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Genetic structure of *Plasmodium vivax* in Nicaragua, a country in the control phase, based on the carboxyl terminal region of the merozoite surface protein-1

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ABSTRACT

Malaria is still a grave public health problem in tropical areas of the world. The greater genetic diversity of *Plasmodium vivax* at geographic sites with less control over infection evidences the importance of genetic studies of these parasites. The present genetic study compares *P. vivax* in Nicaragua, which is still in the control phase, with this species in several other countries. In Nicaragua, *P. vivax* causes over 80% of malaria cases, most occurring in two remote northern regions. *Plasmodium* asexual blood-stage antigens, implicated in reticulocyte invasion, are possible molecular markers for analyzing parasite population genetics and for developing vaccines. The aim of this work was to investigate the genetic structure of *P. vivax* based on the 42 kDa merozoite surface protein-1 (PvMSP-1_{42}), which may represent a sensitive marker for evaluating malaria transmission control. From blood samples of patients with *P. vivax*, we amplified PvMSP-1_{42}, obtained the nucleotide sequences, and compared them to homologous sequences of parasites from other geographic sites, retrieved from the GenBank. The 92 nucleotide sequences of *P. vivax* resulted in the resolution of eight haplotypes, six exclusive to Nicaragua. The great nucleotide diversity ($\pi = 0.020$), the minimal recombination events (Rm = 11), and the *dN–dS* values were similar to other control phase countries. F_{ST} values between parasites were low (0.069) for Nicaragua versus Brazil but higher for Nicaragua versus other regions (0.134–0.482). The haplotype network revealed five lineages: two were very frequent in Nicaragua and closely related to American parasites; three have been detected in multiple geographic sites around the world. These results suggest that *P. vivax* in Nicaragua is a differentiated and genetically diverse population (mainly due to mutation, positive balancing selection and recombination) and that PvMSP-1_{42} may be a sensitive marker for evaluating sustained reduction in malaria transmission and for developing vaccines.

1. Introduction

Plasmodium vivax is widely distributed in Latin-America, Asia, the Middle East and Oceania (Guerra et al., 2010). An estimated 25 million people in the Americas are considered at high risk. Thirteen countries, including Nicaragua, reported a decline of over 75% in laboratory-confirmed malaria cases between 2000 and 2012 (PAHO, 2012). In 2012, the American continent reported 469,380 confirmed malaria cases and 108 malaria deaths (WHO, 2013).

Currently, several Central American countries are in the control phase, while El Salvador, Costa Rica and Belize are in the pre-elimination phase. In the North Atlantic Autonomous Region (RAAN) in Nicaragua, *P. vivax* and *Plasmodium falciparum* are transmitted throughout the year, and on the North Pacific coast transmission is sporadic with periodic outbreaks. The RAAN is adjacent to Honduras, and there is a large territorial extension covering both sides of the border where most malaria transmission takes place. In 2012, Honduras and Nicaragua reported 6434 and 1235 malaria cases, respectively (PAHO, 2012). The current strategy and Plan of Action for Malaria (2011–2025) prioritizes different actions including malaria prevention, surveillance, early case detection, outbreak containment, monitoring and evaluation (Martinelli, 2014). To support these actions, genetic population studies are necessary to identify the haplotypes circulating at each geographic site and determine how they originate, persist and disperse in the region. The degree of genetic diversity and multiple clone infections might determine the transmission intensity and complexity of *Plasmodium* species (Arnott et al., 2012).

Although the Mesoamerican region (from central Mexico to northern Costa Rica) shares ecological and socioeconomical conditions, there might be a restriction on parasite circulation. For instance, the circumsporozoite repeat genotype vk247 highly prevalent in southern

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Mexico (Rodriguez et al., 2000) and Colombia (Hernandez-Martinez et al., 2011) has not been reported in Guatemala (Mendizábal-Cabrera and Padilla, 2006), Nicaragua (Gonzalez-Ceron et al., 2013) or Honduras (Lopez et al., 2012). In the southern part of Mexico bordering with Guatemala, the different *P. vivax* lineages identified show several hybridization events followed by diversification (Cerritos et al., 2014).

Gene markers with high evolutionary dynamics may be useful for explaining how *P. vivax* populations evolve and produce new haplotypes, and for elucidating the changes in haplotype frequency (Barry et al., 2015). The gene encoding for the merozoite surface protein-1 (MSP-1) plays an important role in the invasion of the host blood reticulocyte and is an important vaccine candidate (Ahlborg et al., 2002; Han et al., 2011; Thakur et al., 2008). It is synthesized as a precursor of the 200 kDa protein during schizogony, and its processing produces four polypeptides of approximately 83, 30, 38 and 42 kDa (Kang et al., 2010; Miahipour et al., 2012; Putaporntip et al., 2002). During the invasion process, the C-terminal 42 kDa is further processed into two fragments of 33 kDa (MSP-1_{33}) and 19 kDa (MSP-1_{19}), although only the 19 kDa fragment remains on the merozoite surface (Holder et al., 1992; Kang et al., 2010). In *P. vivax*, it has been reported that PvMSP-1_{33} is highly polymorphic, while PvMSP-1_{19} is better conserved. Both fragments are capable of inducing blocking antibodies, but PvMSP-1_{42} is more immunogenic than PvMSP-1_{19} (Bastos et al., 2007; Wickramarachchi et al., 2007; Zeyrek et al., 2010).

The aim of the present study was to analyze the genetic structure of *P. vivax* in Nicaraguan parasites based on the gene fragment *pvmsp-1_{42}*. The corresponding nucleotide sequences from parasites of other geographic sites, retrieved from the GenBank data base, were used to compare genetic diversity, natural selection and recombination, and to determine the degree of genetic differentiation and the genealogical relationships.

2. Methods

This study was approved by the Ethical Committee of the National Center for Diagnosis and Reference (CNDR) of the Health Ministry in Nicaragua, and the Ethical Committee of the Mexican National Institute of Public Health.

2.1. Blood samples and geographic origin

Following informed consent, infected blood samples were obtained from symptomatic patients seeking malaria diagnosis during 2012 and 2013 in the sentinel laboratory network established by the Nicaraguan Health Ministry at head municipalities: Waspam (50 above sea level (asl), 14° 43.994′ N, 83° 56.458′ W), Rosita (66asl, 13° 52.004′ N, 84° 23.467′ W), Puerto Cabezas (16 asl, 14° 1.828′ N, 83° 23.089′ W), Bonanza (220asl, 14° 1.800′ N, 84° 35.715′ W), Siuna (200 asl, 13° 44.114′ N, 84° 46.653′ W) and Prinzapolka (22 asl, 13° 30.589′ N, 84° 13.200′ W) in the North Atlantic Autonomous Region (RAAN); Chinandega (65 msnm, 12° 37.474′ N, 87° 7.802′ W) and El Viejo (50 asl, 12° 39.769′ N, 87° 9.958′ W) on the North Pacific side. The diagnosis of *P. vivax* was carried out by microscopic examination of stained thick blood smears, and then patients were asked to donate capillary blood to impregnated filter paper (Whatman #2). After *P. vivax* infected blood samples were dried in silica gel, they were preserved in that state and protected from light until analyzed. Whole genomic DNA was extracted from dried blood spots using a commercially available QIAamp DNA blood Minikit (Qiagen, USA) following the manufacturer's instructions. DNA obtained from three punches (0.5 cm in diameter) was eluted in 50 μL of water and stored at −20 °C until use.

2.2. PCR amplification and sequencing

From total genomic DNA, *pvmsp-1_{42}* was amplified by polymerase chain reaction (PCR) using oligonucleotides: For 5′-GCC GAG GAC TAC GAC AAA G-3′ and Rev 5′-CCT CCA GCT TCC TAA GCT TG-3′. The PCR reaction was prepared as follows: 2.6 μL of each primer (10 pM), 2.6 μL of the dNTP mixture (1.25 mM), 5.1 μL of 10× PCR buffer, 1.3 μL of $MgSO_4$ (50 mM), 0.2 μL Taq Platinum DNA polymerase (Invitrogen Corporation, Carlsbad, CA), and 4 μL of extracted DNA for a final volume of 50 μL. The PCR reaction conditions were as follows: 5 min at 95 °C, 1 min at 95 °C, 1 min at 60 °C, and 75 s at 72 °C for 35 cycles; afterwards, there was a final extension of 72 °C for 10 min, all run in a MyCycler (BioRad, Hercules, CA, USA).

The amplified products were examined in agarose gels at 1%, and stained with 0.2 μg/mL ethidium bromide using an electrophoresis chamber Midicell primo (Thermo EC330, New York, USA). The 100 bp ladder (Invitrogen Corporation, Carlsbad, CA, USA) was used as molecular marker. The amplified PCR product was purified using a MiniElute PCR Purification Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The purified products were Sanger sequenced using forward and reverse primers at the High Throughput Genomics Unit, Department of Genome Sciences, University of Washington, Seattle, WA, USA. The quality of pherograms with the forward and reverse nucleotide sequences was verified manually and by using Bioedit v7.1.3 software. The consensus sequences obtained for each gene fragment were submitted to the NCBI-Gen Bank [accession numbers: KR871926–KR872017].

2.3. Data analysis

The nucleotide sequence of *pvmsp-1_{42}* for the Sal 1 strain (XM_001614792.1) was used as a reference. For genetic analysis, nucleotide sequences from other geographic sites were extracted from the NBCI, GenBank. For South Korea (SK), n = 200: JQ446312–JQ446322 (Kang et al., 2012), HQ171934 − HQ171941 (Han et al., 2011), AF435635–AF435638. For Vanuatu (VAN), n = 2: AF435632, and AF435634 (Putaporntip et al., 2002). For Turkey (TUK), n = 30: AB564559–AB564588 (Zeyrek et al., 2010). For Thailand (THL), n = 93: AF435595, AF435615 (Putaporntip et al., 2002), GQ890917–GQ890974 (Jongwutiwes et al., 2010), AF199393–AF199404, and AF199408–AF199410 (Putaporntip et al., 2000). For Singapore (SNG), n = 50: GU971656 − GU971705 (Ng et al., 2010). For India–Bangladesh (IND–BNG), n = 35: EU430452–EU430479, KF612323; AF435639 (Putaporntip et al., 2000), and AF435616–AF435620 (Putaporntip et al., 2002). For Brazil (BRZ), n = 11: AF435622–25,27,29,30,31, and AF199405,6,7 (Putaporntip et al., 2000). For Sri Lanka (SLK), n = 106: AJ292349–AJ292359, and GU175174–GU175268 (Dias et al., 2011). For Myanmar (MYN), n = 28: JX490129–JX490156. For China (CHN), n = 2: JX993754–JX993755 (Zhou and Chen, unpublished). For Cambodia (CAM), n = 27 (Parobek et al., 2014). For Mexico (MEX), n = 35:KP759850-KP759884 (Gonzalez-Ceron et al., unpublished).

2.4. Population genetic analysis

The genetic diversity was measured by calculating π and θ indexes for the complete DNA sequence and by using 100 pb windows assuming different levels of variation across the *pvmsp-1_{42}* fragment. The mean of pairwise nucleotide changes detected along the sequences (using the Jukes–Cantor correction) is represented by π (Nei and Li, 1979), while θ is the number of segregating sites in a group of sequences (Watterson, 1975). The number of polymorphic sites (S) and haplotypes (H) and the minimal number of recombination events (Rm) were calculated. These analyses were computed using DnaSP software (Librado and Rozas, 2009).

To test whether positive selection shaped the evolution of the *pvmsp-1_{42}* subfragment in Nicaraguan parasites and to compare them to those from other geographic sites, the number of synonymous (*s*) and non-synonymous (*ns*) nucleotide changes and the difference between the rate of non-synonymous versus synonymous changes (*dN–dS*) were determined by using the Nei-Gojobori proportion method