 2018;67(7):1-28. https://doi.org/10.15585/mmwr.ss6707a1
37. Rodrigues PT, Valdivia HO, de Oliveira TC, Alves JMP, Duarte A, Cerutti-Junior C, *et al.* Human migration and the spread of malaria parasites to the New World. Sci Rep. 2018;8:1993. https://doi.org/10.1038/s41598-018-19554-0
38. Secretaría de salud. Norma Oficial Mexicana NOM-032-SSA2-2014, para la vigilancia epidemiológica, prevención y control de las enfermedades transmitidas por vectores. Mexico City: Secretaría de Salud, 2014.
39. Pan American Health Organization. Situación de la malaria en la región de las Américas, 2000-2016. Washington DC: PAHO, 2016.
40. Gonzalez-Ceron L, Montoya A, Corzo-Gomez JC, Cerritos R, Santillan F, Sandoval MA. Genetic diversity and natural selection of *Plasmodium vivax* multi-drug resistant gene (pvmdr1) in Mesoamerica. Malar J. 2017;16:261. https://doi.org/10.1186/s12936-017-1905-x
41. Gonzalez-Ceron L, Rodriguez MH, Sandoval MA, Santillan F, Galindo-Virgen S, Betanzos AF, *et al.* Effectiveness of combined chloroquine and primaquine treatment in 14 days versus intermittent single dose regimen, in an open, non-randomized, clinical trial, to eliminate *Plasmodium vivax* in southern Mexico. Malar J. 2015;14:426. https://doi.org/10.1186/s12936-015-0938-2
42. Gonzalez-Ceron L, Martinez-Barnetche J, Montero-Solis C, Santillan F, Soto AM, Rodriguez MH, *et al.* Molecular epidemiology of *Plasmodium vivax* in Latin America: polymorphism and evolutionary relationships of the circumsporozoite gene. Malar J. 2013;12:243. https://doi.org/10.1186/1475-2875-12-243
43. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, *et al.* Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. Nature. 2008;455:757-63. https://doi.org/10.1038/nature07327
44. Jovel IT, Mejia RE, Banegas E, Piedade R, Alger J, Fontecha G, *et al.* Drug resistance associated genetic polymorphisms in *Plasmodium falciparum* and *Plasmodium vivax* collected in Honduras, Central America. Malar J. 2011;10:376. https://doi.org/10.1186/1475-2875-10-376
45. Sridaran S, Rodriguez B, Soto AM, Macedo De Oliveira A, Udhayakumar V. Molecular analysis of chloroquine and sulfadoxine-pyrimethamine resistance-associated alleles in *Plasmodium falciparum* isolates from Nicaragua. Am J Trop Med Hyg. 2014;90:840-5. https://doi.org/10.4269/ajtmh.13-0214
46. Lee WJ, Kim HH, Choi YK, Choi KM, Kim MA, Kim JY, *et al.* Analysis of the dihydrofolate reductase-thymidylate synthase gene sequences in *Plasmodium vivax* field isolates that failed chloroquine treatment. Malar J. 2010;9:331. https://doi.org/10.1186/1475-2875-9-331
47. Imwong M, Pukrittayakamee S, Renia L, Letourneur F, Charlieu JP, Leartsakulpanich U, *et al.* Novel point mutations in the dihydrofolate reductase gene of *Plasmodium vivax:* evidence for sequential selection by drug pressure. Antimicrob Agents Chemother. 2003;47:1514-21. https://doi.org/10.1128/AAC.47.5.1514-1521.2003
48. Samudio F, Santamaria AM, Obaldia N 3rd, Pascale JM, Bayard V, Calzada JE. Prevalence of *Plasmodium falciparum* mutations associated with anti-malarial drug resistance during an epidemic in Kuna Yala, Panama, Central America. Am J Trop Med Hyg. 2005;73:839-41. https://doi.org/10.4269/ajtmh.2005.73.839
49. Lu F, Lim CS, Nam DH, Kim K, Lin K, Kim TS, *et al.* Mutations in the antifolate-resistance-associated genes dihydrofolate reductase and dihydropteroate synthase in *Plasmodium vivax* isolates from malaria-endemic countries. Am J Trop Med Hyg. 2010;83:474-9. https://doi.org/10.4269/ajtmh.2010.10-0004
50. Sharifi-Sarasiabi K, Haghighi A, Kazemi B, Taghipour N, Mojarad EN, Gachkar L. Molecular surveillance of *Plasmodium vivax* and *Plasmodium falciparum* dhfr mutations in isolates form southern Iran. Rev Inst Med Trop Sao Paulo. 2016;58:16. https://doi.org/10.1590/S1678-9946201658016
51. Das S, Banik A, Hati AK, Roy S. Low prevalence of dihydro folate reductase (dhfr) and dihydropteroate synthase (dhps) quadruple and quintuple mutant alleles associated with SP resistance in *Plasmodium vivax* isolates of West Bengal, India. Malar J. 2016;15:395. https://doi.org/10.1186/s12936-016-1445-9
52. Bareng AP, Espino FE, Chaijaroenkul W, Na-Bangchang K. Molecular monitoring of dihydrofolatereductase (dhfr) and dihydropteroatesynthe-

tase (dhps) associated with sulfadoxine-pyrimethamine resistance in *Plasmodium vivax* isolates of Palawan, Philippines. Acta Trop. 2018;180:81-7. https://doi.org/10.1016/j.actatropica.2018.01.006

53. Asih PB, Marantina SS, Nababan R, Lobo NF, Rozi IE, Sumarto W, *et al.* Distribution of *Plasmodium vivax* pvdhfr and pvdhps alleles and their association with sulfadoxine-pyrimethamine treatment outcomes in Indonesia. Malar J. 2015;14:365. https://doi.org/10.1186/s12936-015-0903-0

54. Huang B, Huang S, Su XZ, Tong X, Yan J, Li H, *et al.* Molecular surveillance of pvdhfr, pvdhps, and pvmdr-1 mutations in *Plasmodium vivax* isolates from Yunnan and Anhui provinces of China. Malar J. 2014;13:346. https://doi.org/10.1186/1475-2875-13-346

55. Auliff A, Wilson DW, Russell B, Gao Q, Chen N, Anh le N, *et al.* Amino acid mutations in *Plasmodium vivax* DHFR and DHPS from several geographical regions and susceptibility to antifolate drugs. Am J Trop Med Hyg. 2006;75:617-21. https://doi.org/10.4269/ajtmh.2006.75.617

56. Urdaneta L, Plowe C, Goldman I, Lal AA. Point mutations in dihydrofolate reductase and dihydropteroate synthase genes of *Plasmodium falciparum* isolates from Venezuela. Am J Trop Med Hyg. 1999;61:457-62. https://doi.org/10.4269/ajtmh.1999.61.457

57. Peterson DS, Di Santi SM, Povoa M, Calvosa VS, Do Rosario VE, Wellems TE. Prevalence of the dihydrofolate reductase Asn-108 mutation as the basis for pyrimethamine-resistant falciparum malaria in the Brazilian Amazon. Am J Trop Med Hyg. 1991;45:492-7. https://doi.org/10.4269/ajtmh.1991.45.492

58. Plowe CV, Cortese JF, Djimde A, Nwanyanwu OC, Watkins WM, Winstanley PA, *et al.* Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J Infect Dis. 1997;176:1590-6. https://doi.org/10.1086/514159

59. Cortese JF, Caraballo A, Contreras CE, Plowe CV. Origin and dissemination of *Plasmodium falciparum* drug-resistance mutations in South America. J Infect Dis. 2002;186:999-1006. https://doi.org/10.1086/342946