Plasmodium vivax apical membrane antigen 1_{I-II} from Nicaragua (2012-2013): genetic and antigenic polymorphism

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Abstract

Objective. Genetic and antigenic polymorphism of *P. vivax* apical membrane antigen-1 (pvama1$_{I-II}$) from Nicaragua was examined. **Materials and methods.** Infected blood samples from patients were obtained during 2012-2013. A gene fragment comprising domains I-II was amplified and sequenced, and the genetic parameters, haplotype relationships, genetic structure, and amino acid variation in predicted B cell epitopes were analyzed. **Results.** 65 sequences of pvama1$_{I-II}$ had 19 nonsynonymous and five synonymous nucleotide changes. Nicaraguan parasites had low diversity, high linkage disequilibrium, and few recombination events. Neutrality tests indicate a positive and divergent selection, and three genetic clusters with loss of haplotypes were demonstrated. Amino acid variation predominated in predicted B cell epitopes and was closely related to that in Latin American parasites. **Conclusions.** Nicaraguan *P. vivax* is a moderately differentiated population under contraction and focalization processes, and the antigenic diversity resembles that of Latin American parasites. This information is relevant for vaccine development and epidemiological surveillance

Keywords: *Plasmodium vivax*; Nicaragua; apical membrane antigen 1; genetic structure; B cell epitopes

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Resumen

Objetivo. Se examinó el polimorfismo genético y antigénico del antígeno de membrana apical 1 (*pvama1*$_{I-II}$) en *P. vivax* de Nicaragua. **Material y métodos.** Se obtuvieron muestras de sangre infectada de pacientes durante 2012-2013. Un fragmento génico de los dominios I-II se amplificó y secuenció. Se analizaron parámetros genéticos, relaciones haplotípicas, estructura genética y substitución de aminoácidos. **Resultados.** En 65 secuencias de *pvama1*$_{I-II}$ hubo 19 cambios no sinónimos y cinco sinónimos. Los parásitos nicaragüenses tuvieron baja diversidad y un alto desequilibrio de ligamiento. Las pruebas de neutralidad indicaron una selección positiva y divergente. Se evidenciaron tres grupos genéticos con pérdida de haplotipos. La variación de aminoácidos predominó en epítopos de células B, similar a parásitos de Latinoamérica. **Conclusiones.** *P. vivax* de Nicaragua fue moderadamente diferenciada en proceso de contracción y focalización, mientras que la diversidad antigénica fue similar a la reportada en parásitos de Latinoamérica. Esta información es relevante para el diseño de vacunas y la vigilancia epidemiológica.

Palabras clave: *Plasmodium vivax*; Nicaragua; antígeno apical de membrana 1; estructura genética; epitopos B

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Plasmodium vivax is the malaria species most worldwide distributed and causes the highest morbidity rate in affected areas outside Africa. In Latin America, this species caused 75% of 596 200 malaria cases reported in 2020.[1] Nicaragua experienced a decrease between 2000 and 2010, from 23 878 to just 692 cases, respectively.[2] Regrettably, malaria transmission upsurge and the number of cases increased greatly, reporting 13 220 and 25 505 in 2019 and 2020, respectively. In this country, *P. vivax* has contributed with 79-91% each year from 2015 to 2019, and with 52% in 2020.[1]

Studies on the molecular polymorphism and genetic structure of malaria vaccine candidates provide new knowledge on circulating variants and the selective forces acting on these molecules, and might aid to the monitoring transmission of variants and emerging mutants.[3] During the life cycle, *P. vivax* merozoites are released from infected hepatocytes and invade reticulocytes by a series of complex ligand-receptor processes.[4] Following the primary adhesion of the parasite to the reticulocyte surface, PvAMA1 is secreted by micronemes, transported to the parasite surface, and participates in merozoite reorientation prior to reticulocyte invasion. This molecule is coded by a gene from chromosome 9, which includes an ectodomain composed of three domains (I, II, and III) and 16 conserved cysteine residues.[5] Domains I-II contain a ligand-binding site for merozoite-reticulocyte interaction.[6] The *P. vivax*, the ectodomain is highly immunogenic,[7] and high antibody titers are developed after the natural infection or in immunized mice.[8] Antibodies against PvAMA1 attained inhibition of the reticulocyte invasion.[9] Because of that, this molecule depicts a promising candidate for vaccines anti-disease, which anticipates to reduce blood infection and disease complications.

In here, the nucleotide and haplotype diversity of *P. vivax* apical membrane antigen 1 domains I and II were studied in parasites from Nicaragua during 2012-2013, and the selective forces acting on the coding gene, and the genetic relationships between Nicaraguan and global parasites were analyzed.

Materials and methods

The Ethics Committees of the National Institute of Public Health of Mexico (CI1042) and the National Center for Diagnosis and Reference of the Ministry of Health of Nicaragua approved the study. The patients' personal information was encrypted and their municipality of origin was used.

P. vivax samples. Infected blood samples were obtained from patients during 2012-2013.[10,11] Symptomatic patients sought diagnosis at the sentinel laboratories in the North Atlantic Coast Autonomous Region (RACCN) and in the North Pacific (NP), National Center for Diagnosis and Reference, Ministry of Health in Nicaragua. RACCN comprises the Miskito region shared with Honduras, where free flow of this ethnic population does take place.[12] The diagnosis of malaria was carried out by the microscopic analysis of the thick blood smear.[13] Patients testing positive for *P. vivax* were asked to donate 2-3 drops of capillary fresh blood to soak filter paper Whatman #2. Samples were dried out, preserved in silica gel, and in the dark.

DNA amplification and sequencing. DNA extraction was performed using the commercial QIAmp DNA Blood Minikit (Qiagen CA, USA) following the manufacturer's instructions. DNA from ~30 μL of each sample was dissolved in 50 μL of water. A nested PCR was used to amplify a gene fragment containing domains I and II ($pvama1_{I\text{-}II}$). The reaction mixture consisted of GoTaqFlexi 1X Buffer, 2 mM magnesium chloride, 0.2 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 0.5 μM of each oligonucleotide (PvPvama1F-5′ TCCAGCTGGAAGATGTCCTG 3′ and Pvama1R1-5′ CCGCCCTTTTCTCTACACAG 3′), 1 U GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA), and 2-4 μL of template DNA in 20 μL final volume. Amplification conditions started with a denaturation cycle at 95°C for five minutes, followed by 35 cycles: denaturation at 95°C for 60 seconds, alignment at 61°C for 60 seconds, and extension at 72°C for 75 seconds. A final extension was carried out at 72°C for 10 minutes. The nested amplification was run using primers Pvama1F and Pvama1R2-5′ CGCAGGGACATTTGATACTCTCC 3′ and 2 μL of the primary PCR reaction. PCR conditions were similar as indicated above. The DNA purification was carried out using the MiniElute PCR Purification Kit (Qiagen, CA, USA) following the manufacturer's instructions, and purified product was quantified using the NanoDrop ND200 (Thermo Scientific Inc., USA). Then, gene fragments were sequenced using forward and reverse oligonucleotides by Sanger method, in the High Throughput Genomics unit, Department of Genome Sciences, Washington University, Washington DC, USA. The sequences were revised manually in BioEdit v7.1.3,[14] and consensus sequences were obtained, and deposited at NBCI with accession numbers: ON730710-ON730774.

Genetic and peptide analysis. $Pvama1_{I\text{-}II}$ sequences were aligned using the ClustalW in BioEdit. Synonymous and non-synonymous nucleotide changes and amino acid substitutions were identified, using as reference the Salvador 1 sequence (XM_001614792). The number of segregating sites, number of mutations, haplotype

diversity, nucleotide and genetic diversity, the minimal number of recombination events (Rm) and the R^2 index of Linkage disequilibrium (LD) were calculated in dnaSP v6.12.[15] Neutrality tests of Tajima's D, Fu & Li´s D* and F*, and the McDonald-Kreitman (MK) test were estimated in dnaSP, and using *P. cynomolgi* sequence (X86099) as a related species. The number of synonymous and non-synonymous mutations, and the difference between the rates of synonymous and non-synonymous substitutions (*dN-dS*) were estimated, using the Nei-Gojobori method with Jukes-Cantor correction and 1 000 bootstrap in MEGA v11.0.[16] The F_{ST} statistical analysis between *P. vivax* groups was performed by pairwise comparisons using the Kimura 2 model in dnaSP, values range from 0 to 1, were 0 indicates that the two populations are identical. The genealogical relationships of *Pvama1*$_{I-II}$ haplotypes from Nicaragua and other geographical sites were analyzed by Median-joining networks using Population Analysis with Reticulate Trees (PopART) v1.7.[17] Structure analysis using admixture model, that assign individuals to one or more clusters was carried out using Structure v2.3.4.[18] Tests were run 20 times at 50 000 burn in period and 100 000 iterations for K from 2 to 5. The most probable number of clusters were estimated using Structure Harvester.[19]

To make genetic comparisons, homologous gene sequences from other geographical areas were obtained from NBCI:[20] India, *n*=111 EF025187-EF025197[21] and MH657021-MH657120;[22] Sri Lanka, *n*=23 EF218679-EF218701;[23] Venezuela: *n*=73 EU346015-EU346087;[24] Thailand, *n*=231 FJ784891-FJ785121;[25] Iran: JF682785-JF682790, KF435081-KF435083,[26] JX624732-JX624760[27] KF422636-KF422681,[28] and KF181626-KF181642;[29] Papua New Guinea (PNG), *n*=102 KC702402-KC702503;[30] Korea, *n*=67 KM230319-KM230384;[31] Brazil, *n*=40 MH049550-MH049589;[32] China, *n*=73 KX495505-KX495577;[33] and Uganda, *n*=17 KU893334-KU893350*. Other 22, 19 and 13 homologous sequences from Colombia, Peru and Mexico, respectively, were extracted from the PlasmoDB website.[34,35]

BepiPred was used to identify potential peptides participating in B cell epitopes in PvAMA1 at the Immune Epitope Database website.[36] BepiPred Linear Epitope Prediction uses a combination of a hidden Markov model and a propensity scale method (using a default value of 0.35),[37] while BepiPred 2.0 uses a Random Forest algorithm trained on epitopes and non-epitope amino acids determined from crystal structures (default threshold of 0.5).[38]

Results

Sixty-five consensus sequences of 915 bp (codons 106-410) were obtained, when compared to the Sal I sequence had 24 nucleotide changes; 19 were non-synonymous and five were synonymous (table I). *P. vivax ama-1*$_{I-II}$ from Nicaragua had nucleotide and haplotype diversity lower than parasites from other sites (table II). The Rm value for Nicaraguan parasites was low and similar to parasites from Brazil, Peru and Mexico, but lower than parasites from Colombia or Venezuela (table I). Nicaraguan *pvama-1*$_{I-II}$ sequences showed the second highest LD index (R^2= 0.299), and positive values for Tajima´s D and Fu & Li´s D* and F* (1.232, 0.744 and 1.098, respectively), similar to parasites from Mexico, Venezuela and PNG. The neutrality index of MK was high (8.33) similar to parasites from other locations (table II). While Z values of selection were low positive (0.15) for Nicaraguan parasites, and higher positive values were estimated for Colombia, Peru and Brazil (0.82, 1.26, 0.81, respectively).

Median Joining Network constructed with Nicaraguan sequences, displayed 10 haplotypes separated from 1 to 26 mutational steps among them (figure 1A). Haplotype Nh1 of high frequency (57%) was detected in all municipalities (figure 1B). Nh9 from Rosita was separated by two mutational steps from Nh1. Nh10 and Nh2 (10.7%) were separated by 9 and 16 mutational steps from Nh9, respectively. While Nh6 and Nh3 (10.7%) were separated from Nh2 by 2 and 4 mutational steps, respectively. Nh2 and Nh3 were detected in municipalities of Bonanza, Rosita and Siuna. Haplotype Nh7 from Waspam and was separated by two mutational steps from Nh3. Furthermore, Nh4 (9.3%) was separated from Nh6 by four mutational steps, while Nh5 and Nh8 were separated from Nh4 by 4 and 1 mutational steps, respectively. Six isolates from the NP region had Nh4. Haplotypes Nh5, Nh6, Nh7, Nh8 and Nh10 from Waspam, were interspersed in the network. In addition, Networks using global sequences had complex and countless connections, with little origin biased clustering, as reported by Kale and colleagues.[22] Origin-based exclusivities of 387 global haplotypes, showed that three haplotypes from Nicaragua including Nh1 were exclusive, and seven haplotypes were shared with parasites from Latin America. A median joining network including 234 sequences from Latin America displays haplotypes separated from one to numerous mutational steps among them (figure 2A). Haplotype Nh9 from Rosita was shared with parasites from different origins, and Nh1/Nh9 clustered with haplotypes from Venezuela and Mexico; some of them were exclusive. Haplotypes from different origins were scattered in the network, and presumably conforming different genealogical groups (figure 2B). Diversification processes were observed mainly in South America.

Table I

P. vivax ama1$_{I-II}$: comparison of parameters of diversity, recombination and natural selection between Nicaraguan parasites and those from other geographic origins. Nicaragua, 2012-2013

Geographic Origin	N	Diversity indexes						Rm	LD	Tajima´s D	Fu & Li´s		MK: Fixed differences				MK: Neutrality Index	Z test of selection (Z value)
													Between species		Within species			
		S	M	H	Hd	π	Θ-w		Index R^2		D*	F*	S	NS	S	NS		
Nicaragua	65	24	24	10	0.651	0.00797	0.00572	3	0.2992	1.2325	0.7443	1.09812	68	31	5	19	8.33‡	0.15
Mexico	13	23	24	5	0.782	0.01154	0.00838	1	0.3666	1.38383	0.82383	1.11259	68	30	5	19	8.61‡	0.11
Colombia	22	31	32	15	0.965	0.00961	0.00962	6	0.1729	-0.12316	0.21892	0.13430	68	29	5	27	12.66‡	0.82
Venezuela	73	23	24	17	0.907	0.00908	0.00535	8	0.2247	1.8122	1.0821	1.6641	68	30	5	16	10.88‡	0.25
Peru	19	28	29	10	0.924	0.00943	0.00906	3	0.2628	0.01718	-0.23287	-0.1853	68	31	5	24	7.83‡	1.26
Brazil	40	38	38	17	0.913	0.00954	0.01011	4	0.2391	-0.6598	-0.7744	-0.6818	69	33	7	25	6.69§	0.81
Iran	101	66	73	75	0.992	0.01357	0.01439	18	0.0879	-0.7674	-1.9245	-1.5866	65	28	10	42	9.75‡	0.03
India	111	40	43	61	0.971	0.01251	0.00857	10	0.1130	0.9947	0.8347	1.1378	68	29	6	30	11.72‡	-0.01
Sri Lanka	23	32	33	15	0.949	0.01079	0.00981	8	0.1905	0.3685	0.7890	0.7313	68	30	4	25	14.16‡	0.93
Thailand	231	49	54	93	0.929	0.01294	0.00921	14	0.0956	1.1164	0.0034	0.4387	63	25	11	31	7.10‡	-0.72
Korea	67	35	35	18	0.886	0.00588	0.00829	2	0.1151	-0.9439	-0.1457	-0.5315	67	34	13	22	3.34*	-0.39
PNG	102	36	39	80	0.994	0.01109	0.00784	10	0.1122	0.73883	1.2513	1.3572	66	30	6	28	10.27‡	0.65
China	73	37	40	35	0.923	0.01121	0.00861	10	0.1291	0.6621	0.5647	0.7202	67	28	10	30	7.18‡	-0.87
Uganda	17	26	27	11	0.949	0.00700	0.00870	2	0.2556	-0.9133	-0.6694	-0.8549	68	34	9	18	4.00§	-0.36

N: number of sequences
S: number of polymorphic sites
M: number of mutations
H: number of haplotypes
Hd: haplotype diversity
ϖ: nucleotide diversity
Θ-w: genetic diversity
Rm: minimal number of recombination events
R^2: index of linkage disequilibrium
MK: McDonald-Kreitman test. The test used *Plasmodium cynomolgi* (X86099) as related species (* $p< 0.01$, ‡ $p< 0.0001$, § $p< 0.001$)
S: synonymous
NS: nonsynonymous
PNG: Papua New Guinea
Z test: HA:dN>dS, Nei Gojobori method (jukes Cantor) 1 000 bootstrap
A sequence comprising from 316 to 1 199 nucleotides

The Structure analysis assumed K=3 (Delta K= 1.4088) as the most probable parasite subpopulations. Parasites from Nicaragua were constituted by two subpopulations, and individuals with admixture ancestry. Subpopulations A-B were present in most sites, while in Brazil A and C predominated. F_{ST} value between Nicaragua and Colombia or Venezuela was moderate (0.113 or 0.118, respectively), followed by Mexico or Peru or countries outside the continent such as Iran and India (table II). F_{ST} values between parasites from Nicaragua or Venezuela vs those from Brazil were higher (0.342 and 0.282, respectively) than those between Brazil and Mexico or Peru or Colombia. The highest differentiation values were between parasites from different origins and those from Korea or Uganda (table II).

Amino acid polymorphism in *PvAMA1*$_{I-II}$ from Nicaragua exposed peptide regions associated to B cell epitopes (pBCE) and named with letters A-H. Of the 17 variable amino acid residues, one was trimorphic (residue 189) and others were dimorphic. Twelve of them were associated to pBCE while other three were nearby (figure 3). The pBCE-A comprises 26 residues (112-127) and four of them were dimorphic (R112T, K120R, N130K, N132D) surrounded by other 3; D107A, L140I, A141E. Other pBCEs (B, C, D, F, I) had one or two variable residues.

Discussion

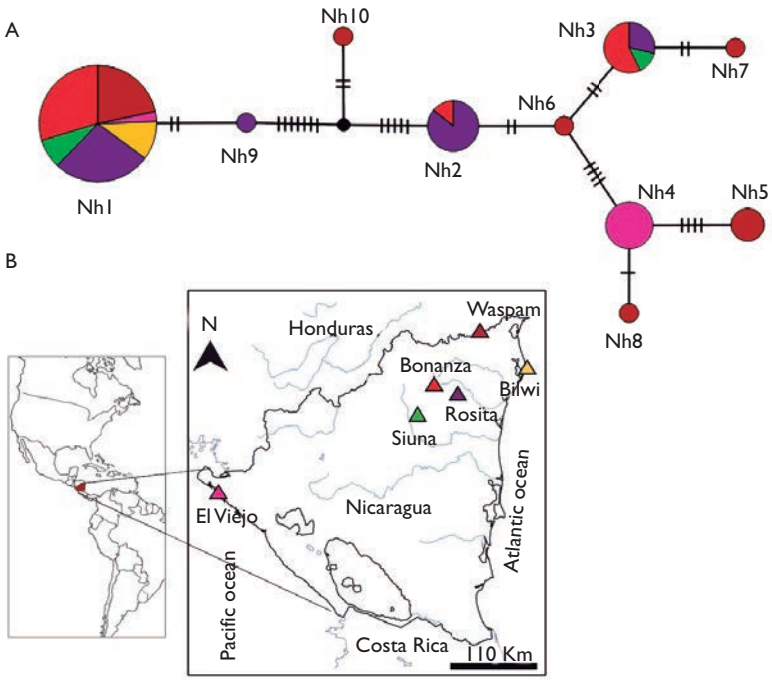
P. vivax population from Nicaragua was moderately differentiated, as depicted by the presence of high frequent

Table II

F_{ST} INDEX OF DIFFERENTIATION BETWEEN *P. VIVAX AMA* 1_{I-II} FROM NICARAGUA AND PARASITES FROM OTHER GEOGRAPHICAL SITES. NICARAGUA, 2012-2013

	Nicaragua	*Mexico*	*Colombia*	*Peru*	*Brazil*	*Venezuela*	*Iran*	*India*	*Sri Lanka*	*Thailand*	*Korea*	*PNG*	*China*
Mexico	0.132												
Colombia	0.113	0.090											
Peru	0.136	0.116	-0.007										
Brazil	0.342	0.169	0.187	0.197									
Venezuela	0.118	0.110	0.023	0.038	0.282								
Iran	0.150	0.031	0.085	0.097	0.162	0.108							
India	0.182	0.050	0.122	0.128	0.232	0.121	0.007						
Sri Lanka	0.241	0.116	0.245	0.275	0.405	0.225	0.105	0.069					
Thailand	0.230	0.169	0.161	0.165	0.290	0.132	0.129	0.126	0.194				
Korea	0.557	0.422	0.471	0.468	0.431	0.505	0.362	0.382	0.536	0.619			
PNG*	0.307	0.190	0.211	0.190	0.290	0.227	0.106	0.091	0.213	0.172	0.389		
China	0.222	0.163	0.141	0.143	0.286	0.098	0.133	0.132	0.230	0.033	0.476	0.192	
Uganda	0.556	0.437	0.482	0.470	0.455	0.509	0.380	0.396	0.537	0.469	0.003	0.404	0.484

* Papua New Guinea



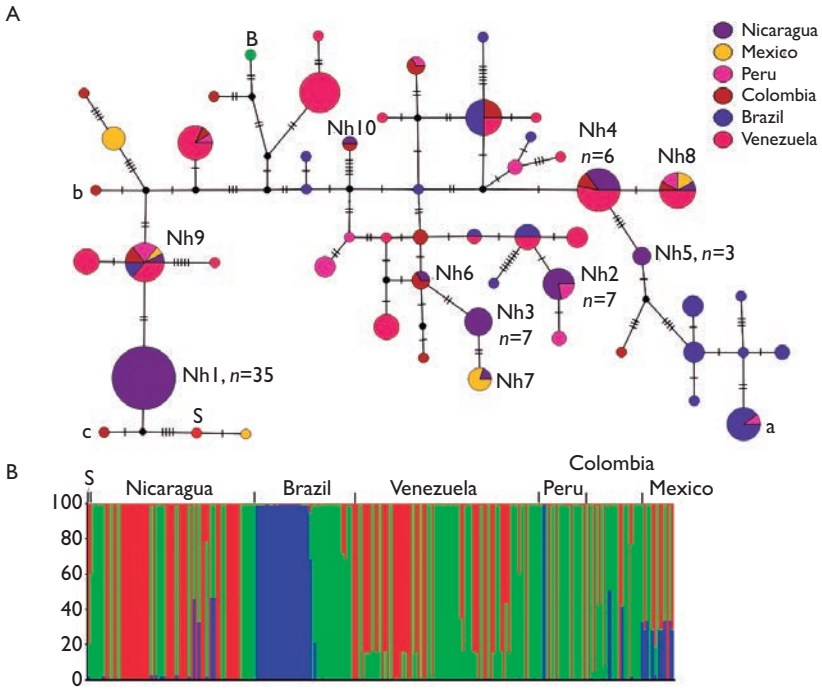
A. The haplotype network shows 10 haplotypes and 65 isolates; the number of mutational steps between them is indicated by vertical strips (haplotypes not sampled or extinct). The color indicates the municipality of origin and the size of the disc corresponds to the frequency of each haplotype. B. Shows a map of Nicaragua, indicating the head of each municipality.

FIGURE 1. MEDIAN JOINING NETWORK AND GEOGRAPHICAL DISTRIBUTION OF *PVAMA* 1_{I-II} HAPLOTYPES IN NICARAGUA, 2012-2013

and exclusive haplotypes (Nh1) dispersed country-wide. The low genetic and haplotype diversity, the high R2 of LD, high positive values of neutrality tests and the low Rm value suggest that *P. vivax* from Nicaragua suffered a population contraction. This concord with the sustained reduction of malaria cases in Nicaragua during 2000-2010.[39] In 2010 few *P. vivax* cases were reported, and during sample gathering (2012-2013) cases were increased to about two fold (~900) per year.[1]

The significant reduction of malaria cases might have render haplotype´s focalization. Under this scenario, the most adapted haplotypes would persist, as observed in southern Mexico by analyzing $Pvama1_{I\text{-}II}$ or $Pvmsp1_{42}$.[40,41] The municipalities from Las Minas (Rosita, Bonanza and Siuna) had *P. vivax* haplotypes (Nh2, Nh3 and Nh9) not detected in other sites. A low genetic flow between these areas was also suggested previously for *P. falciparum* when examining microsatellites; one haplotype was exclusive to Siuna and Rosita.[42] Similarly, on the North Pacific coast one *pvama1* haplotype was exclusive (Nh4). The exclusivity of a parasite genotype was reported earlier in this area by analyzing $pvmsp1_{42}$ and *pvmdr1*.[10,11] Which confirms a predominant haplotype present in the outbreak whipping this region in 2012.[10,11,43]

The presence of various exclusive and divergent *P. vivax* haplotypes in Waspam also suggests a greater pool circulated in the past, and the effect of a continuous parasite flow in bordering municipalities of Nicaragua-Honduras.[42,44] During 2010-2012, Honduras reported higher *P. vivax* cases (from 8 759 to 5 856) than Nicaragua, and the analysis of samples from Honduras obtained in 2010-2011 including the Miskito area, *pvama1* domain I haplotypes had no geographic structure.[45] In 2015, both countries had comparable number of cases unlike the following years, when transmission intensity turn around; malaria incidence in Honduras went down and reported 596 *P. vivax* cases in 2020.[1] However, *P. vivax* cases in Nicaragua were expanding in numbers and spatially, mainly in Puerto Cabezas municipality.[12] *P. vivax* haplotype Nh9 from Rosita might be an ancestral haplotype, supported by its presence in different Latin American sites and the connections to highly frequent haplotypes. The emergency of distinct allelic families and high Index of MK test is suggestive of divergent



A. Median Joining Network: three haplotypes from Nicaragua were exclusive, while others were shared with parasites of different origins in the continent. Lines are connecting 53 haplotypes and the vertical stripes indicate haplotypes not sampled or extinct. Each circle corresponds to one haplotype; the color represents the country of origin and their size is proportional to the frequency of each haplotype. Haplotypes from Nicaragua: Nh1-Nh10 are indicated. Strains: S, Sal1; B, Belem. B. Structure analysis shows the most likely clustering of haplotypes by their ancestry (K= 3); A: green, B: red, C: blue. A gene fragment of 884bp (nucleotides: 316-1 199) was used. Haplotypes a and c were shared with parasites from Iran, and b with those from Iran, Sri Lanka and India.

Figure 2. Genealogical relationships and genetic structure of Pvama1I-II from Nicaragua and Latin America. Nicaragua, 2012-2013

BepiPred-predicted peptides participating in B cell epitopes are underlined and those predicted by BepiPred v2.0 are indicated by a red overlie on the Sal I sequence (S). Conserved cysteine residues are pink. The AA substitutions are in color according to their physicochemical characteristics. In red, D (Asp) and E (Glu) are both polar and negatively charged; in bright blue, K (Lys) and R (Arg) are polar and positively charged. Brown and green indicate polar and non-polar residues, respectively.

FIGURE 3. AMINO ACID POLYMORPHISM OF *P. VIVAX* AMA1I-II FROM NICARAGUA, 2012-2013

selection were distinct haplotypes became dominant in a particular geographic area.[46]

All Nicaraguan haplotypes and more than 90% from Latin America were exclusive. The large number of haplotypes shared by Latin American countries suggest that the genetic pool came from a larger population conformed by different past migrations as suggested previously.[24,47] The haplotype network and structure analysis suggest that Latin American haplotypes seemed to belong to three genealogical groups, and *P. vivax ama1*$_{I-II}$ from Nicaragua was discreetly differentiated from parasites in southern Mexico, south America and Iran. The exclusivity of frequent haplotypes also suggests origin-biased diversification, which might have been implicated human migration, immune responses, local vector species, among other factors.[32,43,44,48]

Antibodies induced by natural infections against PvAMA1$_{I-II}$ can block merozoite invasion,[9] and the effectiveness of a vaccine depends on the conservation of certain amino acid residues.[49] Residue variation was mostly detected in or nearby predicted linear peptides participating in B cell epitopes. A recent study reported that polymorphic residues highly frequent in Latin America or other sites, and now detected in Nicaraguan parasites are exposed on the active face of the molecule.[22,32] Furthermore, two polymorphic sites (130-132) are likely involved in RON2.[50] In Brazil, two divergent PvAMA1 haplotypes detected IgG specific antibodies in 88 and 60.6% of patient's sera samples, and some differences in detecting homologous vs heterologous antibody were observed.[32]

A recent study demonstrate that *P. falciparum* genetic pool was restricted to few genotypes in parasites collected from Nicaragua and Honduras (2018-2020),[44] however, *P. vivax* has predominated and produces hypnozoites which serve as a genetic pool reserve.[12,51] The analysis of the secondary source of malaria records from Nicaragua, showed few relapse episodes in 2013-2014, however, relapse episodes increased enormously with transmission specially in the Miskito region.[12] To setback the *P. vivax* genetic reserve in the Miskito region, fighting malaria transmission simultaneously in either country border is mandatory. Further molecular studies in Nicaragua might uncover emerging and persistent *P. vivax* population and to understand the impact of control efforts on malaria transmission. Current results might contribute as baseline for epidemiological surveillance and for vaccine development.

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Declaration of conflict of interests. The authors declare that they have no conflict of interests.

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