



Article/Artigo

Molecular epidemiology of hepatitis B virus among the indigenous population of the Curuçá and Itaquai Rivers, Javari Valley, State of Amazonas, Brazil

Epidemiologia molecular do vírus da hepatite B em população indígena em torno dos Rios Curuçá e Itaquai, Vale do Javari, Estado do Amazonas, Brasil

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ABSTRACT

Introduction: Hepatitis B virus (HBV) infection is one of the most serious public health problems in the world. In Brazil, HBV endemicity is heterogeneous, with the highest disease prevalence in the North region. **Methods:** A total of 180 samples were analyzed and subjected to polymerase chain reaction (PCR) and semi-nested PCR of the HBV S-gene, with the aim of determining the prevalence of HBV-DNA (deoxyribonucleic acid) in indigenous groups inhabiting the areas near the Curuçá and Itaquai Rivers in the Javari Valley, State of Amazonas, Brazil. **Results:** The prevalence of the HBV-DNA S-gene was 51.1% (92/180). The analysis found 18 of 49 (36.7%) samples from the Marubo tribe, 68 of 125 (54.4%) from the Kanamary, and 6 of 6 (100%) from other ethnic groups to be PCR positive. There was no statistically significant difference in gender at 5% ($p=0.889$). Indigenous people with positive PCR for HBV-DNA had a lower median age ($p<0.001$) of 23 years. There was no statistical difference found in relation to sources of contamination or clinical aspects with the PCR results, except for fever ($p<0.001$). The high prevalence of HBV-DNA of 75% (15/20) in pregnant women ($p=0.009$) demonstrates an association with vertical transmission. **Conclusions:** The results confirm the high prevalence of HBV-DNA in the Javari Valley, making it important to devise strategies for control and more effective prevention in combating the spread of HBV.

Keywords: Hepatitis B virus. Diagnosis of HBV-DNA by PCR. Prevalence of HBV-DNA. Indigenous populations.

RESUMO

Introdução: A infecção pelo vírus da hepatite B (VHB) é um dos mais sérios problemas de saúde pública do mundo. No Brasil, a endemicidade do VHB é heterogênea, sendo a doença mais prevalente na região norte do país. **Métodos:** Neste estudo, foram investigadas 180 amostras de sangue por meio da técnica da reação em cadeia da polimerase (PCR) e PCR semi-nestada para o vírus da hepatite B, gene S, com o intuito de determinar a prevalência do DNA (ácido desoxirribonucleico) do vírus da hepatite B em povos de etnias indígenas habitantes dos Rios Curuçá e Itaquai no Vale do Javari, Estado do Amazonas, Brasil. **Resultados:** A prevalência encontrada para o DNA-VHB gene S foi de 51,1% (92/180). Entre as amostras positivas 18/49 (36,7%) pertenciam à etnia Marubo, 68/125 (54,4%) à Kanamary e 6/6 (100%) a outras etnias. Não houve diferença significativa ao nível de 5% em relação ao gênero ($p=0,889$). Os indígenas com PCR positiva para DNA-VHB apresentaram mediana de idade menor de 23 anos ($p<0,001$). Não foi constatado nenhuma diferença estatística em relação às fontes de contágio e o resultado da PCR, como também aos aspectos clínicos, com exceção da febre ($p<0,001$). A alta prevalência do DNA-VHB de 75% (15/20) em gestantes ($p=0,009$) demonstra associação com a transmissão vertical. **Conclusões:** Os resultados comprovam a alta prevalência do DNA-VHB no Vale do Javari, tornando-se importante traçar estratégias de controle e prevenção mais eficazes no combate à disseminação do VHB.

Palavras-chaves: Vírus da hepatite B. Diagnóstico do DNA-VHB PCR. Prevalência do DNA-VHB. População indígena.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem. Although it is preventable through vaccination, an estimated two billion people are infected around the world, with more than 350 million showing serological markers of active infection¹.

The Brazilian Amazonian region is characterized as having one of the highest levels of HBV occurrence in the world, with the basins of the Juruá-Purus and Middle-Solimões Rivers showing the most elevated rates²⁻⁴. Seroepidemiological studies among the indigenous population of this area have shown a rate of 9.7% for hepatitis B surface antigen (HBsAg) in chronic carriers⁵.

Research has demonstrated that in some cases serological markers are insufficient in detecting viral activity and that, in these instances, molecular tests are more sensitive and specific^{6,7}. Deoxyribonucleic acid (DNA) detection of the virus is considered the most reliable evidence that replication and infectiousness exist⁸.

In the Javari Valley, the presence of HBV constitutes a particularly serious public health problem, because the indigenous population lives in isolation, often at great distances from health centers that can diagnose the disease. The non-indigenous population living in the Valley has provoked numerous disease epidemics, beyond simply hepatitis. Studies in this region are scarce, and many cases are not reported.

This study sought to determine the prevalence of HBV-DNA among indigenous peoples in the Javari Valley of the Brazilian State of Amazonas, as well as to show the epidemiological aspects of HBV prevalence among this population.

METHODS

A descriptive cross-sectional study was undertaken among indigenous ethnic groups that inhabit the Curuçá and Itaquai river basins in the

Javari Valley (Amazonas, Brazil). The prevalence of HBV-DNA was estimated using polymerase chain reaction (PCR).

The indigenous lands of the Javari Valley are located in the municipality of Atalaia do Norte, near the western limits of Amazonas on the border with Peru (Figure 1). The study population was composed of inhabitants of the indigenous communities of São Sebastião (S 05°51'37.3" W 072°04'53.2"), Volta Grande (S 05°37'21.6" W 072°03'26.8"), and Pedro Lopes (S 04°32'22.6" W 071°24'04.3") on the Curuçá River, and the communities of Massapé (S 06°14'26.8" W 070°36' 32.6"), Remancinho (S 05°57'55.5" W 070°37'07.9"), and Bananeira (S 05°56'28.4" W 070°34'52.1") on the Itaquai River.

Sample composition

Allowing for a 5% sample error and a 15% error on HBV prevalence, a sample size of 180 was calculated. A stratified random sampling method was used, and inclusion into the sample was based simply on the order of arrival at the collection site, contingent upon the consent of the indigenous community.

Participants in the study included young and adult indigenous persons of both sexes, all of whom provided informed consent. In the case of minors, consent was given by a parent or guardian.

Samples were collected by a health technician appointed by the National Health Foundation in Atalaia do Norte/AM upon confirmation of the informed consent forms translated from the Kanamary, Kulina, Marubo, and Mayoruna languages used in these communities. Venous blood (5ml) was collected using disposable

syringes and needles, and was stored in a Vacutainer tube containing ethylenediaminetetraacetic acid (EDTA).

The indigenous communities of São Sebastião, Volta Grande, and Pedro Lopes on the Curuçá River were visited in September 2009; the samples from the communities of Massapé, Remancinho, and Bananeira on the Itaquai were collected in March 2010.

A form was used to obtain data on the different variables in the study: age, sex, ethnic group, indigenous community, possible sources of infection, and clinical aspects.

The molecular laboratory procedures were carried out in the virology laboratory (Coordination of Health Sciences Research of the National Institute for Research in the Amazon).

Laboratory procedures

The samples were centrifuged at 2,000rpm for 5min for plasma separation, then identified with a registration number and stored at -20°C. For each laboratory phase, HBV-DNA positive and negative controls were used.

DNA extraction was completed using the phenol-chloroform method as described by Karasawa et al.⁹ and modified by Oliveira¹⁰.

The method for identifying viral DNA was PCR, followed by semi-nested PCR, with specific initiators that amplified segments of the HBV S-gene. For PCR the initiators 2821(+) 5'-GGGTCACCATATTCTTGGGAACA-3' and 783(-) 5'-CTC ACGATGCTGTACAGACTT-3' were used, which amplified the whole S-gene genomic segment, creating a product of 1,200bp.

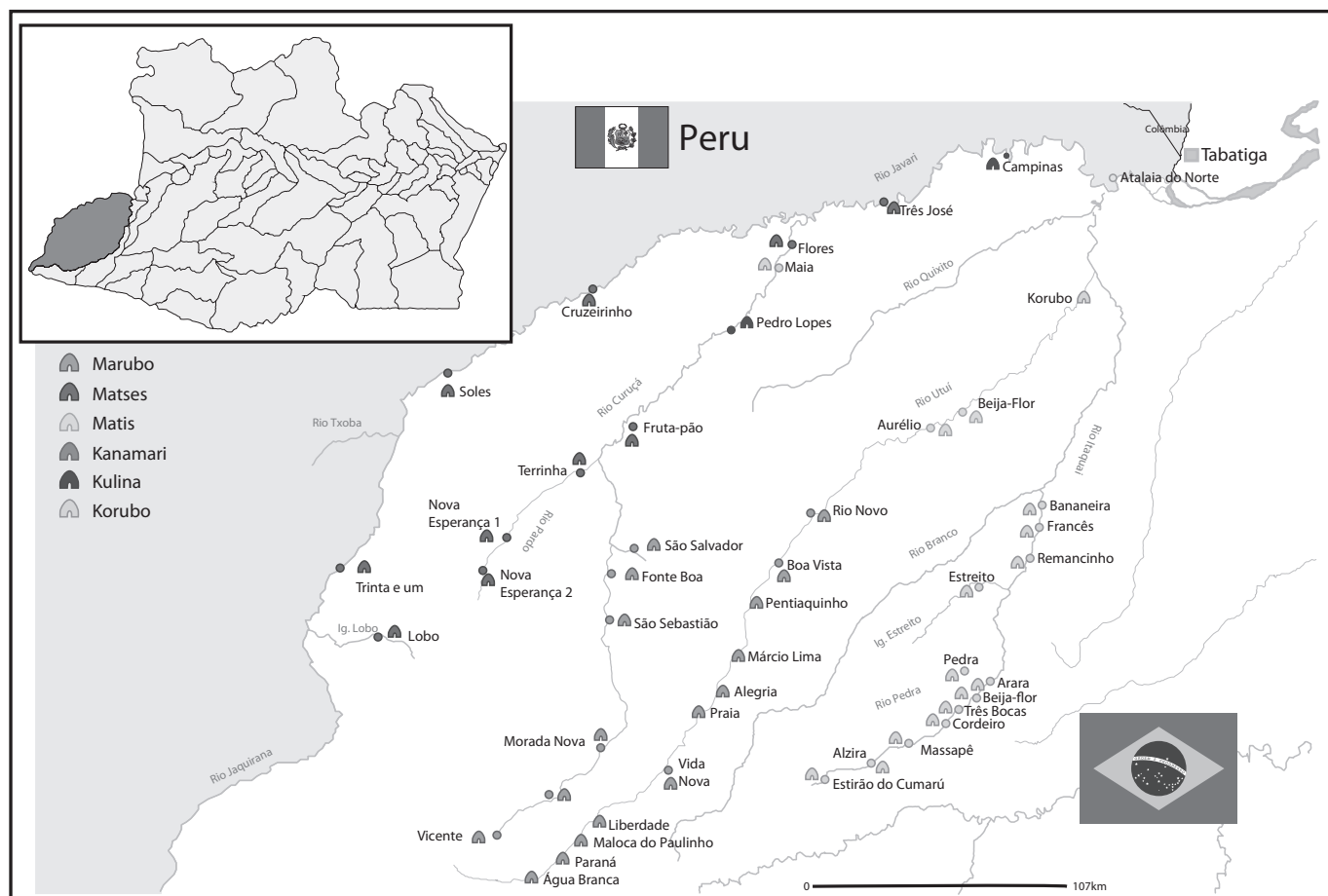


FIGURE 1 - Map of Javari Valley, State of Amazonas, Brazil (Adapted of FUNASA, 2008)

The result of the first PCR reaction was submitted to semi-nested PCR, with the substitution of the 2821(+) initiator with P1(+)
5'-TGCCTCTCACAT CTCGTCAA-3', giving a product of 680bp¹⁰.

The amplification products were submitted to agarose gel electrophoresis (1.5%) in a tris-borate-EDTA buffer (pH 8.3), at 100 volts for one hour. A standard of 100bp (Invitrogen) was used, stained with ethidium bromide (1.0µg/mL). DNA fragments were visualized using a transilluminator and were registered with digital equipment.

Analysis of the results

The absolute and relative frequencies were calculated for the data collected. For prevalence estimation, a confidence interval of 95% (CI95%) was used. For the age analysis, medians and quartiles (Q_2) were selected because normal distribution was not seen at 5% significance. In the comparison of the double entry tables, a Pearson chi-square test was used, and where it was not possible to apply, the Fisher exact test was employed. For the comparison of median age relative to PCR results, the non-parametric Mann-Whitney test was applied¹¹.

The software utilized in the analyses was Epi-Info version 3.5.1 for Windows⁸, and a 5% level of significance was designated for all tests.

Ethical considerations

This research is part of a wider study known as Molecular Epidemiology of Hepatitis B Virus in the Indigenous Population of Javari Valley, State of Amazonas, approved by the National Commission for Research Ethics on April 24, 2009 (registry no. 15276, evaluation no. 216/2009), and by the Research Ethics Committee of the National Institute of Amazon Research on December 5, 2008 (protocol no.189/08).

RESULTS

A total of 180 samples taken from the areas adjacent to the Curuçá and Itaquai Rivers in the Javari Valley were analyzed. Of these samples, 49 (27.2%) came from members of the Marubo ethnic group, 125 (69.4%) from the Kanamary, and 6 (3.3%) from other groups. The study population consisted of 87 (48.3%) men and 93 (51.7%) women.

The median age of the individuals studied was 27 years, with a range of 11 to 77 years. The age group with the highest frequency was that of 15 to 20 years (20.4%), and quartiles Q_1 - Q_3 were between 20 and 39. A total of 43 participants could not inform us of their age.

Of the 180 samples analyzed, 92 were PCR positive for HBV S-gene DNA, resulting in an estimated prevalence of 51.1% (Figure 2).

Table 1 shows the distribution of PCR positivity for HBV-DNA broken down by ethnic group.

In the tests for the HBV-DNA S-gene, 50.6% of males and 51.6% of females were PCR positive. Table 2 shows the distribution of the socio-demographic data of the positive and negative cases for HBV-DNA. Of the samples studied, no statistically significant difference was found in relation to sex at 5% ($p = .889$). However, when the mean age was compared with PCR results it was observed that PCR-positive indigenous persons showed a lower median age at 23 years ($p < 0.001$) than the PCR-negative group at 33 years.

The frequency distribution of possible sources of infection did not show statistical significance relative to the PCR results at 5% for contact with persons suspected of having hepatitis B ($p = 0.164$).

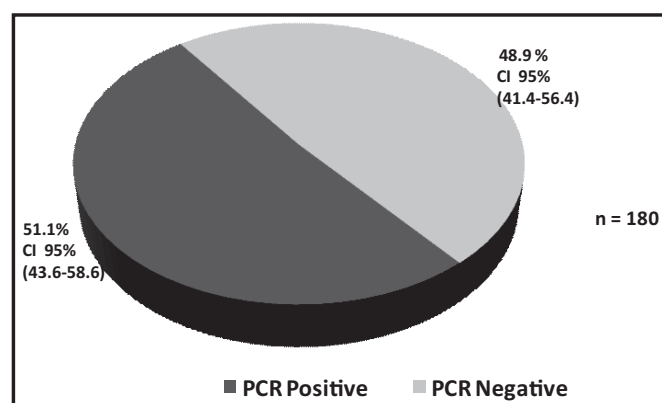


FIGURE 2 - Distribution of PCR results for HBV-DNA in the indigenous population of Javari Valley, State of Amazonas, Brazil.

PCR: polymerase chain reaction; HBV-DNA: hepatitis B virus-deoxyribonucleic acid; CI 95%: confidence interval 95%; n: number.

TABLE 1 - Distribution of participants by frequency of HBV-DNA PCR positivity in relation to the ethnic groups in the indigenous population of Javari Valley, State of Amazonas, Brazil.

Ethnic group	f/n	%	CI 95%
Marubo	18/49	36.7	23.4 - 51.7
Kanamary	68/125	54.4	45.3-63.3
Others	6/6	100.0	-

HBV-DNA: hepatitis B virus-deoxyribonucleic acid; PCR: polymerase chain reaction; f: frequency of PCR positive cases; n: number; CI 95%: confidence interval 95%.

TABLE 2 - Distribution of participants by socio-demographic data relative to the PCR results for HBV-DNA in the indigenous population of Javari Valley, State of Amazonas, Brazil.

Variables	PCR				Total	p
	positive		negative			
	f _i	%	f _i	%		
Gender (n=180)						0.889*
male	44	50.6	43	49.4	87	
female	48	51.6	45	48.4	93	
Age						<0.001**
median	23.0		33.0			
Q_1 - Q_3	18-30		22-44			

PCR: polymerase chain reaction; HBV-DNA: hepatitis B virus-deoxyribonucleic acid; Q_i: quartile; f_i: simple frequency; *Pearson chi-square test; **Mann-Whitney non-parametric test; p-value in bold italics indicates statistical difference at a level of 5%.

The same was true for having a suspected case of hepatitis in the family, tattoos ($p = 0.420$), dental extraction ($p = 0.781$), sharing of undergarments, sharing of toothbrushes ($p = 0.364$), sharing of razors, skin wounds, and frequent departure from the village ($p = 0.271$).

In the group of participants that declared they had recently been febrile, 80% (12/15) were HBV-DNA positive and 20% (3/15) were negative, with a statistical difference at 5% ($p < 0.001$). Among those that presented no fever, 28.6% (12/42) and 71.4% (30/42) presented positive and negative results, respectively, for HBV DNA.

During the study, 20 (21.5%) women indicated they were pregnant, with 75% (15/20) positive and 25% (5/20) negative for HBV-DNA, showing a statistical difference (5%) of $p = 0.009$ in relation to the PCR results. Among those who were not pregnant, 40.4% (21/52) were HBV-DNA positive and 59.6% (31/52) were negative. However, a statistically significant difference was not seen

for having received hepatitis B vaccine ($p=0.748$), or for malaise ($p=0.799$), nausea ($p=0.550$), jaundice, pain in the liver area ($p=0.146$), myalgia ($p=0.552$), arthralgia ($p=0.832$), or darkened urine ($p=0.438$).

As for the distribution by frequency of HBV-DNA positivity for each river and indigenous community of the Javari Valley, 24 (43.6%) of 55 samples from the Curuçá River were positive, with 8 (21.6%) of 37 coming from the indigenous community of São Sebastião, 10 (83.3%) of 12 from Volta Grande, and 6 (100%) of 6 from Pedro Lopes. Of the samples from the Itaquai River, 68 of 125 were positive for HBV DNA, for a 54.4% prevalence. Broken down by community, 32 (57.1%) of 56 were positive in Massapê, 13 (34.2%) of 38 in Remancinho, and 23 (74.2%) of 31 in Bananeira.

DISCUSSION

Epidemiological studies on hepatitis B in Brazil are rare, despite the fact that it is a major public health issue. Its frequency is still underestimated, considering that many infected individuals are asymptomatic and symptomatic infections are underreported¹².

The Amazon region presents the highest endemicity of HBV in Brazil, especially among indigenous peoples^{5,13,14}. However, the epidemiological aspects and the transmission of HBV infection in the Amazon region are not clear, particularly the conditions that favor high prevalence^{3,15}.

The results of this study confirm the high prevalence of HBV-DNA, estimated at 51.1%, in the indigenous communities of the Curuçá and Itaquai Rivers in the Javari Valley of Amazonas state. This information is in line with other findings in which similar prevalence figures were found^{16,17}. Barros-Junior et al.¹⁸ reported a lower prevalence in 51 (17.6%) patients of the Brazilian Amazon. In studies conducted in the States of Bahia and Mato Grosso do Sul the prevalence was 18% and 24.7%^{19,20}, respectively, showing that HBV-DNA prevalence is quite heterogeneous in Brazil.

Oliveira¹⁰, in a study done among outpatients of the Tropical Medicine Foundation in the municipality of Lábrea, Amazonas, found an 85% prevalence of HBV-DNA in patients with serological confirmation of hepatitis B. We believe that this difference in prevalence is due to the fact that the samples were chosen from patients who tested positive for HBsAg, hepatitis B e antigen (HBeAg), or total antibody against hepatitis B core antigen (anti-HBc), as opposed to the present study in which participants were included randomly. According to Fonseca²¹, serological markers assist in the diagnosis of HBV infection.

A number of works in the literature have used serological markers in indigenous peoples^{2,3,5,15,22}. All these studies also report a high prevalence of HBV.

This study shows a 54.4% HBV-DNA prevalence in the Kanamary tribe, 36.7% in the Marubo, and 100% in other ethnic groups. The Marubos have been in contact with non-indigenous cultures for 150 years and the Kanamary for around 35. The border area between Brazil and Peru facilitated access to Javari Valley, leading to environmental exploitation and the dissemination of diseases^{23,24}. It is still up for discussion whether this is an element for the propagation of HBV in the villages.

In studies conducted by Braga et al.⁵ and Ferreira et al.²⁵ in different tribes of the Amazon, HBV infection was shown to be less prevalent in indigenous groups that have little contact with non-

indigenous cultures, which is backed up by our research. It is worth mentioning, however, that the majority of Marubos live in settlements near the Curuçá River and that the collection of samples in this area was done in September 2009, coinciding with the entry of influenza A (H_1N_1) in Brazil. This event caused considerable fear and mistrust among indigenous persons, and as a result fewer of them consented to participate in the study, thereby resulting in the collection of only 55 samples.

Analysis of the gender of the study population showed more female participants largely due to their being in charge of the domestic workload and small farms. Thus, women were more present in the villages, with the men either hunting or being farther from home. The research of Viana et al.²⁶ of Amerindians and non-Amerindians in the western part of the State of Acre was also characterized by a predominance of women relative to men.

As for the results of PCR positivity for HBV-DNA in the study, we found no statistically significant difference by gender. This goes against some studies that indicate greater prevalence among men^{17,19} and other studies that show a higher caseload among women²². It is the view of the researchers, however, that men are probably more exposed to sources of infection due to behavioral aspects such as drug use, promiscuity, infrequent prophylactic use, greater frequency of travel outside of the indigenous areas, and decorative body piercings.

In relation to the age groups of the study participants, younger indigenous persons (median age 23) were found to have higher positivity for HBV-DNA/PCR. This outcome shows that sexual transmission is significant in this group with many members at a sexually active age. Several studies reinforce the effect of this behavior^{10,27,28}.

With regard to the sources of infection and the presence of HBV-DNA, no statistical difference was found. It has been suggested that the transmission of HBV between family members is important in areas of high endemicity. Research done by Lobato et al.²⁹ on pregnant women participating in the pre-natal program in the municipality of Rio Branco, State of Acre, Brazil, confirms intrafamilial transmission of HBV between brothers/sisters, parents, sexual partners, children, and those who share the use of a toothbrush. Several studies also suggest the transmission of HBV by mosquitoes³⁰⁻³².

In the survey found was the presence of some variables with missing values, since the indigenous people could not inform us some variables, which resulted in the impossibility of identifying other sources of HBV infection.

In the evaluation of the clinical aspects related to PCR results for HBV-DNA, this study did not find any statistical significance for the following variables: malaise, nausea, jaundice, liver pain, myalgia, arthralgia, and darkened urine. On the other hand, fever was statistically significant among participants who had this symptom and were PCR positive (80%). This suggests the occurrence of other febrile diseases not exclusive to hepatitis B. In the Amazon, infection with HBV and *Plasmodium falciparum* is quite common, as are co-infections³². A study undertaken by Viana et al.²⁶ also relates case histories of malaria and jaundice association.

In the Javari Valley, malaria is highly endemic^{23,33}. We believe it is necessary to conduct other studies of diseases that present the same pattern of fever, especially arboviruses, that are present in the Amazon.

In areas where hepatitis B is highly endemic, the virus is frequently transmitted during childhood, either by HBV-carrier mothers to their newborns or during pregnancy/childbirth. The chances of transmission increase as the pregnancy comes to term and are much higher in acute carriers than in chronic ones³⁴.

Our research showed that 75% of the pregnant women involved in the study were HBV-DNA positive by PCR. This outcome suggests the existence of vertical HBV transmission in the Javari Valley. Unfortunately, however, it was not possible to verify this occurrence since the study did not include samples from newborns and children. The age range of the study group was only 11 to 77 years of age. The percentage of HBV-DNA found in the pregnant women reveals the vulnerability to viral transmission and infection despite the availability of hepatitis B vaccination.

This vaccine is the most effective way to prevent acute or chronic infection and to eliminate transmission in all age groups³⁵. In the present study, it was not possible to evaluate the effects of vaccine, since the indigenous people could not inform us whether they had taken three doses, reporting only that they were vaccinated and there was no information of their anti-HBs (antibody to HBsAg), notwithstanding efforts by the National Health Foundation to develop immunization campaigns in Javari Valley³³.

In the present study, the prevalence of HBV-DNA in the Curuçá and Itaquai river basins was 43.6% and 54.4%, respectively. It is interesting to note that the indigenous communities closest to the City of Atalaia do Norte had the highest rates, as seen in the community of Pedro Lopes (100%) on the Curuçá River and Bananeira (74.2%) on the Itaquai. This suggests the influence of environmental, geographical, and epidemiological factors in the dissemination of HBV, especially contact with neighboring non-indigenous population groups. Several studies back this hypothesis^{5,25,36}.

The results of this study provide evidence of the high prevalence of HBV-DNA in the Javari Valley and highlight the importance of developing more effective control and prevention strategies to combat the spread of HBV. Further research will be needed in the future to verify the association between HBV and HDV (hepatitis D Virus), as well as to identify the HBV genotypes present in the Javari Valley so as to better evaluate the association between genotype, clinical evolution of infection, treatment response, and ethnic origin.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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