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Frequency of *Plasmodium vivax* circumsporozoite protein genotypes in humans and anopheline mosquitoes in an endemic area of southeastern Pará State, Brazil

Frequência de genótipos da proteína circunsporozoíta de *Plasmodium vivax* em seres humanos e mosquitos anofelinos em área endêmica da região sudeste do Estado do Pará, Brasil

Frecuencia de genotipos de la proteína circunsporozoíta de *Plasmodium vivax* en seres humanos y mosquitos *Anopheles* en área endémica de la región sudeste del Estado de Pará, Brasil

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ABSTRACT

The objective of this study was therefore to investigate the frequency of circumsporozoite protein (CSP) genotypes in human blood and their correlation with parasitemia, as well as to evaluate the presence of these genotypes in *Anopheles* in the Municipality of Goianésia do Pará, an endemic area of southeastern Pará State, Brazil from 2012-2013. Blood samples were collected from 118 patients with *Plasmodium vivax* and 369 anopheline mosquitoes. The CSP gene was genotyped using the polymerase chain reaction/restriction fragment length polymorphism technique, and the infectivity of the anophelines was determined using ELISA. Parasitemia ranged from 5-70,000 parasites/mm^3, and the three genotypes (VK210, VK247, and *P. vivax*-like) were detected both in single and mixed infections. No sample exhibited mixed infection with all three genotypes. The most frequent genotype was VK210 followed by VK247 and the latter associated with the highest parasitemia values ($p < 0.0001$). Among the identified mosquitoes, only 11 specimens were infected; of the seven *Anopheles darlingi* specimens four were infected with *Plasmodium falciparum*, two with VK210, and one with VK247. The three *Anopheles albitarsis* specimens were infected with VK247, and one *Anopheles nuneztovari* specimen was infected with VK210. The VK210 genotype continues to be the most prevalent in southeastern Pará; however, a new evidence shows the adaptation of VK247. The species *An. darlingi*, *An. albitarsis*, and *An. nuneztovari* play an important role in the transmission of CSP genotypes in the study area. This finding may be a public health concern due to the possibility of resurgence of *P. vivax* malaria epidemics in susceptible communities.

Keywords: Malaria; *Plasmodium vivax*; Genotyping Techniques; *Anopheles*.

INTRODUCTION

Plasmodium falciparum, *Plasmodium malariae*, and *Plasmodium vivax* are the main species that cause malaria in Brazil, and the latter parasite is responsible for 80% of the malaria cases registered. Furthermore, 99.7% of reported cases are detected in Brazilian Amazon[1,2,3]. *P. vivax* malaria rarely progresses to severe disease; however, studies reinforce the idea that this species may be involved in clinical complications and fatalities, making it a matter of public health concern[4,5].

P. vivax sporozoites are covered by circumsporozoite protein (CSP), which is involved in hepatocyte invasion mechanisms and is highly immunogenic[6]. Genetic variants exist in the central repeat domain of the CSP gene that are characterized as three genotypes, termed VK210, VK247 and *P. vivax*-like[7,8,9]. Serological and molecular studies have shown that these genotypes circulate nationwide, mainly in Brazilian Amazon[10,11,12,13,14]. The VK210 genotype is the most common in the investigated areas, and the VK247 and *P. vivax*-like genotypes had been found only in mixed infections with other genotypes[13,14]. However, a decade after the first genotypic study in five different states of

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Brazilian Amazon, this distribution profile has changed, with the VK247 and *P. vivax*-like genotypes also detected as single infections in a single area of Pará State[15].

Human malaria is transmitted by mosquitoes of the genus *Anopheles*, which includes 465 recognized species and more than 50 unidentified species[16]. Transmission occurs in a human-vector-human manner, and the main malaria-transmitting species in Brazil is *Anopheles darlingi*, although other anopheline species have been detected to be naturally infected with *P. vivax* and/or *P. falciparum* and can therefore act as secondary vectors[17,18,19,20,21,22].

The sexual reproduction of the parasite occurs during the cycle of the vector, and genetic changes in the plasmodium may occur during this stage[23]. Additionally, the variability of the repeat region of the CSP gene has also been used to determine infectivity of *Plasmodium* species in *Anopheles*[24,25]. Previous studies using monoclonal antibodies against VK210 and VK247 CSP in non-endemic areas of the Atlantic Forest, São Paulo State, detected the presence of *Anopheles* infected with both genotypes[26]. In endemic areas of Brazilian Amazon, 15% of *Anopheles* were found to be infected with this genotype in Acre State using anti-*P. vivax*-like antibodies[27]. In Pará State, it has been observed that the *Anopheles aquasalis* and *An. darlingi* species are susceptible to infection by VK210 and VK247[28]. This demonstrates the importance of studying the genetic diversity of *P. vivax* in endemic areas. Thus, it is essential to investigate the epidemiology of *P. vivax* CSP genotypes and to detect naturally infected mosquitoes to understand immunity mechanisms as well as differences in parasite load, drug resistance, transmission dynamics, and natural selective pressure of the parasite among different local communities[29,30]. The objective of the current study was to investigate the frequency of CSP genotypes in human blood and its correlation with parasitemia, as well as to evaluate the presence of these genotypes in *Anopheles* in an endemic area of southeastern Pará State, Brazil.

MATERIALS AND METHODS

STUDY AREA

This descriptive epidemiological study was conducted in Municipality of Goianésia do Pará (03°50'33"S, 49°05'49"W) located in Pará State southeastern mesoregion and Paragominas microregion, bordering the Municipalities of Breu Branco, Novo Repartimento, Dom Eliseu, Ipixuna do Pará, Jacundá, and Rondon do Pará. This region has a land area of 7,021 km^2 and an estimated population of 29,161 inhabitants, of whom 52% are over 14 years old. This area has population density of 4.5 inhabitants/km^2, and its distance from Belém, the State capital, is approximately 350 km[31].

STUDY POPULATION

Patients

The sample was formed by patients living in the localities of Santa Paula, Rouxinol, and Ararandeua that concentrate the highest number of malaria cases in Goianésia do Pará Municipality[32] and which sought the healthcare services in their own localities during 2012-2013 and had a positive diagnosis by *P. vivax*. Thus, a total of 118 blood samples were collected from patients with *P. vivax* diagnosed by Thick Smear technique and confirmed by nested PCR as described by Kimura et al[33]. The following individuals were excluded from the study: pregnant women, children under 15 years old, and patients who were infected with other *Plasmodium* species, displayed mixed infection, or who did not agree to participate. This study was approved by the Ethics Committee for Research on Human Beings of Instituto Evandro Chagas under number 00141/10 – CAAE: 0014.0.0.072.000-10, and participants signed an informed consent form.

Mosquitoes

The adult mosquitoes were collected from the same localities as the patients, and three collections/year (one of 12 h and two of 4 h by locality) were performed. The mosquitoes were identified by dichotomous keys[18,34], and their natural infectivity was determined using ELISA protocol[35]. This method detects natural infection in mosquitoes caused by *P. falciparum*, *P. malariae*, and the VK210 and VK247 genotypes of *P. vivax*.

P. vivax GENOTYPING

DNA was extracted from peripheral blood samples using the Easy-DNA™ (Invitrogen, Carlsbad, California, USA) and the QIAamp® DNA Blood Kit (Qiagen, Inc., Chatsworth, California, USA) extraction/purification kits. The *P. vivax* CSP genotypes were determined using polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP)[36].

STATISTICAL ANALYSIS

Differences between the *P. vivax* CSP genotypes were tested using the Mann-Whitney and Kruskal-Wallis tests. The significance level for statistical tests was set at $p < 0.05$.

RESULTS

P. vivax CSP GENOTYPES

Of the 118 blood samples collected and diagnosed as infected with *P. vivax*, 110 were successfully amplified and genotyped. The three genotypes were detected both as single and mixed infections (Table 1). There were no mixed infections containing all three genotypes. The most frequent genotype in Goianésia do Pará Municipality was VK210, followed by VK247. Genotyping revealed that 75.5% of the samples were single infections, with only 24.5% mixed infections.

RELATIONSHIP BETWEEN PARASITEMIA AND CSP GENOTYPES

Parasitemia ranged from 5-70,000 parasites/mm^3 (geometric mean ± standard deviation: 1,299.95 ± 8:51 parasites/mm^3). The mean parasitemia was 569.42 parasites/mm^3 (± 10:52) for the VK210 genotype, 2,563.76 parasites/mm^3 (± 4.13) for the VK247 genotype, and 3,307 parasites/mm^3 (± 5.64) for mixed VK210 and VK247 infection.

Table 1 – Distribution of *P. vivax* genotypes in Municipality of Goianésia do Pará, Pará State, Brazil, 2012-2013

	Single infections n (%)			Mixed infections n (%)			
Locality	1	2	3	1 + 2	1 + 3	2 + 3	1+2+3*
Goianésia do Pará	52 (47.3%)	28 (25.5%)	3 (2.7%)	25 (22.7%)	1 (0.9%)	1 (0.9%)	–

*1: VK210; 2: VK247; 3: *P. vivax*-like. Conventional sign used: – Numeric data not equal to zero due to rounding.

The *P. vivax*-like genotype was excluded from the analysis due to its low frequency. A significant association was observed between the presence or absence of VK247 in the infections and parasitemia. Regardless of whether single or mixed infection was observed, individuals infected with this genotype were associated with the highest parasitemia values (Mann-Whitney, $p < 0.001$) compared with those without the VK247 genotype (Figure 1).

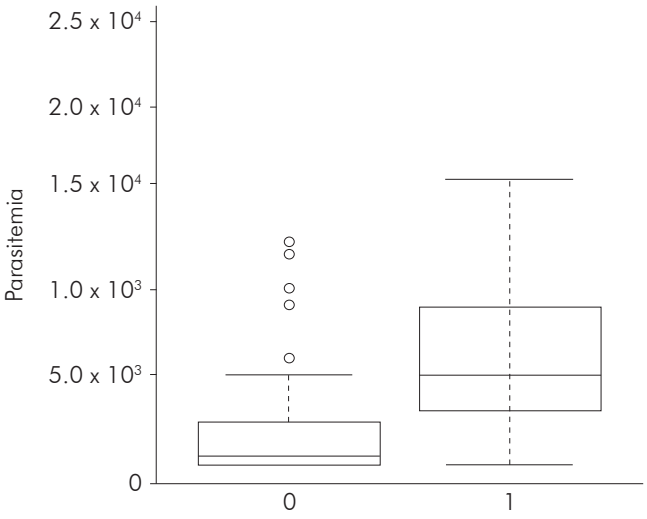

Figure 1 – Statistical association between the VK247 genotype and parasitemia significant difference ($p < 0.001$) between individuals with the VK247 (1) variant and those without the VK247 (0) genotype with parasitemia (number of parasites/mm^3)

NATURAL INFECTION OF MOSQUITOES

Of the 369 adult anopheline species found in that Municipality, namely *An. darlingi, An. albitarsis* s.l., *Anopheles triannulatus*, *Anopheles strodei*, *Anopheles galvaoi*, and *Anopheles nuneztovari*, only 11 specimens were infected: seven *An. darlingi* (four with *P. falciparum*, two with VK210, and one with VK247); three *An. albitarsis* s.l. (with VK247); and one *An. nuneztovari* (with VK210).

DISCUSSION

The municipal government of Goianésia do Pará has implemented diagnostic training measures and treatment adherence campaigns, and has introduced insecticide-treated nets to decrease the time between onset of symptoms and the start of treatment, and to reduce or prevent human-vector contact. Due to the significant drop in the number of cases reported in recent years (2011 – 2,856 registered cases, 2012 – 1,136, and 2013 – 192), it is believed that these measures are effective. However, it should be emphasized that clarification of the geographic distribution of genotypes and of mosquitoes naturally infected with different *Plasmodium* species can provide new opportunities for understanding vector/parasite interaction and the local epidemiology of malaria.

The detection of three *P. vivax* CSP genotypes circulating in the municipality confirms previous evaluations conducted in Brazilian Amazon in the States of Pará, Rondônia, Amapá, Acre, and Mato Grosso[13,15]. However, there was no evidence of mixed infections with the three CSP genotypes in Goianésia do Pará Municipality, which contrasts with previous data from Novo Repartimento in Pará State, a Municipality located in the same mesoregion of southeastern Pará[15]. The VK210 genotype remains the most prevalent, most likely because of the great susceptibility of the *An. darlingi* vector, which is the most abundant in the region, to this variant[28]. The *P. vivax*-like genotype had a frequency of only 2.7% of the genotyped samples; this low frequency could be due to its recent introduction into the region or due to differences in the development of this genotype in the vectors present in the area[12,13]. Interestingly, the VK247 genotype appeared for the first time as a single infection ten years ago in Municipality of Novo Repartimento[15]. In this study, this genotype was also found to have naturally infected humans in isolation and was observed in all mosquitoes of the species *An. albitarsis* and in one *An. darlingi* specimen.

Another relevant observation is the association of patients infected with the VK247 genotype and the high parasitemia. At the end of the 1990s, Machado and Póvoa[13] observed that the VK210 genotype was associated with high levels of parasitemia in the City of Belém (350 km from Goianésia do Pará). This profile change may be related to the evolution of *P. vivax*. González-Cerón et al[37] observed in samples of *P. vivax* from Mexico, Nicaragua, and Peru that the genetic diversity of the CSP gene is restricted mainly to the central repeat domain and 3'-terminal portion. These authors also stressed that this variation occurs due to changes in the type of nucleotides and number of repeats of the repeat region. The authors noted that the VK247 genotype displays high identity at the

carboxy-terminal end with the reported sequence for *Plasmodium cynomolgi* CSP, and it is possible that the repeat region of VK247 is more stable than VK210. Despite of the association between high parasitemia and the VK247 genotype in this study can not be causal, the data obtained here contribute to the understanding of the molecular epidemiology of *P. vivax* in Brazil and suggest that the introduction of the VK247 and VK210 genotypes may have occurred at different times according to the endemic area of Brazilian Amazon.

Naranjo-Díaz et al[38] showed evidence that the importance and distribution of human malaria vectors may vary depending on location. In fact, these authors observed *An. nuneztovari* infected with the VK210 and VK247 genotypes in Colombia, showing its importance for malaria transmission in areas with anthropic intervention[38]. This pattern was also observed in Brazil, where *An. darlingi* was found to be infected only with VK247 in District of Lourenço, Amapá State[39]. Moreover, in Municipality of Marabá, another mesoregion of southeastern Pará State, *An. darlingi* was found to be infected by *P. falciparum* and VK247, whereas *An. albitarsis* was infected with both VK210 and VK247 genotypes[40]. In turn, there was a 1.22% infection rate in Acre State for VK247 in the *Anopheles oswaldoi* mosquito[24], the main malaria vector in the region[41]. In this descriptive study, the VK247 CSP genotype was detected in one mosquito *An. darlingi* and in three *An. albitarsis*, corresponding to 36.4% (4/11) of infection. As these two species play an important role in malaria transmission in Brazil and because this variant has been detected separately in different locations in this mesoregion of Pará, it was hypothesized in this study an increase in the number of cases of this genotype in the region. This new evidence should be investigated in other locations where different species of anopheline mosquitoes are the main vector, such as *An. aquasalis* in Amazon Region[20] and *Anopheles bellator* and *Anopheles cruzii* in outside Amazon region[3], to determine whether other species may facilitate the transmission process by carrying different CSP genotypes.

In general the distribution profile of CSP genotypes in other countries of Latin America is different from that observed in Brazil. Thus, the possibility of an outbreak of vivax malaria cases by VK247 in Brazil cannot be ruled out. This could lead to higher parasitemia as well as more severe clinical conditions. Although the VK247 variant is ancestral and its CSP repeat region is more stable than that of VK210[42], the distribution of this genotype can be quite heterogeneous in other regions of Brazilian Amazon, most likely due to the absence or presence of VK247 polymorphisms in different locations and this fact may be related to changes in the geographical distribution profile of this variant[37,43].

Another hypothesis that reinforces this idea is related to the immune response. The VK210 genotype is more immunogenic than VK247, which leads to high immune responses against this genotype and facilitates the selection process of VK247 sporozoites. The possible presence of anti-VK210 antibodies may therefore limit the production of VK210 sporozoites and result in a lower frequency of this genotype in some species of mosquito[44]. This may also impact the parasite load in infected individuals. These variations can occur due to physiological incompatibility between host/parasite, defense mechanisms of the mosquito such as the destruction of ookinetes blocking the development of oocysts in the mosquito, or ecological and evolutionary factors that can contribute to the divergence or restriction of gene flow among parasite strains adapted to different local vectors[45,46,47]. It is noteworthy that the detection of CSP in vectors only occurs when the parasite reaches the sporoblast stage[48,49], and for this reason it is possible that the absence of CSP expression in a parasite or even the vector's immune response against this protein may have influenced the detection of genotypes in mosquitoes.

CONCLUSION

Although the VK210 genotype remains the most prevalent in Brazil, a new evidence reveals a strong adaptation of the VK247 variant in southeastern Pará, as well as the association of this genotype with high parasitemia. The species *An. darlingi*, *An. albitarsis* and *An. nuneztovari* play an important role in the transmission of these genotypes in the study area. However this is the second time that *An. albitarsis* has been found in a natural infection with the VK247 genotype in Pará State, and it may be the main vector in the spread/selection of this genotype. This may therefore present a public health concern because it raises the possibility of a resurgence of vivax malaria epidemics in susceptible communities.

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RESUMO

O objetivo deste estudo foi investigar a frequência de genótipos da proteína circunsporozoíta (CSP) em sangue humano e sua correlação com a parasitemia, bem como avaliar a presença desses genótipos em *Anopheles* no Município de Goianésia do Pará, uma área endêmica do sudeste do Estado do Pará, Brasil, de 2012 a 2013. Amostras de sangue foram coletadas de 118 pacientes com *Plasmodium vivax* e 369 mosquitos anofelinos. O gene da CSP foi genotipado usando-se a reação em cadeia da polimerase/polimorfismo de comprimento de fragmento de restrição, e a infectividade dos anofelinos foi determinada pelo ELISA. A parasitemia variou de 5-70.000 parasitas/mm^3, e os três genótipos (VK210, VK247 e *P. vivax*-like) foram detectados tanto em infecções simples quanto em mistas. Nenhuma amostra apresentou infecção mista com todos os três genótipos. O genótipo mais frequente foi o VK210, seguido pelo VK247 e o último associado com os valores mais altos de parasitemia ($p < 0,0001$). Entre os mosquitos identificados, somente 11 espécimes foram infectados; de sete espécimes *Anopheles darlingi*, quatro foram infectados por *Plasmodium falciparum*, dois por VK210 e um por VK247. Os três *Anopheles albitarsis* foram infectados por VK247 e um *Anopheles nuneztovari* por VK210. O genótipo VK210 continua sendo o mais prevalente no sudeste do Pará; entretanto, novas evidências indicam a adaptação do VK247. Os espécimes *An. darlingi*, *An. albitarsis* e *An. nuneztovari* desempenham um importante papel na transmissão dos genótipos CSP na área de estudo. Essa descoberta pode ser um problema de saúde pública devido à possibilidade de ressurgimento de epidemias de malária por *P. vivax* em comunidades suscetíveis.

Palavras-chave: Malária; *Plasmodium vivax*; Técnicas de Genotipagem; *Anopheles*.

Frecuencia de genotipos de la proteína circunsporozoíta de *Plasmodium vivax* en seres humanos y mosquitos *Anopheles* en área endémica de la región sudeste del Estado de Pará, Brasil

RESUMEN

El objetivo de este estudio fue de investigar la frecuencia de genotipos de la proteína circunsporozoíta (CSP) en sangre humana y su correlación con la parasitemia, bien como evaluar la presencia de esos genotipos en *Anopheles* en el Municipio de Goianésia do Pará, un área endémica del sudeste del Estado de Pará, Brasil, de 2012 a 2013. Se recolectaron muestras de sangre de 118 pacientes con *Plasmodium vivax* y 369 mosquitos *Anopheles*. El gen de la CSP fue genotipado usando la reacción en cadena de la polimerasa/polimorfismo de longitud de fragmento de restricción, y la infectividad de los *Anopheles* se determinó por el método ELISA. La parasitemia varió de 5-70.000 parásitos/mm^3, y los tres genotipos (VK210, VK247 y *P. vivax*-like) fueron detectados tanto en infecciones simples como en mixtas. Ninguna muestra presentó infección mixta con todos los tres genotipos. El genotipo más frecuente fue el VK210, seguido por VK247 y el último asociado con los valores más altos de parasitemia ($p < 0,0001$). Entre los mosquitos identificados, solamente 11 especímenes fueron infectados; de siete especímenes *Anopheles darlingi*, cuatro fueron infectados por *Plasmodium falciparum*, dos por VK210 y uno por VK247. Los tres *Anopheles albitarsis* fueron infectados por VK247 y un *Anopheles nuneztovari* por VK210. El genotipo VK210 sigue siendo el más prevalente en el sudeste de Pará; mientras que nuevas evidencias indican la adaptación del VK247. Los especímenes *An. darlingi*, *An. albitarsis* y *An. nuneztovari* desempeñan un importante papel en la transmisión de los genotipos CSP en el área de estudio. Ese hallazgo puede ser un problema de salud pública debido a la posibilidad de resurgimiento de epidemias de malaria por *P. vivax* en comunidades susceptibles.

Palabras clave: Malaria; *Plasmodium vivax*; Técnicas de Genotipaje; *Anopheles*.

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