




# Applicability of oral fluid samples for tracking hepatitis B virus mutations, genotyping, and phylogenetic analysis

Moyra Machado Portilho<sup>1,2</sup> · Cristianne Sousa Bezerra<sup>1,3</sup> · Ana Carolina da Fonseca Mendonça<sup>1</sup> · Vanessa Alves Marques<sup>1</sup> · Leticia Cancelli Nabuco<sup>4</sup> · Cristiane Alves Villela-Nogueira<sup>4</sup> · Cláudia Alexandra Pontes Ivantes<sup>5</sup> · Lia Laura Lewis-Ximenez<sup>1</sup> · Bárbara Vieira do Lago<sup>1,6</sup> · Livia Melo Villar<sup>1</sup> 

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## Abstract

Little is known about the usefulness of saliva samples for hepatitis B virus (HBV) genotyping and mutation analysis. The aim of this study was to evaluate the usefulness of oral fluid samples to determine HBV genotype distribution, S/polymerase mutations, and HBV subpopulation diversity among chronically HBV-infected individuals. Serum and oral fluid samples were obtained from 18 individuals for PCR and nucleotide sequencing of the HBV surface antigen gene. Biochemical analysis of liver enzymes (ALT, AST, GGT) and HBV, HCV, and HIV serological tests were also performed. All serum samples were HBsAg (+), anti-HBc (+), and anti-HBs (–); 55.6% were HBeAg (+)/anti-HBe (–), and 11.1% were anti-HIV (+). The mean HBV DNA viral load was  $6.1 \pm 2.3$  log IU/mL. The HBV genotype distribution was as follows: A, 72.2%; D, 11.1%; E, 5.6%; F, 11.1%. A concordance of 100% in genotype classification and 99.8% in sequence similarity between paired oral fluid and serum samples was observed. HBsAg mutations were detected in all samples, but no resistance mutations were found in the polymerase gene. This study demonstrates that oral fluid samples can be used reliably for tracking HBV mutations, genotyping, and phylogenetic analysis. This could be important for molecular epidemiology studies with hard-to-reach populations.

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Institution at which the work was performed: Laboratory of Viral Hepatitis, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro.

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✉ Livia Melo Villar  
lvillar@ioc.fiocruz.br

- <sup>1</sup> Laboratory of Viral Hepatitis, Oswaldo Cruz Institute, FIOCRUZ, Helio and Peggy, Ground Floor, Room B09, Av. Brasil, 4365, Manguinhos, Rio de Janeiro, RJ 210360-040, Brazil
- <sup>2</sup> Gonçalo Moniz Research Institute, FIOCRUZ, Salvador, BA, Brazil
- <sup>3</sup> Federal Institute of Education, Science and Technology, Fortaleza, CE, Brazil
- <sup>4</sup> Clementino Fraga Filho Hospital, University of Rio de Janeiro, UFRJ, Rio de Janeiro, RJ, Brazil
- <sup>5</sup> Center of Guidance and Advisor (COA), Curitiba, PR, Brazil
- <sup>6</sup> Immunobiological Technology Institute (Bio-Manguinhos), FIOCRUZ, Rio de Janeiro, RJ, Brazil

## Introduction

About 257 million people are living with hepatitis B virus (HBV) infection worldwide, making this a global public health concern. HBV is a small, partially double-stranded DNA virus that causes acute and chronic hepatitis in humans. It has been classified phylogenetically into nine genotypes (A–I), with a putative tenth genotype, "J", isolated from a single individual. There are also several sub-genotypes [1, 2].

HBV genotypes have a distinct geographical distribution: genotype A is predominant in Africa, Southeast Asia, Europe, the Americas, and Japan; genotypes B and C are common in the Pacific region of Asia; genotype D is predominant throughout the world, mostly in Africa, Europe, the Mediterranean region, and India; genotype E is restricted to West Africa; genotype F is found in South and Central America; genotype G has been found in France, Germany, and the Americas, mainly among men who have sex with men; and genotype H is detected in Central America and Mexico [1, 3, 4]. More recently, genotypes I and J were identified in Vietnam and Japan, respectively [5–7]. In Brazil, according to a recent study using samples from five different

geographic regions of the country, seven HBV genotypes (A–G) were found circulating, but genotype A was the most prevalent (58.7 %), followed by genotypes D (23.4 %) and F (11.3 %) [8].

In Brazil, peginterferon alfa-2a and the first-line nucleos(t)ide analogues (NAs) entecavir and tenofovir are available for treatment. In rare cases, the long-term use of NAs may lead to the emergence of resistant strains, with increasing signs of liver injury, cirrhosis, and hepatocellular carcinoma [9].

Polymorphisms in the S gene, which partially overlaps with the viral polymerase gene, are frequently associated with escape from vaccine-induced immunity, impaired HBsAg detection, and a poor response to immunoglobulin therapy [10–12]. HBV DNA can be detected and quantified using oral fluid samples [13–15]. These samples are easier to collect, the procedure is less invasive and painless, and the risk of exposure to needles is eliminated [16, 17]. Therefore, oral fluid samples may be useful in molecular epidemiology studies in children, the elderly, and hard-to-reach populations, allowing broader population adherence to be achieved compared to blood sampling [18].

To our knowledge, no study has evaluated the applicability of using oral fluid for HBV DNA sequencing, detection of resistance/escape mutations, and phylogenetic analysis. In this study, we evaluated the usefulness of oral fluid samples for HBV genotyping, detection of S/polymerase mutations, and analysis of HBV subpopulation diversity compared to the gold standard, serum samples.

## Material and methods

### Study population

A total of 18 paired oral fluid and serum samples from chronically HBV-infected individuals (positive HBsAg in serum) were included in this study. They were recruited at public health centers in South/Southeast Brazil from July 2013 to December 2015. Informed consent was obtained from all participants prior to sample collection. The study was approved by the ethics committee (number CAAE 18281313.4.0000.5248). Information regarding treatment status and biochemical markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) were obtained.

### Sample collection

Blood samples were collected by venipuncture, and oral fluid samples were obtained using a Salivette collector (Sarstedt, Nümbrecht, Germany) as described previously [14, 19]. For oral fluid collection, patients were asked not to consume

food or water for at least one hour beforehand to avoid sample contamination that could impair DNA amplification. All oral fluid samples were checked visually for blood contamination and excluded if it was observed.

### Detection of biochemical and serological markers of HBV infection

A 12-h overnight fasting blood sample was drawn to determine serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, and  $\gamma$ -glutamyltransferase (GGT), using an automated immunochemiluminometric assay (ICMA) (Liason, Diasorin, Varceli, Italy). Serum samples were tested using commercial enzyme immunoassays (ELISA) for the detection of HBsAg (BioElisa HBsAg 3.0, BioKIT, Spain), total anti-HBc (BioElisa anti-HBc, BioKIT, Spain), anti-HBc IgM (ETI CORE IgMK Plus, DiaSorin, Italy), anti-HBs antibodies (BioElisa anti-HB, BioKIT, Spain), HBeAg, and anti-HBe (e411 Cobas, Roche Diagnostics, Mannheim, Germany) according to the instructions from each manufacturer. Samples were also tested for antibodies against hepatitis C virus (anti-HCV) (Murex anti-HCV 4.0, DiaSorin, South Africa) and human immunodeficiency virus (anti-HIV) (DS-EIA-HIV-AGAB-SCREEN, RPC, Diagnostic System, Russia).

### Amplification of HBV DNA from serum and oral fluid samples

HBV DNA in serum samples was quantified using an Abbott RealTime HBV assay (Abbott Laboratories, USA). HBV DNA was extracted from serum samples using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) and from oral fluid using an RTP® DNA/RNA Virus Mini Kit (Strattec, Germany) following the manufacturer's instructions except that the oral fluid sample volume was increased by twofold ( $n = 400 \mu\text{L}$ ) [19]. For HBV amplification, optimized PCR, using oligonucleotide primers specific for the S/polymerase gene, resulting in an amplicon of ~900 base pairs [20], was done as described by Portilho et al. [14].

### Sequence analysis

Sequence analysis of positive serum and oral fluid samples was performed using PCR products after purifying them using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Direct nucleotide sequencing was done in both directions using a Big Dye Terminator Kit (version 3.1, Applied Biosystems, Foster City, CA, USA) and external and internal primers [20]. Sequencing reactions were analyzed on an ABI3730 automated sequencer (Applied Biosystems). MEGA 7.0 software was used to

align and analyze nucleotide sequences and to construct a phylogenetic tree by the maximum-likelihood method. The reliability of the phylogenetic tree was assessed by bootstrap test (1000 replicates).

Consensus sequences of each HBV isolate (serum and oral fluid) were submitted to a web-based computer program for subtyping and prediction of phenotypic resistance mutations in the polymerase gene (RT mutation) and vaccine escape mutations in the S gene (Max-Planck-Institut für Informatik, Germany, at <http://hbv.geno2.pheno.org/index.php>).

### Statistical analysis

Descriptive statistical analysis was performed by calculating the mean and standard deviations. The influence of laboratory parameters on the probability of obtaining an HBV sequence was evaluated using an unpaired *t*-test with Welch correction for continuous variables and Fisher's exact test for categorical variables. Statistical analysis was determined using GraphPad InStat software.

## Results

### Study population

A total of 18 individuals were evaluated. Most were male (12/18; 66.7%), and the mean age was  $42.72 \pm 14.14$  years. Four individuals reported previous HBV treatment (one patient was treated with tenofovir, two with entecavir, and one with tenofovir + lamivudine during pregnancy).

### Biochemical and serological tests

None of the subjects had a high ALT serum level (based on reference value higher than 41 U/mL). The median ALT level was 18 U/mL, ranging from 7 to 42 U/mL. For AST levels, the median was 22.6 U/mL, ranging from 8 to 52 U/mL. For total bilirubin, the median was 0.17 U/mL (0.01 to 2.25 U/mL), for alkaline phosphatase, it was 47.5 U/L (22 to 321 U/mL), and for GGT, it was 25.5 IU/L (8 to 1059 U/mL). Regarding the serological results, all serum samples were anti-HBc total (+), one was anti-HBc IgM (+), 10 were HBeAg (+)/ anti-HBe (-), eight were anti-HBe (+), and none were anti-HBs (+). None of the samples were anti-HCV (+), and two were anti-HIV (+) (Table 1).

**Table 1** Demographic, clinical, and laboratory characteristics of HBV serum samples

Sample	Age	Gender	ALT (U/mL)	AST (U/mL)	Anti-HIV	HBeAg/ anti-HBe	Viral load (log UI/mL)	Genotype/ subgenotype	Sequenced in oral fluid	Treatment
P01	42	M	12	11	+	+/-	7.8	A1	Yes	No
P02	52	F	42	19	-	-/+	7.09	D3	Yes	No
P03	51	M	20	17	-	-/+	6.04	F2	Yes	No
P04	31	F	9	19	-	+/-	9	A2	Yes	TDF + LAM
P05	36	M	8	12	+	-/+	3.38	A1	No	No
P06	24	M	12	31	-	+/-	7.54	A1	Yes	No
P07	40	F	18	36	-	+/-	8.34	A2	Yes	No
P08	40	M	15	23	-	-/+	3.2	A1	No	No
P09	26	M	14	19	-	+/-	8.67	E	Yes	No
P10	25	F	26	42	-	+/-	9	A1	Yes	No
P11	54	F	27	26	-	+/-	3.79	A1	No	No
P12	64	F	12	14	-	-/+	2.72	A1	Yes	No
P13	53	M	7	21	-	+/-	7.3	A1	No	No
P14	57	M	12	11	-	-/+	3.33	A1	No	No
P15	49	M	14	52	-	+/-	6.66	F2	No	ETV
P16	47	M	12	34	-	-/+	2.53	A1	No	ETV
P17	32	M	13	8	-	-/+	4.8	A1	No	No
P18	46	M	12	13	-	+/-	8.66	D3	Yes	TDF

LAM, lamivudine; TDF, tenofovir; ETV, entecavir

## Molecular tests and genotyping

The mean HBV DNA viral load in serum was  $6.1 \pm 2.3$  log IU HBV DNA/mL, and genotype distribution was as follows: A, 13/18; D, 2/18; E, 1/18; and F (2/18). Regarding subgenotype distribution, among the 13 patients with genotype A, 11 (84.6%) were classified as subgenotype A1 and two as subgenotype A2 (15.4%). The genotype D and F isolates were classified as D3 and F2, respectively. The HBV genotype distribution is shown in Table 1.

In the 13 patients with genotype A, the mean viral load was  $5.59 \pm 1.927$  log UI HBV DNA/mL, and six of them were HBeAg(+)/anti-HBe(-). Among the patients with genotypes D (n = 2) and F (n = 2), one with each genotype was HBeAg(+)/anti-HBe(-). The mean viral load was of  $7.87 \pm 0.78$  log UI HBV DNA/mL for genotype D and  $6.35 \pm 0.09$  log UI HBV DNA/mL for genotype F. The HBV genotype E sample had a serum viral load of 8.67 log UI HBV DNA/mL and was HBeAg reactive. Demographic, laboratory, and clinical characteristics of the paired oral fluid and serum samples are shown in Table 1.

Of the 18 oral fluid samples with detectable HBV DNA, it was possible to sequence and analyze an HBV S/pol gene fragment from 10 samples. A comparison between the oral fluid samples that could and could not be sequenced is shown in Table 2. In the oral fluid samples that were successfully sequenced, genotype A was the most frequent (6/10; 60%), with four classified as subgenotype A1 and two as A2. The other oral fluid samples were classified as genotype D (subgenotype D3) (n = 2), E (n = 1), and F (subgenotype F2) (n = 2) (Fig. 1). All samples showed high sequence similarity to the corresponding serum samples, with pairwise genetic distances between 0.00 and 0.023 (Table 3) and 100% agreement in the genotype/subgenotype classification.

## Mutations

It was possible to identify amino acid mutations in the polymerase and/or S gene in all 18 HBV sequences from serum and in all 10 from oral fluid. No primary antiviral resistance

mutations were found. The self-compensatory V173L mutation was detected in one treatment-naïve patient. HBV polymerase/S gene polymorphisms, when present, were found in both the serum and oral fluid samples from the same patient (Table 3). However, amino acid substitutions were found in greater numbers in oral fluid samples.

The most frequently detected polymorphisms in the polymerase were 122H/Y, M129L, V163I, and I253V, which were observed in 12, 13, 11, and 9 serum samples, respectively, and in their paired oral fluid samples, when available.

One or more escape mutations were detected in the S gene of five serum samples and four paired oral fluid samples. Most of these isolates had amino acid substitutions within the 'a' determinant region (aa 124-147) of the major hydrophilic region (MHR). The mutation Y100C was observed in two subjects. Furthermore, the mutations 109V, 126N, M133T, 144A, and 145R were found in one isolate each.

## Discussion

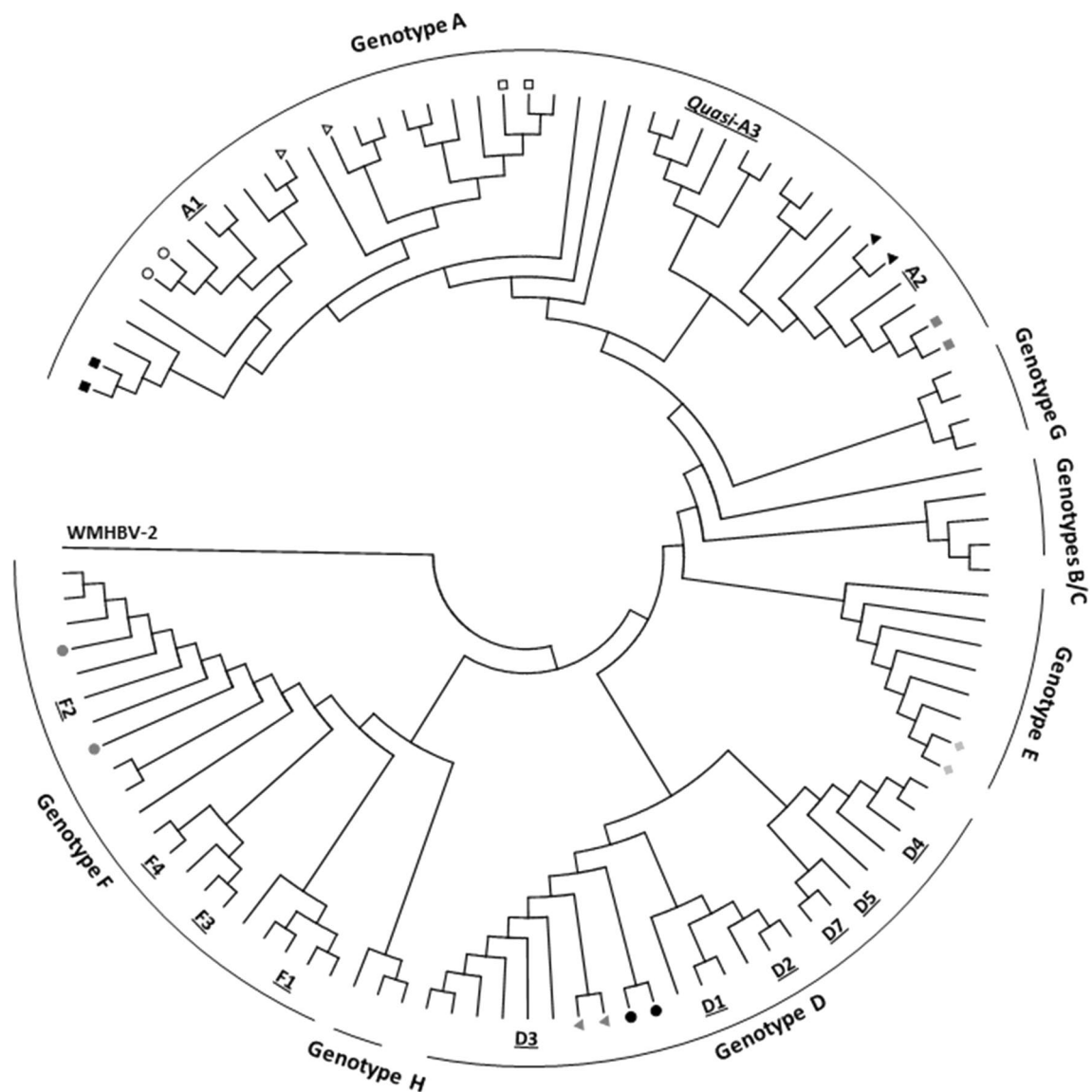
Previous studies have suggested that oral fluid samples are a safe, less-invasive, and economical alternative to serum samples for molecular surveillance of infectious diseases [18]. This study demonstrated the usefulness of oral fluid for sequencing purposes, for genotyping, and for identification of clinically relevant mutations in HBV S/Pol.

In this study, a concordance of 100% in genotype/subgenotype classification between paired oral fluid and serum samples was observed. HBV genotypes A, D, E, and F were detected. Phylogenetic analysis revealed that most of the samples (61.1%) were classified as subgenotype A1, clustering with other Brazilian samples in the HBV/A1 Asia-American clade [21]. HBV subgenotypes A2, D3, and F2 were also detected in a proportion of 11.1% each and were phylogenetically related to samples from Belgium, Italy, and Venezuela, respectively. Subgenotypes A1, A2, D3, and F2 are highly prevalent in Brazil and appear to be linked to the different ethnic groups (Africans, Europeans, and Amerindians) that contributed to the current Brazilian demographic composition [8, 22]. Genotype E (5.6%) was detected in one patient who was born in Angola (Africa) but had recently moved to Brazil. This genotype had been reported previously in Brazil, being mostly associated with immigrants or refugees from East Africa [8, 22], a region that has a high prevalence of genotype E strains [23]. The successful detection, not only of Brazilian endemic genotypes but also of an imported genotype, demonstrates that the use of oral fluid for sequencing applications may be effective for molecular analysis of genetically heterogeneous populations.

It has been suggested that intra-host HBV subpopulations may evolve into quasispecies according to the selective pressure imposed by the infected cellular reservoirs [24]. In this

**Table 2** Laboratory parameters of oral fluid samples according to HBV sequencing

Variable	HBV sequenced (n = 10)	HBV not sequenced (n = 8)	<i>p</i>
Mean viral load in serum (copies/mL $\pm$ SD)	$7.486 \pm 1.919$	$4.373 \pm 1.73$	0.0026
HBeAg positivity	7	3	0.34
ALT mean (U/mL)	$17.7 \pm 9.91$	$13.5 \pm 6.11$	0.28
AST mean (U/mL)	$22.1 \pm 10.51$	$23.37 \pm 14.46$	0.83



**Fig. 1** Phylogenetic tree comparing serum and oral fluid sequences. The phylogenetic tree, constructed by the maximum-likelihood method, incorporates sequences from 10 paired serum and oral fluid

samples from this study and 99 reference sequences retrieved from the GenBank database. Symbols in the tree indicate the serum and oral fluid pairs.

study, the sequences for paired serum and oral fluid samples were over 99.8% identical. These results suggest that the HBV subpopulations present in lymph nodes and oral mucosa (oral fluid samples) strongly reflect those circulating in the blood. However, molecular investigations using liver biopsies would be useful to determine if serum and oral fluid accurately represent the major viral subpopulation in the liver. To our knowledge, this is the first study comparing HBV subpopulation characteristics present in serum and oral fluid samples. Since the diversity of HBV quasispecies may fluctuate depending on the cellular reservoir, managing viral subpopulations at extrahepatic sites may be the key to a successful antiviral therapy.

It has been demonstrated that both host immune background and antiviral selective pressure can drive HBV evolution by boosting the emergence of mutations in the Pol/S gene [25]. In this study, HBV sequence analysis identified amino acid mutations in all 18 HBV serum samples and 10 oral fluid samples in both the polymerase and S genes. Most of the polymorphisms occurred in the MHR of HBsAg, which encompasses the major B and T cell epitopes and is an important region for binding of neutralizing antibodies [12, 26].

HBsAg is a target for immune system cells and acts as a template for vaccine-mediated immunity. Therefore, mutations in the MHR may seriously impact vaccine efficacy



**Table 3** Mutations in HBV polymerase and surface proteins found in paired serum and oral fluid samples

Sample	Genetic distance	Polymerase mutations	HBsAg mutations
P1	0.001%	I53L*, V112I, N122H, Y126C, M129L, V142A, I53H# V163I, Q215H, I253V	S45T#, V96A, M103I, T118A, K122R, F134L, D144A, V168A, V180A, S207T
P2	0.001%	L91I, F122L, Q130P, N131H, Y135S, V173L#, C256S, D263E, I266V, V278I,	G44E#, T127P, E164D#, S207N
P3	0.007%	S74P#, N123D*, M129L, S137T, L151F*, S213T, L220I*, N246S, T259S, D271E, Q288H	N40S*, C76Y*, Q101H#, N204K
P4	0.000%	L217R	L209V
P6	0.001%	N122H, M129L, I53N#, V163I	S45T#, S207N
P7	0.000%	L217R	L209V
P9	0.000%	M267L	T57I, P62Q
P10	0.000%	N122H, M129L, W153R, V163I, I253V, V278I	Q101H#, S207N
P12	0.023%	I53V#, N122H, M129L, W153R#, S159T#, V163I, S219A#, I253V, V278I#	L49R#, I68T#, Y100C#, Y161F#, A194V*, S207N, I208T, S210R#
P18	0.000%	N53K, F122L, Q130P, Y135S, D263E, I266V, V278I	T45S, T127P

\* mutations found only in serum samples; # mutations found only in oral fluid samples

and facilitate immune evasion [10, 12]. In this study, HBV quasispecies with the MHR amino acid mutations Y100C, L109V, M133T, D144A, and G145R in the S gene were detected in eight serum/oral fluid sample pairs (Table 3). These mutations are frequently associated with vaccine escape, failure in HBsAg detection, and a poor response to immunoglobulin therapy [11, 12, 27]. Despite these mutations, HBsAg detection in the samples from this study using a commercial assay was not impaired. Similar results were observed by Avellon and colleagues [11] in a study of Spanish chronic HBV carriers. Despite the detection of escape mutants in 12.5% of the subjects, detection of HBsAg was not suppressed in the three commercial assays evaluated by the authors. As suggested previously, the impact of a specific mutation on reactivity in a diagnostic test might be affected by other polymorphisms present in the sample [11]. In this context, the existence of other mutations that may have secondary effects on HBsAg detection should be investigated further.

The impact of HBsAg mutations in the epidemiological setting is currently unclear. Although no influence on diagnostic assays was observed in this study, the effects of these polymorphisms, as reported by others [11, 27], may represent a challenge in HBV vaccine efficacy and in the outcome of immunotherapy. Although four individuals were undergoing antiviral therapy, no primary antiviral resistance mutations were detected. In contrast, the rtV173L mutation, which is a self-compensatory mutation associated with enhanced viral replication in lamivudine-resistant patients [28, 29], was found in a treatment-naïve patient. Studies have demonstrated that the use of **tenofovir disoproxil fumarate** (TDF) was strongly associated with undetectable/low HBV DNA levels [30]. However, the two patients from this study who

were previously treated with TDF had surprisingly high viral loads (Table 1), and no relationship with HBe/anti-HBe seroconversion was observed.

This study has some limitations, such as the small sample size, the lack of data regarding HBV subpopulations in the liver, and the unavailability of cloning or next-generation sequencing data for the HBV subpopulations present in the serum and oral fluid samples that could shed light on the overall diversity of HBV quasispecies present in different cellular reservoirs. In addition, sequence data from oral fluid could not be obtained from the samples with the lowest viral loads (generally HBeAg negative), preventing inferences about sequencing accuracy in these samples.

In conclusion, this study demonstrates the usefulness of oral fluid samples in tracking HBV mutations, genotyping, and phylogenetic analysis compared to the gold standard, serum samples. Since collection of oral fluid is a safe, convenient, and non-invasive method, its use may be an important tool in molecular epidemiology studies, especially in remote areas and hard-to-reach populations.

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**Author contributions** MMP, BVL, and LMV prepared and revised the manuscript. MMP, CSB, and ACFM did molecular tests, including sequencing. BVL and MMP performed the phylogenetics analysis. LLLX, CAVN, LCN, and CAPI contributed to recruiting and medical care of patients. VAM, CSB, MMP, and ACFM worked on data curation, performed the molecular assays, also providing the interpretation of the molecular results obtained. LMV supervised and managed the project and worked on funding acquisition. All authors revised the final version of this manuscript.

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**Availability of data and material** The datasets generated and/or analysed during the current study are not publicly available, in order to maintain the privacy and confidentiality of the subjects, but are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** This study was approved by the Fiocruz Ethics Committee (number CAAE 18281313.4.0000.5248).

**Consent to participate** All individuals gave formal consent to participate in this study.

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