Molecular epidemiology of hepatitis B virus mutants associated with vaccine escape, drug resistance and diagnosis failure

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Funding information
Consejo Nacional de Investigaciones Científicas y Técnicas, Grant/Award Number: PIP1122015-0100595; Universidad de Buenos Aires, Grant/Award Number: UBACyT 20020130100505BA 2014-2017

Summary
The massive implementation of the vaccine and antiviral agents against hepatitis B virus (HBV), targeting the envelope and viral polymerase genes, induces a selection pressure that might lead to the emergence of variants that impair the effectiveness of the vaccine, diagnostic methods and antiviral therapy. The aim of this study was to evaluate the prevalence of HBV vaccine escape mutants (VEMs), diagnostic failure mutants (DFMs) and treatment resistance mutants (ARMs) among individuals from Buenos Aires, Argentina. HBV surface antigen and polymerase sequences obtained from serum samples of 530 HBV-infected individuals were analysed. Samples belonged to genotypes A (28.1%), D (13.6%) and F (58.3%). VEMs, DMFs and ARMs were present in 40 (7.5%), 57 (10.7%) and 27 (5.1%) samples within the studied population. Additionally, eight nonpreviously reported VEMs and nine DMFs were identified. VEMs and DMFs were biased by genotype, being higher in genotype D (33.3% and 33.3%) compared to genotype A (6% and 17.4%) and genotype F (2.3% and 2.3%) (P > 0.001). On the contrary, there was no association between the presence of ARMs and HBV genotype (P = 0.324). VEMs, DMFs and ARMs create public health concerns. The current study provided valuable information about mutants in surface antigen and polymerase in HBV-infected patients from Argentina where HBV-F is the most prevalent genotype. Consequently, it constitutes an important reference for Latin American clinicians in order to optimize the management of HBV-infected patients.

KEYWORDS
antiviral resistance, diagnostic failure, hepatitis B virus, vaccine escape mutant

INTRODUCTION

Hepatitis B virus (HBV) infection and HBV-related complications remain a major global public health problem since an estimated 260 million people are chronically infected and more than 800,000 deaths occur yearly, mostly from complications, including cirrhosis and hepatocellular carcinoma.

Hepatitis B virus has a small partially double-stranded relaxed circular DNA genome, which has a very compact coding organization with four partially overlapping open reading frames.

Based on genetic divergence, HBV has been classified into 9 genotypes designated A-I defined by >8% divergence at the nucleotide level and several subgenotypes, while another putative genotype, “J,” has been proposed after isolation from one individual.
Genotypes and subgenotypes have a restricted ethnic-geographical distribution.6 

Due to the absence of proofreading activity, the HBV polymerase/RT leads to the introduction of random mutations into HBV genome, creating a genetic variability described as viral quasispecies. These variants include vaccine escape mutants (VEMs), diagnostic failure mutants (DFMs) in the routine screening and antiviral drug resistance mutants (ARMs).5 

Hepatitis B virus vaccine was introduced in the early 1980s, and currently, the global coverage with three doses is estimated at 82%.6,10 In Argentina, vaccination against HBV was finally incorporated into the calendar for newborns since 2000.

The current recombinant hepatitis B surface antigen (HbsAg) used in HBV vaccine and diagnostic assays contains a highly conserved antibody-neutralizing epitope cluster, which spans amino acids (aa) 124-147 within the major hydrophilic region (MHR, aa 99-169), and is referred to as a"a" determinant. It is known that neutralizing antibodies produced after vaccination against HBV are targeted to the conformational epitopes of the "a" determinant.7-9 

Despite the high efficacy of HBV vaccine, breakthrough infections due to VEMs have been reported in vaccinated individuals, which highlights the importance of these escape mutants. Additionally, these variants may also provide false-negative results in serological tests, which are known as false occult HBV infection (OBI).7,10 

Furthermore, in the last decades several oral nucleos(t)ide analogues (NAs) were approved for HBV chronic infection treatment. The viral target of these antiviral agents is the RT domain of the HBV polymerase.11 Under selective pressure imposed by the administration of antiviral agents, minor HBV quasispecies converge on a dominant mutant that can escape selection pressure, creating a drug-resistant HBV strain. 

As mentioned before, the HBV genome is organized in such a way that the surface antigen gene is completely overlapped with the polymerase one.12 Therefore, polymerase gene mutations selected during the course of antiviral NA therapy may affect neutralization epitopes within the HbsAg.

Most epidemiological data of HBV surface and polymerase mutants have been based on studies performed in Asia or in Europe, in patients infected with genotypes B and C or A and D, respectively, with a paucity of information regarding infections with other genotypes.13-16 Particularly, information about genotype F, characteristic of Native American populations of Alaska, South and Central America and likely originated in Amerindian populations, was scarcely addressed.17-19 

Additionally, there is no information about VEM, DFM and ARM prevalence in Argentina. Thus, we aimed to evaluate the prevalence of HBV vaccine escape, diagnostic failure and drug resistance mutants in HBV chronically infected individuals from Buenos Aires, Argentina.

2 | MATERIALS AND METHODS

2.1 | Study population

In a retrospective cross-sectional study, 530 HbsAg carriers who attended a tertiary care centre located in the city of Buenos Aires, Argentina, between December 1999 and December 2017 were included.

2.2 | Laboratory determinations

Hepatitis B virus serological markers were analysed with AxSYM; Abbott Diagnostics, USA (samples before 2010), and Architect Abbott system, Abbott Diagnostics, Wiesbaden, Germany (samples since 2010).

2.3 | HBV-DNA extraction, amplification and sequencing

DNA was extracted from 200 μL of serum using the High Pure Viral Nucleic acid Kit (Roche Diagnostics, Germany). The HbsAg gene was amplified with primers HBVS1 (sense, 5’ TCA CCA TAT TCT TGG GAA CAA GA 3’, 2821-2843) and HBVS2 (antisense, 5’ AAA ACC CAA AAG ACC CAC AAT 3’, 1017-997) and HBVS3 (sense, 5’ CTG CTG GTG GCT CCA GTT C 3’, 57-75) and HBVS4 (antisense, 5’ CAA AAG AAA ATT GGT AAC AGC GG 3’, 816-794) for the second round. The first PCR round encompasses the entire S region and the Pol region from amino acid 178 to 637 (rtPol aa 1 to 289), while the second PCR round encompasses the S region from amino acid 1 to 213 and the Pol region from amino acid 331 to 569 (rtPol aa 1-221). For the first round of PCR amplification, 3 μL of extracted DNA and 0.25 μmol/L of each primer were added to AmpliTaq Gold® 60 Master Mix in a final volume of 25 μL. For the second round, 2 μL of first round product was added to 40 μL final volume of PCR mix. The cycling protocol was denaturing at 94°C for 5 minutes, followed by 40 cycles in the first round and 25 cycles in the second one of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute, and a final extension at 72°C for 5 minutes.

The PCR product of the first round (1416 nt) or the second round (759 nt) was purified using QiAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and submitted to direct nucleotide sequencing reaction in both directions (Unidad de Genómica, INTA, Castelar, Buenos Aires, Argentina) with the same primers used in amplification stage.

2.4 | HBV typing

Genotyping was assessed by phylogenetic analysis. Seventy-one nucleotide sequences spanning about 759 nt from HbsAg region representing the different HBV genotypes were retrieved from GenBank and used as references. Sequences obtained in this study and HBV sequences from GenBank database were aligned with ClustalX (v2.1) software20 and edited with BioEdit (v7.1.3.0) software.21 Phylogenetic trees were constructed using the maximum likelihood method performed with RAxML (v 0.8.24) program.22 Evolutionary models were inferred according to the Akaike information criterion (AIC) statistics obtained with jModeltest (v2.1) software.23 Robustness of the reconstructed phylogenies was evaluated by bootstrap analysis (1000 replicates). In order to differentiate among subgenotypes, phylogenetic analyses were combined with amino acid alignments.
acid and nucleotide patterns characteristic of each subgenotype within the HBsAg; this was assessed by VisSPA v1.6.2 program.\textsuperscript{24} It was established that the amino acid pattern characteristic of each subgenotype would be formed by at least 90% of the amino acids present in the sequences from the group analysed and in less than 10% of the samples in the reference group.

2.5  Mutational analysis

Nucleotide sequences were aligned, and occurrence of each amino acid at each position of the alignment was analysed using the Positional Aminoacid Numerical Summary tool included in BioEdit (v7.1.3.0) followed by visual inspection. In order to search for most significant HBV surface mutants, aa 99-169 within HBsAg gene were examined. Fourteen positions related to VEMs (116, 120, 126, 129, 130, 131, 133, 134, 141, 142, 143, 144 and 145) and thirty related to DFM s (100, 101, 115, 116, 118, 120, 121, 122, 123, 126, 128, 129, 130, 131, 133, 134, 135, 137, 138, 141, 142, 143, 144, 145, 146, 147, 148, 154, 155 and 157) were analysed in this work according to previous reports.\textsuperscript{16-18,25-28} Additionally, eleven positions in the polymerase gene (rtL80, rtI169, rtV173, rtL180, rtA181, rtS184, rtA194, rtS202, rtM204, rtN236T and rtM250V) were also investigated in order to evaluate ARMs for the most widely used antivirals.\textsuperscript{29,30} Mutations at positions rtN236 and rtM250V were determined in 356 out of the 530 samples.

2.6  Statistical analysis

Frequencies were compared using the chi-squared test or Fisher’s test. Student’s t test and the Mann-Whitney U test were used for comparing continuous variables. The statistical analysis was carried out using the SPSS statistical software package release 19.0 (IBM SPSS Inc, Chicago, IL, USA).

2.7  Nucleotide sequence accession numbers

Nucleotide sequences for the HBV have been deposited in GenBank under accession numbers MH763038-MH763567.

2.8  Ethical aspects

Written informed consents to participate in this study were obtained from the patients. The study protocol was approved by the ethics committee from “Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires” (record number 02032015-2/2015) in accordance with the 1975 Helsinki Declaration.

3  RESULTS

3.1  Characteristics of the study population

Serum samples from 530 HBsAg- and anti–HBc-positive patients were analysed. Median (Q1-Q3) age was 44 (36-57) years, and 375 (70.7%) were male. Fourteen patients asserted having received antiviral treatment, 8 with entecavir, 5 with lamivudine and 1 with adefovir. Epidemiological characteristics of the study cohort are shown in Table 1.

Phylogenetic analysis of HBsAg gene showed supported clusters (bootstrap >70) for each genotype (data not shown). The overall genotype distribution was as follows: HBV-A 149 (28.1%), HBV-D 72 (13.6%) and HBV-F 309 (58.3%). In the same way, HBV subgenotypes were in the following proportions: HBV-A1: 5.7%; HBV-A2: 22.4%; HBV-D1: 3.8%; HBV-D2: 2.5%; HBV-D3: 5.8%; HBV-D4: 1.5%; HBV-F1b: 39.8%; and HBV-F4: 18.5%. The patient’s age was evenly distributed among the different genotypes: HBV-A 45 (37-56), HBV-D 44 (36-58) and HBV-F 44 (34-57), \( P = 0.900 \).

3.2  Vaccine escape mutant analysis

Forty-four VEMs were detected in 40 out of 530 samples (7.5%). In this regard, single mutations were observed in 36 cases and double mutations in 4 cases. All VEMs were observed in 11 out of 14

| TABLE 1  Epidemiological characteristic of the study population |
|------------------|-----------------|--------|
| Characteristics   | Population, N = 530 | %     |
| Age median, years | 44 (36-57)        |
| Gender            |                  |       |
| Male              | 375              | 70.3  |
| Female            | 155              | 29.7  |
| Genotype          |                  |       |
| A                 | 149              | 28.1  |
| D                 | 72               | 13.6  |
| F                 | 309              | 58.3  |
| HBeAg\textsuperscript{a} |          |       |
| Positive          | 269              | 70    |
| Negative          | 115              | 30    |
| ALT\textsuperscript{a} |            |       |
| Normal            | 67               | 7.5   |
| High              | 317              | 92.5  |
| Antiviral treatment |                  |       |
| Yes               | 14               | 2.6   |
| No                | 516              | 97.4  |
| VEMs              |                  |       |
| Detected          | 40               | 7.5   |
| Not detected      | 490              | 92.5  |
| DFM s             |                  |       |
| Detected          | 57               | 10.7  |
| Not detected      | 473              | 89.3  |
| ARMs              |                  |       |
| Detected          | 27               | 5.1   |
| Not detected      | 503              | 94.9  |

\textsuperscript{a}Available for 384 patients.
aa residues analysed, while three positions (T116, Q129 and K141) were unchanged in all cases. Additionally, 4 nonreported mutations, namely G130R (2), M133I and P142T, were observed. Table 2 shows the mutations at each analysed position by genotype and subgenotype.

The most frequent VEMs were as follows: T118A/V (2.26%), M133L/T (1.13%), P143L (0.94%) and D144A/E/G (0.94%). Patients infected with HBV-D showed a higher prevalence of VEMs (33.3%) than patients infected with HBV-A (6%) or HBV-F (2.3%), respectively (P < 0.001). Moreover, one patient infected with HBV-D (1.4%) and 3 infected with HBV-A (2%) presented more than one VEM (P < 0.005). The age of the patients with VEMs was 49 (38-61) vs 44 (36-57) in those without VEMs (P = 0.286).

Since it was reported that subgenotypes other than A2 (present in the vaccine antigen) produce a suboptimal protection against infection, VEMs were also analysed according to the subgenotype. In this sense, subgenotypes F1b, F4 and A1 presented a low frequency of VEMs (1.9%, 3.1% and 3.3%), respectively. Subgenotypes D1 and A2 presented an intermediate frequency of VEMs (5% and 6.7%), and subgenotypes D2, D3 and D4 presented very high frequencies of VEMs (100%, 25.8% and 25%), respectively (P < 0.001). The prevalence of VEMs was significantly higher in patients with normal ALT than in those with elevated ALT levels [14.9% vs 5.4% (P < 0.005)] and in HBeAg-negative patients compared to HBeAg-positive patients [16.5% vs 3% (P < 0.001)].

3.3 | Diagnostic failure mutant analysis

Seventy-two DFMs were detected in 57 out of 530 samples (10.7%). Those changes included 43 isolates with a single mutation, 13 isolates with double mutation and 1 isolate with a triple mutation. Forty-one out of 72 DFMs were shared with the VEMs, given that 14 of the 30 analysed positions overlap with those of the VEMs. Additionally, thirty-one DFMs were detected: Y100C (14), Q101K (3), A128V (10), G130R (2), M133I and N146S. The most frequent DFMs were the same previously mentioned for VEMs plus Y100C and D144N (36-57) in those without ARMs (P = 0.286).

On the other hand, 14 positions (T115, T116, C121, KR122, Q129, T123, P135, C137, C138, K141, C147, T148, S155 and A157) remained unchanged in all cases. Furthermore, nine nonreported substitutions in six of the 30 positions, namely Y100W (3), Q101P, P120A, M133L (2), P142T and S154A, were detected.

As observed for VEMs, patients infected with HBV-D showed a higher prevalence of DFMs (33.3%) than patients infected with HBV-A (17.4%) or HBV-F (2.3%), respectively (P < 0.001). Moreover, 11 patients infected with HBV-D (15.3%) harboured two or more DFMs simultaneously, while only 3 infected with HBV-A (2%) and none infected with HBV-F presented more than one DFM (P < 0.001). Additionally, it has been described that several combinations of DFMs displayed lower reactivity with at least one commercial diagnostic assay (Y100C/P120T, S113T/G130N, P120S/S143L, T123N/I143S, F134V/D144G, T126S/G145R, P142L/G145R, P142S/G145R, D144A/G145R and P120Q/T131K/G145R). However, none of these combinations was observed in our samples. Finally, patient’s age and ALT levels were not associated with the frequency of DFMs [45 (38-61) in patients with DFMs versus 44 (36-56) in those without DFMs (P = 0.235) and 11.9% vs. 9.1% in patients with normal or elevated ALT levels, respectively]. On the other hand, the number of DFM was associated with the presence of HBeAg. In this regard, the prevalence of DFM was higher in HBeAg-negative patients than in HBeAg-positive patients (15.7% vs 7.1%, P = 0.009, respectively).

Lastly, twelve out of 28 (42.8%) mutational types observed in the HBsAg gene had different frequency according to genotype (Table 2). Among them, it is worth mentioning that Y100C was highly prevalent in HBV-A (almost exclusive in HBV-A1, 13 out of 14 mutational types) and A128V, G130N, F143N and S143L were mainly detected in genotype D (12.5%, 2.8%, 2.8% and 6.9%, respectively).

3.4 | Antiviral-resistant mutations analysis

ARMs were detected in 27 samples (5.1%) in 6 out of 11 aa residues analysed. The 27 mutated isolates included 5 isolates with a single mutation, 13 isolates with double mutations, 8 with a triple mutation and 1 with four mutations (Table 3).

The most frequent ARMs were rtL180M (3.8%) and rtM204V/I (3.8%). Mutations of rtL80 (0.8%), rtV173L (1.2%), rtT184 (0.8%) and rtS202 (1%) had a lower pooled incidence. Five positions (rtI169, rtA181, rtA194, N236 and M250) remained conserved in all cases. In contrast with VEM and DFM observations, prevalence of ARMs was independent from HBV genotype [HBV-A (6.7%), HBV-D (6.9%) and HBV-F (3.9%), P = 0.324]. The prevalence of ARMs, as expected, was significantly higher in those patients who reported having received antiviral treatment (78.6%) than in naïve patients (3.1%, P < 0.001). Moreover, all observed mutations were related to the received treatment.

Finally, patient’s age and HBeAg status were not associated with the presence of ARMs. In this regard, age was 50 (30-64) in patients with ARMs versus 44 (36-57) in those without ARMs (P = 0.376), and the ARMs were present in 5.9% of HBeAg-positive patients and 3.5% of HBeAg-negative patients (P = 0.318).

4 | DISCUSSION

The present work represents, to our knowledge, the first study that estimates the prevalence of vaccine escape mutations, diagnostic failure mutations and antiviral resistance mutations in a size representative cohort of HBV-infected patients from Buenos Aires, Argentina, and the largest study analysing these mutants in genotype F.

Since the beginning of the 90s, the emergence and increment of VEMs due to vaccine implementation have been described.18,25,31,32 HBV strains carrying VEMs represent an epidemiological concern since they have the potential to infect even immunized population. Frequency of VEMs in literature covers a wide range from <5% to more than 40%.14,16,19,25,27,31,33 Different
| Variant     | Number (%) | A n:149 | A1 n:30 | A2 n:119 | D n:72 | D1 n:20 | D2 n:13 | D3 n:31 | D4 n:8 | F n:309 | F1b n:211 | F4 n:98 | VEM | DFM | P |
|------------|------------|---------|---------|---------|-------|--------|--------|--------|-------|--------|--------|-------|-----|-----|---|
| Y100C      | 14 (2.6)   | 14 (9.4) | 13 (43.3) | 1 (0.8) |       |        |        |        |       |        |        |       | C   | C   | <0.001 |
| Y100W      | 3 (0.6)    | 3 (2.0)  | 3 (2.6)  |         |       |        |        |        |       |        |        |       | C   | NR | 0.021 |
| Q101K      | 3 (0.6)    | 3 (2.0)  | 3 (2.6)  |         |       |        |        |        |       |        |        |       | C   | NR | 0.021 |
| Q101P      | 1 (0.2)    | 1 (0.7)  | 1 (0.8)  |         |       |        |        |        |       |        |        |       | C   | NR | 0.278 |
| T118A      | 3 (0.6)    | 3 (2.0)  | 3 (2.6)  |         |       |        |        |        |       |        |        |       | C   | C   | <0.001 |
| T118V      | 9 (1.7)    | 1 (1.4)  | 1 (3.2)  |         |       |        |        |        |       |        |        |       | C   | C   | <0.001 |
| P120A      | 1 (0.2)    | 1 (1.4)  | 1 (7.7)  |         |       |        |        |        |       |        |        |       | C   | C   | 0.041 |
| P120Q      | 3 (0.6)    | 3 (1.0)  | 2 (0.9)  | 1 (1)   |       |        |        |        |       |        |        |       | C   | NR | 0.340 |
| T126S      | 1 (0.2)    | 1 (1.4)  | 1 (3.2)  |         |       |        |        |        |       |        |        |       | C   | C   | 0.041 |
| A128V      | 10 (1.9)   | 1 (0.7)  | 1 (0.8)  | 9 (12.5) | 9 (69.2) |       |        |        |       |        |        |       | C   | C   | <0.001 |
| G130N      | 2 (0.4)    | 2 (2.8)  | 1 (5)    | 1 (3.2)  |         |        |        |        |       |        |        |       | C   | C   | 0.002 |
| G130R      | 2 (0.4)    | 1 (0.7)  | 1 (0.8)  | 1 (1.4)  | 1 (12.5) |       |        |        |        |        |        |       | C   | NR | 0.176 |
| NT131I     | 1 (0.2)    | 1 (1.4)  | 1 (7.7)  |         |       |        |        |        |        |        |        |       | C   | C   | 0.041 |
| M133I      | 1 (0.2)    | 1 (0.7)  | 1 (0.8)  |         |       |        |        |        |        |        |        |       | C   | C   | 0.278 |
| M133L      | 2 (0.4)    | 2 (1.3)  | 2 (1.7)  |         |       |        |        |        |        |        |        |       | C   | NR | 0.077 |
| M133T      | 4 (0.8)    | 3 (2.0)  | 3 (2.5)  | 1 (1.4)  | 1 (12.5) |       |        |        |        |        |        |       | C   | C   | 0.053 |
| FY134L     | 2 (0.4)    | 2 (1.3)  | 2 (1.7)  |         |       |        |        |        |        |        |        |       | C   | C   | 0.077 |
| FY134N     | 2 (0.4)    | 2 (2.8)  | 1 (3.2)  | 1 (12.5) |         |       |        |        |        |        |        |       | C   | C   | 0.002 |
| P142S      | 1 (0.2)    | 1 (0.7)  | 1 (0.8)  |         |       |        |        |        |        |        |        |       | C   | C   | 0.278 |
| P142T      | 1 (0.2)    | 1 (1.4)  | 1 (3.2)  |         |       |        |        |        |        |        |        |       | C   | NR | 0.041 |
| ST143L     | 5 (0.9)    | 5 (6.9)  | 5 (16.1) |         |       |        |        |        |        |        |        |       | C   | C   | <0.001 |
| D144A      | 2 (0.4)    | 1 (0.7)  | 1 (0.8)  |         | 1 (0.3) | 1 (0.5) |         |        |        |        |        |       | C   | C   | 0.727 |
| D144E      | 3 (0.6)    | 1 (0.7)  | 1 (3.3)  | 1 (1.4)  | 1 (7.7)  | 1 (0.3) | 1 (0.5) |         |        |        |        |       | C   | C   | 0.544 |
| D144G      | 1 (0.2)    | 1 (0.7)  | 1 (0.8)  |         |         |        |        |        |        |        |        |       | C   | C   | 0.278 |
| G145A      | 1 (0.2)    | 1 (0.7)  | 1 (0.8)  |         |         |        |        |        |        |        |        |       | C   | C   | 0.699 |
| G145R      | 1 (0.2)    | 1 (0.7)  | 1 (3.3)  |         |         |        |        |        |        |        |        |       | C   | C   | 0.278 |
| N146S      | 1 (0.2)    | 1 (0.3)  |         |         |         |        |        |        |        |        |        |       | C   | -   | 0.699 |
| S154A      | 1 (0.2)    | 1 (0.7)  |         |         |         |        |        |        |        |        |        |       | C   | NR | 0.278 |

Some positions are polymorphic: *"N" is the major aa in genotype A, and "T" is the mayor aa in genotypes D and F. †"Y" is the major aa in genotype D, and "F" is the mayor aa in genotypes A and F. **"T" is the major aa in genotype A, and "S" is the mayor aa in genotypes D and F. P was calculated for differences between genotypes. C, confirmed; NR, not reported.
variables such as cohort size, prevalence of infection, time of introduction or mandatory implementation of vaccination, region...drawbacks of current vaccines, has led to the development of vaccines...itself as genotype D (33%) compared to A (6%) or F (2.3%) ones. This result is in accordance with Ma's study where they observed that HBV genotypes A–D tended to be more prone to harbour VEMs, supporting that genotypes may display different clinical implications on the S gene variability for virus vaccine design. 17 Additionally, in a recent study carried out in Australia, a suboptimal protection against infections caused by subgenotypes other than the antigen present in the vaccine (HBV-A2) was observed. 41 However, in our study, the lower frequencies of VEMs were observed in subgenotypes F1b, F4 and A1, while D1 and D2 showed intermediate prevalence and D3 and D4 presented very high prevalence of VEMs.

Interestingly, more than a half of the samples analysed in this study (58.3%) grouped as genotype F. This represents a relevant fact since data about VEMs for genotype F is very scarce. In this regard, genotype F was frequently associated with hepatitis infections in vaccinated individuals. 55,56 Despite this, in this work we have observed that the prevalence of VEMs for genotype F is very low. The effectiveness of HBV vaccine against different genotypes is a controversial issue. Although many studies have demonstrated that the current vaccine (HBV-A2 based) provides broad protection against the different HBV genotypes, other studies have postulated that protection against more divergent genotypes, such as genotype F, might be a drawback of the current vaccine. 57,58 In the present study, DFMs were observed in 10.7% of the cases, and most of the mutations overlapped with previously described VEMs. Nonetheless, several DFMs outside the 'a' determinant were observed. Likewise, VEMs, DFMs showed a biased distribution by HBV genotypes, with other studies performed in Spain (6.6%), Turkey (8.3%) and China (6.1%). 59–61 The population included in this study was not reached by vaccine implementation and therefore was probably not affected by its selective pressure.

### Table 3

| Variant     | Number (%) | A | A1 | A2 | D | D1 | D2 | D3 | D4 | F | F1b | F4 | Type mutant | P  |
|-------------|------------|---|----|----|---|----|----|----|----|---|-----|----|-------------|----|
| rttL80I     | 2 (0.4)    |   |    |    | 2 (2.8) | 1 (7.7) | 1 (3.2) |  | 2 (0.6) | 1 (0.5) | 1 (1) | ARM | 0.699 |
| rttL80V     | 2 (0.4)    |   |    |    | 2 (1.7) | 1 (1.4) | 1 (7.7) |  | 3 (0.9) | 2 (0.9) | 1 (1) | ARM | 0.917 |
| rttL173L    | 6 (1.2)    | 2 (1.3) | 2 (1.7) | 1 (1.4) | 1 (7.7) |  |  |  |  |  |  |  |  |  |
| rttL180M    | 20 (3.8)   | 8 (5.4) | 2 (6.7) | 6 (5) | 1 (1.4) |  | 1 (12.5) | 11 (3.6) | 7 (3.3) | 4 (1.1) | ARM | 0.331 |
| rttL184A    | 1 (0.2)    | 1 (0.7) | 1 (3.3) |  |  |  |  |  |  |  |  |  |  |  |
| rttL184S    | 3 (0.6)    | 1 (0.7) | 1 (3.3) |  |  |  |  |  |  |  |  |  |  |  |
| rttS202G    | 3 (0.6)    |   |    |    | 1 (1.4) | 1 (3.2) | 2 (0.6) | 2 (2) |  |  |  |  | ARM | 0.278 |
| rttS202I    | 2 (0.4)    | 1 (0.7) | 2 (1.7) |  |  |  |  |  |  |  |  |  |  |  |
| rttM204A    | 3 (0.6)    |   |    |    | 2 (2.8) | 1 (7.7) | 1 (3.2) | 1 (0.3) | 1 (1) |  |  |  | 0.024 |
| rttM204V    | 17 (3.2)   | 6 (4) | 1 (3.3) | 5 (4.2) | 1 (1.4) | 1 (12.5) | 10 (3.2) | 7 (3.3) | 3 (1.1) | ARM | 0.580 |

P was calculated for differences between genotypes.
The prevalence of ARMs observed in the 14 treated patients (78.6%) could be explained as a consequence of selection pressure exerted by the antiviral agent and is consistent with previous reports. Treatment selection pressure of ARMs can lead to virological and biochemical breakthroughs, hepatitis flares, hepatic decompensation and even death. The most frequent mutation (rtM204V/I) was usually accompanied (95% of samples) by a compensatory mutation and could result in false negative, thus increasing the risk of “false” OBI.

Interestingly, ARMs were observed in 3.1% of the 516 treatment-naïve cases. This may be a consequence of either HBV diversity given by replication through an error-prone polymerase or transmission of a mutated variant from patients receiving antiviral therapy to HBV-susceptible persons. The finding of ARMs in naïve antiviral therapy patients with HBV infection has important epidemiologic and clinical implications.

Results in this study corroborate previous findings showing prevalences of ARMs that range between 0 and 5.2%. However, our findings disagree with previous research reporting high rates of polymerase mutations. Such variability, as mentioned above, is likely due to differences in the study design, uncertainty about prior exposure to antiviral therapy, rate of patients on treatment and/or cohort size. Moreover, most of the patients analysed in the present work were HBV-F and none of the previous studies has enrolled such a large number of patients infected with this genotype.

Due to gene overlapping, ARMs induced by antiviral agents, beyond its implication in antiviral therapy efficacy, may impair HBsAg antigenicity and contribute to HBsAg failure detection and vaccine escape.

Finally, some limitations need to be considered. Firstly, the sequence information to detect VEMs, DFMs and ARMs was not determined by next-generation sequencing. The Sanger method was used, so the presence of minor variants at frequencies <15%-20% cannot be excluded. However, there is no information about the importance that, not only the presence of mutations but also the mutations dominancy (>15%-25%), have in the quasispecies infecting a patient on vaccine escape, diagnostic failure or treatment outcomes. Secondly, no data about vaccination were collected in this study. Nevertheless, taking into account the median age of the analysed patients (44 years) and that vaccination programmes in Argentina started in 2000, it is very likely that the great majority of included patients were unvaccinated. Lastly, only one single health centre in the area of Greater Buenos Aires was analysed. It would be advisable to carry out a broader study including other regions of the country to validate the findings at national level. Nevertheless, more than one-third of the Argentinean population lives in the area of Buenos Aires, so the study can be regarded as an acceptable approximation to the current situation.

In conclusion, the current study provides valuable information about mutants in surface antigen and polymerase genes of HBV-infected patients from Argentina. Of particular interest is that HBV-F, the most prevalent in South and Central American countries and the most sparsely characterized genotype, showed a lower prevalence of VEMs and DFMs but similar prevalence of ARMs when compared to HBV-A and HBV-D genotypes. For these reasons, this study constitutes an important reference for Latin American clinicians, who mostly treat patients infected with HBV-F, in order to draw up the treatment guidelines and evaluate the efficacy of vaccine and diagnostic assays, in a region with more than 600 000 000 inhabitants and 5-7 million of HBV-infected people.

ACKNOWLEDGEMENTS

This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (grant number PIP1122015-000595), Universidad de Buenos Aires (grant number UBACyT 20020130100505BA 2014-2017). F.A.D., E.R., R.H.C. and D.M.F. are members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Research Career Program. We would like to thank Mrs. Silvina Heisecke for providing language assistance.

DISCLOSURES

The authors have nothing to disclose with respect to the content of this manuscript.

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REFERENCES

1. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. *Lancet*. 2015;386:1546-1555.

2. WHO. Hepatitis B. http://www.who.int/news-room/fact-sheets/detail/hepatitis-b-accessed-2018. July 2018.

3. McNaughton AL, D’Arrienzo V, Ansari MA, Lumley SF, Littlejohn M, Revill P, McKeating JA, Matthews PC. Insights from deep sequencing of the HBV Genome-UniQue, Tiny, and Misunderstood. *Gastroenterology*. 2018. [Epub ahead of print].

4. Sunbul M. Hepatitis B virus genotypes: global distribution and clinical importance. *World J Gastroenterol*. 2014;20(18):5427-5434.

5. Locarnini SA, Yuen L. Molecular genesis of drug-resistant and vaccine-escape HBV mutants. *Antivir Ther* 2010;15(3 Pt B):451-461.

6. Cassidy A, Mossman S, Olivieri A, De Ridder M, Leroux-Roels G. Hepatitis B vaccine effectiveness in the face of global HBV genotype diversity. *Expert Rev Vaccines*. 2011;10(12):1709-1715.

7. Romano L, Paladini S, Galli C, Raimondo G, Pollicino T, Zanetti AR. *J Viral Hepat*. 2015;22(16):e0172101.

8. Zuckerman JN, Zuckerman AJ. Mutants of the surface protein of hepatitis B virus. *Vaccine*. 2003;60:75-78.

9. Pollicino T, Cacciola I, Saffioti F, Raimondo G. Hepatitis B virus PreS/S gene variants: pathobiology and clinical implications. *J Hepatol*. 2014;61:408-417.

10. Samal J, Kandpal M, Vivekanandan P. Molecular mechanisms underlying occult hepatitis B virus infection. *Clin Microbiol Rev*. 2012;25(1):142-163.

11. Zoulim F. Hepatitis B virus resistance to antiviral drugs: where are we going? *Liver Int*. 2011;31(Suppl 1):111-116.

12. Ahn SH, Park YK, Park ES, et al. The impact of the hepatitis B virus polymerase rtA181T mutation on replication and drug resistance is potentially affected by overlapping changes in surface gene. *J Virol*. 2014;88(12):6805-6818.

13. Kim HS, Chen X, Xu M, et al. Frequency of hepatitis B surface antigen variants (HBsAg) in hepatitis B virus genotype B and C infected East- and Southeast Asian patients: Detection by the Elecsys® HBsAg II assay. *J Clin Virol*. 2018;103:48-56.

14. Stitchi L, Caligiuri P, Cacciani R, Alicino C, Bruzzone B. Epidemiology of HBV S-gene mutants in the Liguria Region, Italy: Implications for surveillance and detection of new escape variants. *Hum Vacc Immunother*. 2013;9(3):569-571.

15. Coppola N, Onorato L, Minichini C, et al. Clinical significance of hepatitis B surface antigen mutants. *World J Hepatol*. 2015;7(27):2729-2739.

16. Avellon A, Echevarria JM. Frequency of hepatitis B virus ‘a’ determinant variants in unscreened Spanish chronic carriers. *J Med Virol*. 2006;78:24-36.

17. Ma Q, Wang Y. Comprehensive analysis of the prevalence of hepatitis B virus escape mutations in the major hydrophilic region of surface antigen. *J Med Virol*. 2012;84(2):198-206.

18. Carman WF. The clinical significance of surface antigen variants of hepatitis B virus. *J Viral Hepat*. 1997;4:11-20.

19. Gencay M, Hübner K, Gohl P, et al. Ultra-deep sequencing reveals high prevalence and broad structural diversity of hepatitis B virus surface antigen mutations in a global population. *PLoS ONE*. 2017;12(5):e0172101.

20. Larkin M, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;23(21):2947-2948.

21. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser*. 1999;41:95-98.

22. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* [Internet]. 2014 [cited 2014 Jul 15];30(9):1312-1313. Available from: http://bioinformatics.oxfordjournals.org/content/early/2014/01/21/bioinformatics.btu033.abstract.

23. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods*. 2012;9(8):772.

24. Korber B, Myers G. Signature pattern analysis: a method for assessing viral sequence relatedness. *AIDS Res Hum Retroviruses*. 1992;8(9):1549-1560.

25. Yan B, Lv J, Feng Y, et al. Temporal trend of hepatitis B surface mutations in the post-immunization period: 9 years of surveillance (2005-2013) in eastern China. *Sci Rep*. 2017;7(1):6669.

26. Hou J, Wang Z, Cheng J, et al. Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. *Hepatology*. 2001;34:1027-1034.

27. Lai MW, Lin TY, Tsaio KC, et al. Increased seroprevalence of HBV DNA with mutations in the s gene among individuals greater than 18 years old after complete vaccination. *Gastroenterology*. 2012;143(2):400-407.

28. Tsai A, Kawai S, Kwei K, et al. Chimeric constructs between two hepatitis B virus genomes confirm transcriptional impact of core promoter mutations and reveal multiple effects of core gene mutations. *Virology*. 2009;387(2):364-372.

29. Hoofnagle JH, Doo E, Liang TJ, Fleisher R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology*. 2007;45(4):1056-1075.

30. Carman WF, Zanetti AR, Karayiannis P, et al. Vaccine-induced escape mutant of hepatitis B virus. *Lancet*. 1990;336(8711):325-329.

31. Hsu HY, Chang MH, Ni YH, Chen HL. Survey of hepatitis B surface variant infection in children 15 years after a nationwide vaccination program in Taiwan. *Gut*. 2004;53(10):1499-1503.

32. Ni YH, Chen DS. Hepatitis B vaccination in children: the Taiwan experience. *Pathol Biol (Paris)*. 2010;58(4):296-300.

33. Sayan M, Sentürk Ö, Akhan SC, Hülügü S, Cekmen MB. Monitoring of hepatitis B virus surface antigen escape mutations and concomitantly nucleos(t)ide analog resistance mutations in Turkish patients with chronic hepatitis B. *Int J Infect Dis*. 2010;14(Suppl 3):e136-e141.

34. Gerlich WH. Do we need better hepatitis B vaccines? *Indian J Med Res*. 2017;145(4):414-419.

35. Jing M, Wang J, Zhu S, et al. Development of a more efficient hepatitis B virus vaccine by targeting hepatitis B virus preS to dendritic cells. *Vaccine*. 2016 Jan 20;34(4):516-522.

36. Fung SK, Lok AS. Hepatitis B virus genotypes: do they play a role in the outcome of HBV infection? *Hepatology*. 2004;40(4):790-792.

37. Purdy MA. Hepatitis B virus S gene escape mutants. *Asian J Transfus Sci*. 2007;1(2):62-70.

38. Velay A, Jeulin H, Eschlimann M, et al. Characterization of hepatitis B virus surface antigen variability and impact on HBs antigen clearance under nucleos(t)ide analogue therapy. *J Viral Hep*. 2016;23:387-398.

39. Lazarevic I, Cupic M, Delic D, Svirilth NS, Simonovic J, Jovanovic T. Prevalence of hepatitis B virus MHR mutations and their correlation with genotypes and antiviral therapy in chronically infected patients in Serbia. *J Med Virol*. 2010;82(7):1160-1167.

40. Wang XY, Harrison TJ, He X, et al. The prevalence of mutations in the major hydrophilic region of the surface antigen of hepatitis B virus varies with subgenotype. *Epidemiol Infect*. 2015;143(16):3572-3582.

41. Cheah BC, Davies J, Singh GR, et al. Sub-optimal protection against past hepatitis B virus infection where subtype mismatch exists between vaccine and circulating viral genotype in northern Australia. *Vaccine*. 2018;36(24):3533-3540.
42. Piñeiro Y, Leone FG, Pezzano SC, Torres C, et al. Hepatitis B virus genetic diversity in Argentina: dissimilar genotype distribution in two different geographical regions; description of hepatitis B surface antigen variants. J Clin Virol. 2008;42(4):381-388.

43. Barbini L, Elizalde M, Torres C, Campos R. Molecular epidemiology and genetic diversity of hepatitis B virus in Mar del Plata city, Argentina. Infect Genet Evol. 2013;19:152-163.

44. Gallego F, Pisano MB, Torres C, et al. Molecular epidemiology of hepatitis B virus in Córdoba, Argentina. J Clin Virol. 2014;63(2):204-210.

45. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol. 2002;10:2059-2073.

46. Devesa M, Loureiro CL, Rivas Y, et al. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. J Med Virol. 2008;10:20-26.

47. Kowalec K, Minuk GY, Borresen ML, et al. Genetic diversity of hepatitis B virus genotypes B6, D and F among circumpolar indigenous individuals. J Viral Hepat. 2013;10:122-130.

48. Mello FC, Souto FJ, Nabuco LC, et al. Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. BMC Microbiol. 2007;10:103.

49. von Meltzer M, Vasquez S, Sun J, et al. A new Amerindian genotype of hepatitis B virus surface protein mutations on the diagnosis of occult hepatitis B virus infection. Hepatology. 2010;52(5):1600-1610.

50. Tacke F, Amini-Bavil-Olyaee S, Heim A, Luedde T, Manns MP, Trautwein C. Acute hepatitis B virus infection by genotype F despite successful vaccination in an immune-competent German patient. J Clin Virol. 2007;38(4):350-357.

51. Tosti ME, Alfonsi V, Lacorte E, et al. Acute hepatitis B after the implementation of universal vaccination in Italy: results from 22 years of surveillance (1993-2014). Clin Infect Dis. 2016;62(11):1412-1418.

52. Sadlier C, Madden K, O’Gorman S, Crowley B, Bergin C. Development of chronic hepatitis B infection in a hepatitis B vaccine responder. Int J STD AIDS. 2017;28(5):526-528.

53. O’Halloran JA, De Gascun CF, Dunford L, et al. Hepatitis B virus vaccine failure resulting in chronic hepatitis B infection. J Clin Virol. 2011;52(2):151-154.

54. Amini A, Varsaneux O, Kelly H, et al. Diagnostic accuracy of tests to detect hepatitis B surface antigen: a systematic review of the literature and meta-analysis. BMC Infect Dis. 2017;17(Suppl 1):698.

55. Gutierrez C, Devesa M, Loureiro CL, Leon G, Liprandi F, Pujol FH. Molecular and serological evaluation of surface antigen negative hepatitis B virus infection in blood donors from Peru with unusual properties. Virus Genes. 2008;10:225-230.

56. Tacke F, Amini-Bavil-Olyaee S, Heim A, Luedde T, Manns MP, Trautwein C. Acute hepatitis B virus infection by genotype F despite successful vaccination in an immune-competent German patient. J Clin Virol. 2007;38(4):350-357.

57. Tosti ME, Alfonsi V, Lacorte E, et al. Acute hepatitis B after the implementation of universal vaccination in Italy: results from 22 years of surveillance (1993-2014). Clin Infect Dis. 2016;62(11):1412-1418.

58. Sadlier C, Madden K, O’Gorman S, Crowley B, Bergin C. Development of chronic hepatitis B infection in a hepatitis B vaccine responder. Int J STD AIDS. 2017;28(5):526-528.

59. O’Halloran JA, De Gascun CF, Dunford L, et al. Hepatitis B virus vaccine failure resulting in chronic hepatitis B infection. J Clin Virol. 2011;52(2):151-154.

60. Amini A, Varsaneux O, Kelly H, et al. Diagnostic accuracy of tests to detect hepatitis B surface antigen: a systematic review of the literature and meta-analysis. BMC Infect Dis. 2017;17(Suppl 1):698.

61. Gutierrez C, Devesa M, Loureiro CL, Leon G, Liprandi F, Pujol FH. Molecular and serological evaluation of surface antigen negative hepatitis B virus infection in blood donors from Peru with unusual properties. Virus Genes. 2008;10:225-230.

62. Thibault V, Servant-Delmas A, Ly TD, Roque-Afonso AM, Laperche S. Detection of in vivo hepatitis B virus surface antigen mutations-A comparison of four routine screening assays. J Viral Hepat. 2018;25(10):1132-1138. [Epub ahead of print].

63. Jardi R, Rodriguez-Frias F, Schaper M, et al. Hepatitis B virus polymerase variants associated with entecavir drug resistance in treatment-naive patients. J Viral Hepat. 2007;14(12):835-840.

64. Han Y, Huang LH, Liu CM, et al. Characterization of hepatitis B virus reverse transcriptase sequences in Chinese treatment naïve patients. J Gastroenterol Hepatol. 2009;24:1417-1423.

65. Ismail AM, Samuel P, Eapen CE, Kannangai R, Abraham P. Antiviral resistance mutations and genotype-associated amino acid substitutions in treatment-naive hepatitis B virus-infected individuals from the Indian subcontinent. Interivirology. 2012;55(1):36-44.

66. Vutien P, Trinh HN, Garcia RT, et al. Mutations in HBV DNA polymerase associated with nucleos(t)ide resistance are rare in treatment-naive patients. Clin Gastroenterol Hepatol. 2014;12(8):1363-1370.

67. Mirandola S, Campagnolo D, Bortoletto G, Franceschini L, Marcolongo M, Alberti A. Large-scale survey of naturally occurring HBV polymerase mutations associated with anti-HBV drug resistance in untreated patients with chronic hepatitis B. J Viral Hepat. 2011;18:e212-e216.

68. Pacheco SR, Dos Santos MMA, Stocker A, et al. Genotyping of HBV and tracking of resistance mutations in treatment-naive patients with chronic hepatitis B. Infect Drug Resist. 2017;10:201-207.

69. Akarsu M, Sengonul A, Tunkurt E, et al. YMDD motif variants in inactive hepatitis B carriers detected by Inno-Lipa HBV DR assay. J Gastroenterol Hepatol. 2006;21:1783-1788.

70. Lee CZ, Lee HS, Huang GT, Yang PM, Sheu JC. Detection of YMDD mutation using mutant-specific primers in chronic hepatitis B patients before and after lamivudine treatment. World J Gastroenterol. 2006;12:5301-5305.

71. Huang ZM, Huang QW, Qin YQ, et al. YMDD mutations in patients with chronic hepatitis B untreated with antiviral medicines. World J Gastroenterol. 2005;11:867-870.

72. Mantovani N, Cicero M, Santana LC, et al. Detection of lamivudine-resistant variants and mutations related to reduced antigenicity of HBsAg in individuals from the cities of Santos and São Paulo, Brazil. Virol J. 2013;10:320.

73. Instituto Nacional de Estadística y Censos (INDEC). Censo Nacional de Población, Hogares y Viviendas 2010 (2010) Available at: http://www.indec.gov.ar/nivel4_default.asp?id_tema_1=2&id_tema_2=41&id_tema_3=135. Last accessed: October 20, 2018.

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How to cite this article: Di Lello FA, Ridruejo E, Martinez AP, Perez PS, Campos RH, Flichman DM. Molecular epidemiology of hepatitis B virus mutants associated with vaccine escape, drug resistance and diagnosis failure. J Viral Hepat. 2019;26:552-560. https://doi.org/10.1111/jvh.13052