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Title: MOLECULAR EPIDEMIOLOGY OF HEPATITIS B VIRUS MUTANTS ASSOCIATED WITH VACCINE-ESCAPE, DRUG-RESISTANCE AND DIAGNOSIS FAILURE

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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 (VEMs), diagnostic failure (DFMs) and treatment resistance mutants (ARMs) among individuals from Buenos Aires, Argentina.

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HBV surface antigen and polymerase sequences obtained from serum samples of 530 HBV-infected individuals were analyzed.

Samples belonged to genotype 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 non-previously reported VEMs and nine DFMs were identified. VEMs and DFMs 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, DFMs 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: Hepatitis B Virus; Vaccine Escape Mutant, Antiviral Resistance, Diagnostic Failure.

Introduction

Hepatitis B virus (HBV) infection and HBV-related complications remain a major global public health problem1 since an estimated 260 million people are chronically infected2 and more than 800.000 deaths occur yearly, mostly from complications, including cirrhosis and hepatocellular carcinoma.

HBV 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" have been proposed after isolation from one individual3. Genotypes and subgenotypes have a restricted ethnic-geographical distribution4.

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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 mutations (ARMs)[5].

HBV vaccine was introduced in the early 1980s and currently, the global coverage with three doses is estimated at 82%6. 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" determinant. It is known that neutralizing antibodies produced after vaccination against HBV are targeted to the conformational epitopes of the "a" determinant7-9.

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 analog (NA) 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 NAs 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,

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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 VEMs, DFMs and ARMs 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.

Materials and Methods

Study population

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

Laboratory determinations

HBV serological markers were analyzed with AxSYM, Abbott Diagnostics, USA (samples before 2010) and Architect Abbott system; Abbott Diagnostics, Wiesbaden, Germany (samples since 2010).

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 to 221). For the first round of PCR amplification, 3 µL of

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extracted DNA and 0.25 µM of each primer were added to AmpliTaq Gold® 360 Master Mix in a final volume of 25 µL. For the second round, 2 µL of first round product were added to 40 µL final volume of PCR mix. The cycling protocol was: denaturing at 94°C 5 min; followed by 40 cycles in the first round and 25 cycles in the second one of 94°C 30 sec, 53°C 30 sec and 72°C 1 min followed by a final extension at 72°C for 5 min.

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.

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) software[20] and edited with BioEdit (v7.1.3.0) software[21]. Phylogenetic trees were constructed using the Maximum Likelihood method performed with RAxML (v 8.0.24) 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 and nucleotide patterns characteristic of each subgenotype within the HBsAg; this was assessed by VisSPA v1.6.2 program[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 analyzed and in less than 10% of the samples in the reference group.

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Mutational Analysis

Nucleotide sequences were aligned and occurrence of each aminoacid at each position of the alignment was analyzed 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 with VEMs (116, 118, 120, 126, 129, 130, 131, 133, 134, 141, 142, 143, 144 and 145) and thirty related with DFMs (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 analyzed in this work according to previous reports[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[29,30]. Mutations at positions rtN236 and rtM250V were determined in 356 out of the 530 samples.

Statistical Analysis

Frequencies were compared using the chi-square test or the Fisher's test. The Student's t-test and the Mann-Whitney U 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).

Nucleotide Sequences Accession Numbers

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

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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.

Results

Characteristics of the study population

Serum samples from 530 patients HBsAg and anti-HBc positive were analyzed. 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: 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 patients 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.

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 aa residues analyzed, while three positions (T116, Q129 and K141) were unchanged in all cases. Additionally, 4 non-reported mutations, namely G130R (2), M133I and P142T were observed. Table 2 shows the mutations at each analyzed position by genotype and subgenotype.

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The most frequent VEMs were: 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 ($p<0.053$). The age of the patients with VEMs was 49 (38-61) versus 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 analyzed 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% ($p <0.001$)].

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 with a triple mutation. Forty-one out of 72 DFMs were shared with the VEMs, given that 14 of the 30 analyzed 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 (2.64%) and A128V (1.88%) (Table 2).

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 non-reported substitutions in six of the 30 positions, namely Y100W (3), Q101P, P120A, M133L (2), P142T and S154A, were detected.

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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%) harbored two or more DFMs simultaneously, while only 3 infected with HBV-A (2%) and none infected with HBV-F presented more than one DFMs (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/143S, 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 those HBeAg-positive (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, Y100C which was highly prevalent in HBV-A (almost exclusive in HBV-A1, 13 out of 14 mutational types) and A128V, G130N, F134N and ST143L, which were mainly detected in genotype D (12.5%, 2.8%, 2.8% and 6.9%, respectively).

Antiviral Resistant Mutations Analysis

ARMs were detected in 27 samples (5.1%) in 6 out of 11 aa residues analyzed. 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 VEMs and DFMs

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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 was 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% in those HBeAg-negative (p=0.318).

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 analyzing these mutants in genotype F.

Since the beginning of the 90s, the emergence and increment of VEMs due to vaccine implementation has 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 less than 5% to more than 40%[14,16,19,25-27,31,33]. Different variables such as cohort size, prevalence of infection, time of introduction or mandatory implementation of vaccination, region analyzed and viral genotypes can affect the rate of VEMs, which hinders comparison of studies.

In this study, VEMs were present in 7.5% of cases and most of them were located in the ''a'' determinant. This result is consistent with other studies performed in Spain (6.6%), Turkey (8.3%) and China (9.01%)[16,25,33]. The population included in this study was not reached by vaccine implementation and therefore was probably not affected by its selective pressure.

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The gradual increase of VEMs over time, as well as other shortcomings of current vaccines, has led to the development of vaccines that include homologous HBsAg subtypes to different regions and neutralizing epitopes of preS1 and preS2 of the predominant HBV strains[34,35].

There is growing evidence that HBV genotypes may play a role in causing different disease profiles in chronic hepatitis B infection[36]. Furthermore, there is evolving evidence that HBsAg variants may influence HBV vaccine and treatment response[37,38]. Several studies have addressed the association of HBV genotype and VEMs[17,39,40]. This association was observed in the cohort analyzed herein, being significantly higher in genotype D (33%) than in 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 harbor 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 A2 showed intermediate prevalence and D2, D3 and D4 presented very high prevalence of VEMs.

Interestingly, more than a half of the samples analyzed in this study (58.3%) grouped as genotype F. This represents a relevant fact since data about VEMs in this genotype, one of the most prevalent in Latin America and Alaska[42-49], is very scarce. In this regard, genotype F was frequently associated with hepatitis infections in vaccinated individuals[50,51]. 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 genotypes6, other studies have postulated that protection against more divergent genotypes, such as genotype F might be a drawback of the current vaccine[52,53].

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Detection of HBsAg is crucial for HBV infection diagnosis and routinely used either for testing of individuals with suspected HBV infection or for screening in blood donors[54].

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 genotype. Twenty-two of the 28 mutation types observed in the analyzed positions had demonstrated low ability to bind antibodies[16-18,25-27]. The most frequent mutation position was Y100C in HBV-A1 (43.3%). This mutation was frequently detected in negative-HBsAg samples and was statistically associated with "false" OBI in other studies[55-57]. In addition, we observed many other mutations in the MHR that reduce the sensitivity of HBV detection assays and could result in false negative, thus increasing the risk of ''false'' OBI[58-60].

The prevalence of DFMs, likewise VEMs, has been increasing since massive introduction of the vaccine[31,32]. Therefore, there is growing public concern regarding assay sensitivity to HBsAg mutants in clinical diagnosis since selection of DFMs carry implicit risk of false negative results[54]. In this regards, there is a concerted effort to understand how mutants affect "a" determinant antigenicity[37].

Fortunately, in recent years, constant development of new enzyme immunoassays with better detection limits has improved the sensitivity and specificity of HBsAg assays. Despite this, HBsAg assays may vary in their ability to detect HBsAg variants[62,63]. For this reason, understanding the prevalence of HBsAg antigenic variation has become a challenge for diagnostic assays design and future changes in the formulation of the vaccine. Consequently, ongoing surveillance of escape mutants is needed.

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The current study found that ARMs were present in 5.1% of cases, with a significantly higher prevalence in patients who reported receiving antiviral treatment.

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[29]. 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 at rtL180M and/or rtV173L, as it has been previously reported[29].

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%[64-69]. However, our findings disagree with previous research reporting high rates of polymerase mutations[70-72]. 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 analyzed 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[73].

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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 was collected in this study. Nevertheless, taking into account the median age of the analyzed patients (44 years) and that vaccination programs in Argentina started in 2000, it is very likely that the great majority of included patients were unvaccinated. Lastly, only one single health center in the area of Greater Buenos Aires was analyzed. 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[73].

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.

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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*		
Positive	269	70
Negative	115	30
ALT*		
Normal	67	7.5
High	317	92.5
Antiviral Treatment		
Yes	14	2.6
No	516	97.4
VEMs		
Detected	40	7.5
No detected	490	92.5
DFMs		
Detected	57	10.7
No detected	473	89.3
ARMs		
Detected	27	5.1
No detected	503	94.9

*Available for 384 patients

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Table 2. Number of VEMs and DFM analyzed variants by genotype and subgenotype, N=530.

Variant	Number (%)	Genotype and Subgenotype N (%)											Mutant type		p
		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	
Y100C	14 (2.6)	14 (9.4)	13 (43.3)	1 (0.8)									-	C	<0.001
Y100W	3 (0.6)	3 (2.0)		3 (2.6)									-	NR	0.021
Q101K	3 (0.6)	3 (2.0)		3 (2.6)									-	C	0.021
Q101P	1 (0.2)	1 (0.7)		1 (0.8)									-	NR	0.278
T118A	3 (0.6)				3 (4.2)		3 (23.1)						C	C	<0.001
T118V	9 (1.7)				9 (12.5)		9 (69.2)						C	C	<0.001
P120A	1 (0.2)									1 (0.3)		1 (1)	C	NR	0.699
P120Q	3 (0.6)									3 (1.0)	2 (0.9)	1 (1)	C	C	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	<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)				NR	C	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)									NR	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)					NR	NR	0.041

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*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 (0.3)		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)										-	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.

C: Confirmed, NR: No Reported

p was calculated for differences between genotypes

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Table 3. Number of ARM analyzed variants by genotype and subgenotype, N=530.

Variant	Number (%)	Genotype and Subgenotype N (%)											Type mutant	p
		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		
rtL80I	2 (0.4)									2 (0.6)	1 (0.5)	1 (1)	ARM	0.699
rtL80V	2 (0.4)				2 (2.8)		1 (7.7)	1 (3.2)					ARM	0.002
rtV173L	6 (1.2)	2 (1.3)		2 (1.7)	1 (1.4)		1 (7.7)			3 (0.9)	2 (0.9)	1 (1)	ARM	0.917
rtL180M	20 (3.8)	8 (5.4)	2 (6.7)	6 (5)	1 (1.4)				1 (12.5)	11 (3.6)	7 (3.3)	4 (4.1)	ARM	0.331
rtT184A	1 (0.2)	1 (0.7)	1 (3.3)										ARM	0.278
rtT184S	3 (0.6)	1 (0.7)	1 (3.3)							2 (0.6)	1 (0.5)	1 (1)	ARM	0.788
rtS202G	3 (0.6)				1 (1.4)			1 (3.2)		2 (0.6)		2 (2)	ARM	0.278
rtS202I	2 (0.4)	1 (0.7)		2 (1.7)									ARM	0.417
rtM204I	3 (0.6)				2 (2.8)		1 (7.7)	1 (3.2)		1 (0.3)		1 (1)	ARM	0.024
rtM204V	17 (3.2)	6 (4)	1 (3.3)	5 (4.2)	1 (1.4)				1 (12.5)	10 (3.2)	7 (3.3)	3 (3.1)	ARM	0.580

p was calculated for differences between genotypes.

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