Journal of Clinical and Translational Hepatology **2023**
DOI: 10.14218/JCTH.2022.00135S

Original Article

High Frequency of Antiviral Resistance Mutations in HBV Genotypes A2 and H: Multidrug Resistance Strains in Mexico

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Received: 30 November 2022 | **Revised:** 4 April 2023 | **Accepted:** 18 April 2023 | **Published online:** Month 00, 2023

Abstract

Background and Aims: Lamivudine (3TC), telbivudine (LdT), entecavir (ETV), adefovir (ADF), and tenofovir (TFV) are drugs used to treat hepatitis B virus (HBV) infection, but specific mutations allow some viruses to become resistant to antiviral drugs or to acquire immune escape capacities. These mutations have not been thoroughly investigated in Mexico. This study aimed to estimate the prevalence of HBV antiviral resistance and escape mutations. ***Methods:*** This cross-sectional study analyzed 158 samples. HBV DNA was extracted, amplified, and sequenced in serum samples using the spin column method, PCR assay, and Sanger's sequencing, respectively. HBV genotypes were determined, and HBV mutations were tested using the Geno2pheno tool. ***Results:*** Overall, 68.4% (108/158) of HBV patients were infected with genotype H, followed by G (11.4%, 18/158), A2 (10.8%, 17/158), F1b (6.9.0%, 11/158), D (1.9%, 3/158), and E (0.6%, 1/158), and 5.1% (8/158) had evidence of recombination. The prevalence of resistance mutations was 8.2% (13/158) and the most common combined mutation was rt180M+rt204V. Notably, we found the combinations rt180M+rt204V+rt173L (n=2) and rt180M+rt204V+rt202G (n=1) that confer multidrug resistance to 3TC, LdT, and ETV. Resistance mutations were found in genotypes A2 (11.8%, 2/17), and H (10.2%, 11/108), and escape mutations were detected in HBV genotypes A2 (11.8%, 2/17), H (10.2%, 11/108), F1b (9.1%, 1/11) and G (5.6%, 1/18). ***Conclusions:*** The highest prevalence of antiviral resistance mutations or escape mutations was detected in HBV genotypes A2 and H. The earliest cases of HBV multidrug resistance were detected in Mexico.

Keywords: Hepatitis B virus; Drug resistance mutations; Multidrug resistance strain; Immune escape.

Abbreviations: 3TC, lamivudine; aa, amino acid; ALT, alanine aminotransferase; ADF, adefovir; DNA, deoxyribonucleic acid; dNTP, deoxynucleotide triphosphate; ELISA, enzyme-linked immunosorbent assay; ETV, entecavir; GTR+G+I, general time reversible with gamma distribution and invariants sites; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IQR, interquartile range; IU, international unit; LdT, telbivudine; MEGA, molecular evolutionary genetics analysis; OBI, occult HBV infection; PCR, polymerase chain reaction; RT, reverse transcriptase; SD, standard deviation; TFV, tenofovir.

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Citation of this article: Jose-Abrego A, Roman S, Rebello Pinho JR, Gomes-Gouvêa MS, Panduro A. High Frequency of Antiviral Resistance Mutations in HBV Genotypes A2 and H: Multidrug Resistance Strains in Mexico. J Clin Transl Hepatol 2023. doi: 10.14218/JCTH.2022.00135S.

Introduction

Hepatitis B virus (HBV) infection is considered one of the most important health problems worldwide, and approximately 30% of the human population has evidence of current or past HBV infection.[1] In 2019, this pathogen caused a total of 820,000 deaths from cirrhosis and hepatocellular carcinoma.[2] Currently, there are five antivirals for the management of chronic HBV infection, lamivudine (3TC), telbivudine (LdT), entecavir (ETV), adefovir (ADF) and tenofovir (TFV).[3,4] The primary goal of treatment is to suppress HBV replication and inhibit the enzymatic functions of the HBV DNA polymerase-reverse transcriptase (RT) domain.[3,4] Most patients experience rapid viral suppression, but some present with treatment failure.[5] That condition occurs when the HBV viral load remains detectable, alanine aminotransferase levels (ALT) are not normalized, and antigen seroconversion is not achieved after treatment.[3,4] The emergence of specific mutations within the HBV polymerase RT region is considered as key for developing treatment failure.[3,4] Some mutations can affect the hepatitis B surface antigen (HBsAg) structure. An alternative open reading frame of the HBV polymerase gene encodes this protein.[6] In conjunction, these variations are known as escape mutations comprising vaccine escape mutations, occult infection, and reduced epitope-antibody binding, and they have been associated with different clinical outcomes.[7–9]

In Mexico, several studies have reported high frequencies of HBV infection in native groups,[10,11] prisoners,[12] sex workers,[13] deferred blood donors,[11] pediatric patients,[14] and patients with human immunodeficiency virus (HIV).[15–17] Sexual promiscuity, injection drug use, and men who have sex with men (MSM) are factors that increase the risk of HBV transmission.[11] In this region, most infections are caused by HBV genotype H, which is frequently asymptomatic with low viral load of <2,000 IU/mL.[18] Occult HBV infection (OBI) is also common in patients with HIV and in native groups.[15,16] Most hepatitis B studies have focused on prevalence, risk factors,

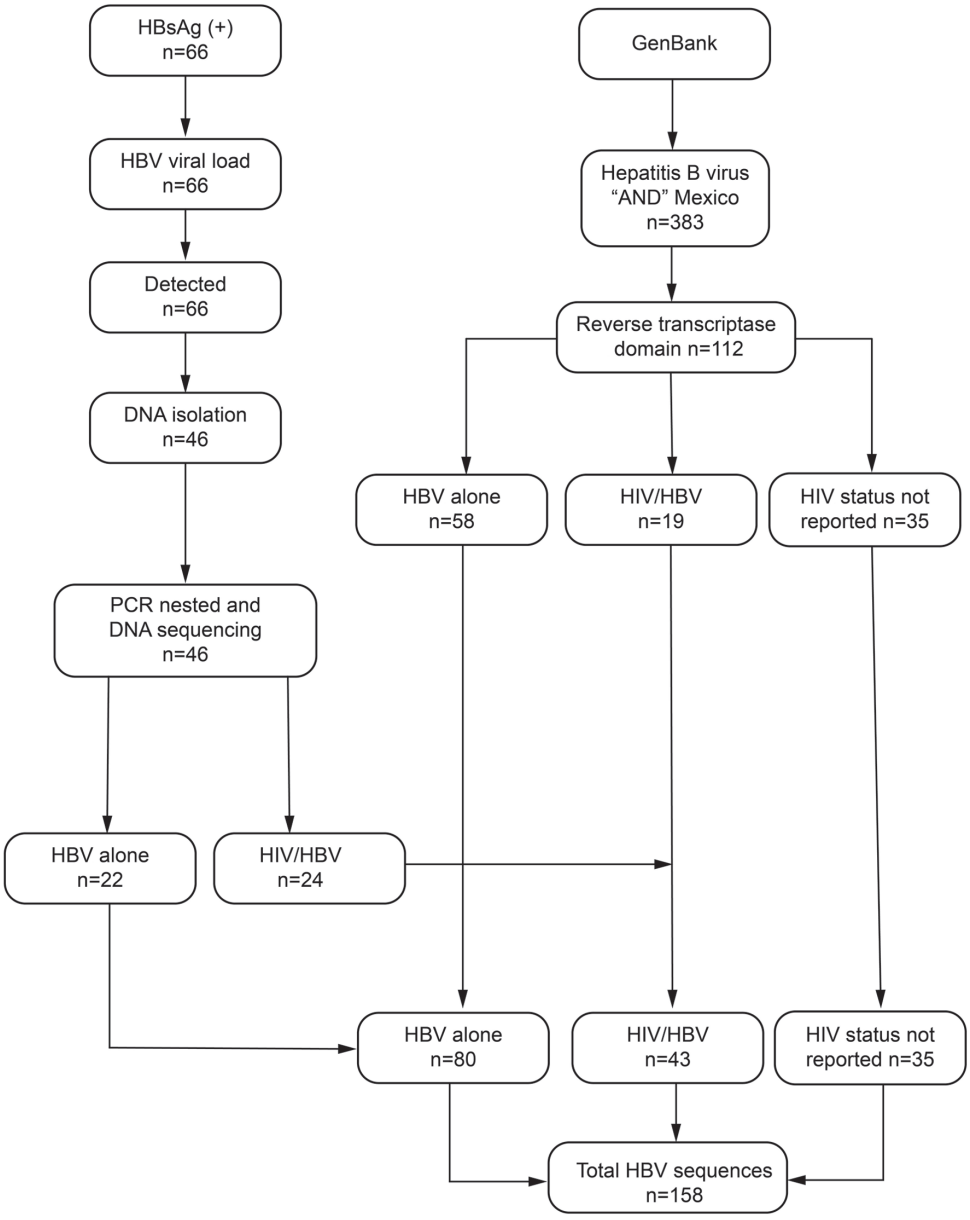

Fig. 1. Flow diagram of the enrollment of the HBV sequences analyzed in this study. On the left, the experimental strategy to obtain HBV sequences of HBV monoinfected and HBV/HIV coinfected patients is shown, while on the right, the selection criteria of GenBank HBV sequences is summarized.

and genotyping. However, little is known about the prevalence of resistance and escape mutations in this region. The surveillance of these mutations is essential to identify which HBV genotype is most likely to be present, select the best treatment alternatives, prevent their spread to other high-risk groups, and avoid the emergence of multidrug-resistant strains.[19–21] Thus, this work aimed to estimate the prevalence of resistance and escape mutations in Mexico.

Methods

Study design and patients

This cross-sectional study was conducted between 2012 and 2018 at the Department of Genomic Medicine in Hepatology, Hospital Civil "Fray Antonio Alcalde" in Guadalajara in collaboration with the Institute of Tropical Medicine and School of Medicine, Department of Gastroenterology, University of São Paulo, and Hospital Israelita Albert Einstein, São Paulo, Brazil. Adult patients of any age with risk factors for viral hepatitis and laboratory evidence of HBV infection (based on HBsAg) were included in the study. The levels of liver enzymes were measured with an AU5800 Clinical Chemistry System (Beckman Coulter Inc. USA). General information including sex, age, and treatment was obtained from the patient's clinical history. Informed consent was obtained from all patients. A total of 158 HBV sequences were studied, 46 were obtained via *de novo* experimental work and 112 were downloaded from GenBank (Fig. 1). Mexican GenBank sequences were included to increase population size and re-

duce bias in prevalence calculations.

Experimental samples and HBV DNA extraction

Of the 66 HBsAg-positive patients recruited, only 46 had detectable HBV viral loads. HBsAg was tested with a second enzyme-linked immunosorbent assay (ELISA, HBsAg, Monolisa PLUS; Bio-Rad, USA), and HBV viral load was assayed with COBAS AmpliPrepCOBAS TaqMan HBV tests, V2.0 (Roche Molecular Systems, Branchburg, NJ) following the manufacturer's instructions, DNA was isolated from 400 µL of serum using QIAamp DNA Mini Kits (Qiagen Science, Hilden, Germany). A 1 µg/µL DNA carrier (Qiagen Science, Hilden, Germany) was used in a final elution volume of 50 µL to improve extraction performance.

PCR amplification

DNA extracts were tested by nested PCR, amplifying an HBV polymerase gene fragments.[22] For the first and second PCR rounds, a final volume of 50 µL of PCR mixture was made up of 31.5 µL of nuclease-free water, 10 µL of reaction buffer (5×), 1 µL of dNTPs (10 mM), 0.5 µL Q5 Hot Star HighFidelity DNA polymerase (2 UI/mL), 1 µL of forward primer (25 pmol/µL), 1 µL of reverse primer (25 pmol/µL), and 5 µL of DNA. Primer PS3132F (5′-CCT CCY GCH TCY ACC AAT CG-'3; nt 31323151) and primer 2920RM (5′-ACG TCC CKC GHA GRA TCC AG-3′) were used in the first PCR (fragment of 1,506 pb). Next, primer PS3201F (5′-CAY CCH CAG GCM ATG CAG TGG-3′; nt 3,201–3,221), and primer P1285R (5′-CWA GGA GTT CCG CAG TAT GG-3′; nt 1,285–1,266) for the second PCR (fragment of 1,306 pb). The DNA amplification was performed under the following conditions. One cycle at 98°C for 1 m, 34 cycles at 94°C for 10 s, 48°C (first round) or 54°C (second round) for 30 s, and 72°C for 50 s, followed by a final elongation step of 72°C at 5 m. The PCR products were visualized by electrophoresis on a 2% agarose gel and stained with SYBR Green (Invitrogen, Carlsbad, CA, USA).

HBV sequencing

DNA sequencing was done with 1 µL of the second PCR product, 4 µL of nuclease-free water, 1 µL of sequencing buffer (5×), 2 µL of forward or reverse primer (0.8 pmol/µL), and 2 µL of BigDye Terminator V3.1 (Applied Biosystems, Foster City, CA, USA). The HBV RT domain of the polymerase gene was sequenced by generating three overlapping fragments: primer PS3132F (5′-CCT CCY GCH TCY ACC AAT CG-3′; nt 3,132–3,151) and HBV477R (5′-GGA CAV ACG GGC AAC ATA CCT T-3′; nt 477– 456) for fragment one; L372 (5′TCG YTG GAT GTR TCT GCG GCG TTT TAT3′; nt 370–396) and RADE2M (5′-TGR CAN ACY TTC CAR TCA ATN GG-3′; nt 989–970) for fragment two; and P781F (5′-GAR TCC CTT TWT RCC KCT RTT ACC-3′; nt 781–804) and P1285R (5′-CWA GGA GTT CCG CAG TAT GG-3′; nt 1,285–1,266) for fragment three. All primers used in this study have been previously described.[17] Cycle sequencing was performed for one cycle at 96°C for 1 m, 24 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 m, followed by a final step of 4°C to preserve the sample. Sequencing reactions were purified using ExoSAP-IT PCR clean-up kits (GE Healthcare, Buckinghamshire, UK) or ethanol/EDTA precipitation following the manufacturer's instructions. The capillary electrophoresis was carried out in the instrument ABI 3500 DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

GenBank samples

To increase the sample size of our study, we downloaded nucleotide sequences from GenBank using the research criteria nucleotide (hepatitis B virus [Organism] OR hepatitis B virus [All Fields]) AND Mexico [All Fields] NOT clone [All Fields] AND viruses [filter]). We found 383 HBV sequences from Mexico, of which four were excluded because they were not isolated from a human host, 196 corresponded to other genomic regions, and 71 had low-quality sequences, resulting in 112 RT domain sequences. The HBV sequences were classified as HBV-alone, HIV/HBV, and HIV status not reported, according to the information in the GenBank or publication.

HBV genotyping

Experimental and downloaded samples were aligned with reference sequences using the ClustalW method implemented in MEGA7 software.[23] The reference sequences used in this study were HE974375 and HE974370 for subgenotype A1; HE576989 and HE576988 for subgenotype A2; AM184126 and AM184125 for subgenotype A3; AF100309 and AB033554 for genotype B; AY123041 and AB014381 for genotype C; Y07587 and AB674425 for subgenotype D1; EU594406 and JN642163 for subgenotype D2; KP090180 and KM519455 for subgenotype D3; KM606755 and KM606754 for subgenotype D4; GQ205388 and GQ205387 for subgenotype D5; KF170740.1 and FJ904442.1 for subgenotype D6; FJ904425.1 for D7; KT192626.1, KF922439.1, and KX186584.1 for genotype E; AY090458 and AY090459 for subgenotype F1a; HM585192 and HM585191 for subgenotype F1b; DQ899145 and DQ899146 for subgenotype F2b; KJ843191 and KC494402 for subgenotype F2a; KP718112.1 and FJ589067 for subgenotype F3; HE981181 and DQ823088 for subgenotype F4; AB625343.1, AB625342.1 and AB064310 for genotype G; AY090457, AB516393.1 and AB516395.1 for genotype H; FJ023670, FJ023673.1 for genotype I; AB486012.1 and EU155829.1 for genotype J; and AF046996 as the out group.

Maximum likelihood and general time reversible with gamma distribution and invariants sites (GTR+G+I) were the methods used to build the phylogenetic tree. Accession numbers submitted to GenBank of the HBV samples identified in this study appear as MF150686, MF150687, MK568525, MK568530, MK568531, MK568535, MN818843, MN818844 for genotype A2; MF150685 was subgenotype D4; MF150688, MF150689, MF150690, MF150693, MF150694 were subgenotype F1b; MK568529, MK568536, MN818845, MN818846, MN818847 were genotype G; MF150691, MF150692, MF150695, MF150696, MK568522, MK568523, MK568524, MK568526, MK568527, MK568528, MK568532, MK568533, MK568534, MN818829, MN818842 were genotype H. For highly divergent sequences from their classification cluster, a recombinant analysis was carried out using three tools, GenBank genotyping tool (https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi), Jumping profile hidden Markov model (http://jphmm.gobics.de/jphmm.html), and HBV phylogenetic typing tool version 2.58 (https://www.genomedetective.com/app/typingtool/hbv/). A recombinant sample was considered when two of the three methods confirmed its result.

Detection of HBV antiviral resistances

Antiviral resistance mutations were detected in the RT domain of the HBV polymerase gene. The mutations were tested using the Geno2pheno tool of the Max Planck Institute Informatik (https://hbv.geno2pheno.org/), then each mutation was confirmed manually in the alignment. All HBV clinical mutations have been previously reported and implemented in HBV clinical guidelines.[3,4] The mutations and their relationship with each drug were classified as follows:

3TC samples with M204V/I, L180M+M204V, L180M+M204V/I±I169T±V173L±M250V, and L180M+M204V/I±T184G±S202I/G substitutions were considered as resistance A181T/V was considered as limited susceptibility. Except for the M204V mutation, these same interpretations were used for LdT. ETV: L180M+M204V/I±I169T±V173L±M250V and L180M+M204V/I±T184G±S202I/G combinations were interpreted as resistance, while M204V/I and L180M+M204V conferred limited susceptibility. ADV: A181T/V/S or N236T presence was considered resistant, while M204V/I and L180M+M204V were considered limited susceptibility. TFV: We also investigated the new quadruple mutation CYEI (S106C+H126Y+D134E+L269I) and A194T associated with TFV resistance. Also, A181T/V and N236T were considered as limited susceptibility.

Detection of HBV surface antigen escape mutations

We investigated 66 escape mutations in the alternative open reading frame of HBsAg (226 aa). The mutations were assessed and interpreted according to the Geno2pheno tool of Max Planck Institute Informatik (https://hbv.geno2pheno.org/).

Statistical analysis

The statistical analysis were performed with SPSS version 21 (IBM Corp., Armonk,. NY, USA and a p-values <0.05 was considered significant. Quantitative variables were reported as medians and interquartile range (IQR) or means with standard deviations (SDs). Categorical variables were reported as frequency and percentage. The significance of differences among groups were determined with Mann-Whitney U-tests, Student's t-tests, or Fisher's exact tests, depending on the variable type.

Ethical approval

The Institutional Review Board of the Health Sciences Center from the University of Guadalajara approved the study (CI-07218). This research was conducted following the ethical principles of the 2013 Declaration of Helsinki.

Results

Characteristics of the study groups

A total of 158 samples were analyzed, 29.1% (46/158) were experimental sequences, and 70.9% (112/158) were sequences obtained from GenBank. Among the experimental samples, most of the patients were men (81.0%, 38/46) with a mean age of 36.7 ± 12.4 years. Among them, 47.8% (22/46) were HBV monoinfected patients, and 52.2% (24/46) were coinfected with HIV (HBV/HIV coinfected). We found no significant differences in clinical characteristics of the study groups (Table 1). Of the HBV/HIV coinfected patients, half had a treatment record, 10 were receiving TFV/emtricitabine, and two received 3TC/TFV. At the time of the study, no HBV monoinfected patients had received HBV treatment. Regarding the GenBank samples, 68.7% of the sequences had a group record (58 HBV-alone and 19 HIV/HBV). Overall, 50.6% (80/158) of the sequences were HBV monoinfected, 27.2% (43/158) were HIV/HBV coinfected, and 22.2% (35/158) did not have HIV status. The samples were from four regions of Mexico, western (69.0%, 109/158), central (25.3%, 40/158), southern (3.8%, 6/158), and northern (1.9%, 3/158).

HBV genotypes

The HBV genotype H was the most prevalent (68.4%, 108/158), followed by G (11.4%, 18/158), A2 (10.8%, 17/158), F1b (6.9%, 11/158), D (1.9%, 3/158), and E (0.6%, 1/158) (Table 2). The distribution of HBV genotypes was similar in HBV monoinfected and HIV/HBV coinfected individuals, except for the proportion of HBV genotype G, which was predominant in the HIV/HBV group (HIV/HBV coinfected 14.0% (6/43) vs. HBV-alone 0.0% (0/80), p=0.003). In addition, 5.8% (8/158) of the HBV sequences had evidence of recombination (Supplementary Fig. 1). The most common HBV recombination was A/G with 50% (4/8), followed by H/G (25%, 2/8), H/F (12.5%, 1/8), and A/D (12.5%, 1/8). The proportion of HBV recombinant genotypes tended to be higher in patients with HIV than in the rest of the groups (HIV/HBV coinfected 9.3% (4/43) vs. HBV-alone 3.8% (3/80), p=0.238; HIV/HBV coinfected 9.3% (4/43) vs. HIV status not reported group 2.9% (1/35), p=0.372; Table 2).

Antiviral resistance mutations

Overall, 8.2% (13/158) of the sequences had at least one HBV resistance mutation (Table 2). The variants were rt180M+rt204V (n=5), rt180M+ rt204V+rt173L (n=2), rt180M+ rt204V+rt202G (n=1), rt204V (n=2), rt204I (n=1), rt181V (n=1), and rt194T (n=1). The most common resistance pattern was rt180M+rt204V (5.1%, 8/158). Based on the interpretation of each mutation, 3TC (7.0%, 11/158) and LdT (5.7%, 9/158) were the most common resistances, whereas that less frequent were to ETV (1.9%, 3/158), ADF (0.6%, 1/158), and TFV (0.6% (1/158).

Drug resistance differed in HIV/HBV coinfected and HBV-alone variants. The prevalence of HBV resistance mutations was 16.3% (7/43) in HIV/HBV coinfected and 5.0% (4/80) in HBV-alone (p=0.0489). Also, resistance to 3TC (16.3%, 7/43), LdT (11.6%, 5/43), and ETV (4.7%, 2/43) were predominant in HIV/HBV coinfected, but resistance to ADV (1.2%, 1/80) and TFV (1.2%, 1/80) was seen in HBV-alone (Table 2). Notably, we found the combinations of rt180M+rt204V+rt173L (n=2) and rt180M+rt204V+rt202G (n=1) that confer multidrug resistance to 3TC, LdT, and ETV. Multidrug stains were detected in 1.9% (3/158) of the total sequences; all were HBV genotype H (HIV/HBV coinfected: MT820157.1, MN818838.1; and HIV status not reported: HM117850.1).

Among the six HBV genotypes detected, resistance mutations were only seen in genotypes A2 (11.8%) and H (10.2%; Fig. 2A). Both HBV genotypes were mainly resistant to 3TC (A2: 11.8%, 2/17; H: 8.3%, 9/108) and LdT (A2: 11.8%, 2/17; H: 6.5%, 7/108) (Fig. 2B–C). Resistant mutations to ETV (2.8%, 3/108), ADF (0.9%, 1/108) and TFV (0.9%, 1/108) were only detected in genotype H (Fig. 2D–F). Those mutations associated with intermediate/reduced susceptibility to ETV (17.6%, 3/17 vs. 9.3%, 10/108, p=0.383) and ADF (17.6%, 3/17 vs. 12.0%, 13/108, p=0.446) tended to be higher in genotype A2 than H.

Escape mutations

The prevalence of HBsAg escape mutations was 9.5% (15/158; Table 2). The variants were s129H (5.1%, 8/158), s144E (1.3%, 2/158), s145R (0.6%, 1/158), s128V (0.6%, 1/158), s126I (0.6%, 1/158), s120T (0.6%, 1/158), and s118K (0.6%, 1/158). The prevalence of escape mutations was significantly higher in HIV/HBV coinfected individuals than in those with HBV-alone [30.2% (13/43) vs. 0.0% (0/80), respectively p=2.9e-07]. Most of the escape mutations were detected in HBV genotypes A2 (11.8%, 2/17), H (10.2%, 11/108), F1b (9.1%, 1/11), and G (5.6%, 1/18; Fig. 3A–H). The mutations s126I (5.9%, 1/17) and s128V (5.9%, 1/17) were predominant in genotype A2. While s118K

Table 1. Overall characteristics of study populations

Characteristics	Total, *n*=158	HBV-alone, *n*=22	HIV/HBV, *n*=24	GenBank, *n*=112	*p*-value
Sex					
Female	8 (19.0%)	6 (28.6%)	2 (9.5%)	NA	
Male	34 (81.0%)	15 (71.4%)	19 (90.5%)	NA	0.238[a]
Age (year)					
Mean (SD)	36.7 (12.4)	35.9 (15.0)	37.6 (9.6)	NA	0.670[b]
ART					
without record	122 (77.2%)	0 (0.0%)	10 (41.7%)	112 (100%)	2.2e-16[a]
3TC+EFV+ABC	1 (0.6%)	0 (0.0%)	1 (4.2%)	0 (0.0%)	0.291[a]
3TC+LPV/r+RAL+TFV+AZT	1 (0.6%)	0 (0.0%)	1 (4.2%)	0 (0.0%)	0.291[a]
ABC+TFV+FTC+LPV/r	3 (1.9%)	0 (0.0%)	3 (12.5%)	0 (0.0%)	0.005[a]
EFV+FTC+TFV	5 (3.2%)	0 (0.0%)	5 (20.8%)	0 (0.0%)	0.001[a]
FTC+LPV/r+TFV	2 (1.3%)	0 (0.0%)	2 (8.3%)	0 (0.0%)	0.041[a]
None	24 (15.2%)	22 (100.0%)	2 (8.3%)	0 (0.0%)	1.85e-26[a]
HBV viral load (IU/mL)					
Median (Q1, Q3)	13,183.0 (1,862.0, 177,828.0)	3,139.5 (353.0, 55,667.5)	26,915.0 (2,884.0, 177,828.0)	NA	0.200[c]
ALT IU/L					
Median (Q1, Q3)	34.0 (23.0, 92.0)	37.5 (25.5, 510.2)	34.0 (23.0, 92.0)	NA	1.000[c]
AST IU/L					
Median (Q1, Q3)	31.0 (25.0, 39.0)	29.0 (22.8, 122.0)	31.0 (27.0, 39.0)	NA	0.821[c]
GGT IU/L					
Median (Q1, Q3)	28.0 (18.5, 69.0)	16.5 (14.0, 46.0)	29.0 (19.8, 69.0)	NA	0.224[c]
Mexico's regions					
North	3 (1.9%)	0 (0.0%)	0 (0.0%)	3 (2.7%)	1.000[a]
Center	40 (25.3%)	0 (0.0%)	0 (0.0%)	40 (35.7%)	1.01E-06[a]
Western	109 (69.0%)	16 (72.7%)	24 (100.0%)	69 (61.6%)	0.001[a]
South	6 (3.8%)	6 (27.3%)	0 (0.0%)	0 (0.0%)	3.80e-061[a]
HBV status					
HBV-alone	80 (50.6%)	22 (100.0%)	0 (0.0%)	58 (51.8%)	
HIV/HBV	43 (27.2%)	0 (0.0%)	24 (100.0%)	19 (17.0%)	
HIV status not reported	35 (22.2%)	0 (0.0%)	0 (0.0%)	35 (31.2%)	

Age was a variable with normal distribution. [a]Pearson's Chi-squared test or Fisher's exact test, [b]Student's t-test, [c]Mann-Whitney U test. 3TC, lamivudine; ABC, abacavir; ALT, alanine aminotransferase; ART, antiretroviral therapy; AST, aspartate aminotransferase; AZT, zidovudine; EFV, efavirenz; FTC, emtricitabine; GGT, gamma-glutamyl transferase; LPV/r, lopinavir and ritonavir; NA, not available; Q1, quartile 1; Q3, quartile 3; RAL, raltegravir; SD, standard deviation; TFV, tenofovir.

Table 2. Major resistance and escape mutations in study populations

Mutation	Total, *n*=158	HBV-alone, *n*=80	HIV/HBV, *n*=43	HIV status not reported, *n*=35	*p*-value
RT substitutions					
180M +204V	5 (3.2%)	2 (2.5%)	3 (7.0%)	0 (0.0%)	0.229[a]
180M+204V+173L	2 (1.3%)	0 (0.0%)	1 (2.3%)	1 (2.9%)	0.242[a]
180M+204V+202G	1 (0.6%)	0 (0.0%)	1 (2.3%)	0 (0.0%)	0.494[a]
204V	2 (1.3%)	0 (0.0%)	2 (4.7%)	0 (0.0%)	0.121[a]
204I	1 (0.6%)	0 (0.0%)	0 (0.0%)	1 (2.9%)	0.222[a]
180M*	3 (1.9%)	0 (0.0%)	3 (7.0%)	0 (0.0%)	0.040[a]
180M+204M+173L*	2 (1.3%)	0 (0.0%)	2 (4.7%)	0 (0.0%)	0.121[a]
181V	1 (0.6%)	1 (1.2%)	0 (0.0%)	0 (0.0%)	1.000[a]
194T	1 (0.6%)	1 (1.2%)	0 (0.0%)	0 (0.0%)	1.000[a]
3TC-response					
Sensitive	141 (89.2%)	77 (96.2%)	31 (72.1%)[b]	33 (94.3%)	2.3e-04[a]
Intermediate/reduced susceptibility	6 (3.8%)	1 (1.2%)	5 (11.6%)[a]	0 (0.0%)	0.014[a]
Resistant	11 (7.0%)	2 (2.5%)	7 (16.3%)[a]	2 (5.7%)	0.015[a]
LdT-response					
Sensitive	143 (90.5%)	77 (96.2%)	33 (76.7%)[a]	33 (94.3%)	0.0024[a]
Intermediate/reduced susceptibility	6 (3.8%)	1 (1.2%)	5 (11.6%)[a]	0 (0.0%)	0.0142[a]
Resistant	9 (5.7%)	2 (2.5%)	5 (11.6%)	2 (5.7%)	0.0998[a]
ADV-response					
Sensitive	141 (89.2%)	77 (96.2%)	31 (72.1%)[b]	33 (94.3%)	2.20e-04[a]
Intermediate/reduced susceptibility	16 (10.1%)	2 (2.5%)	12 (27.9%)[b]	2 (5.7%)	6.68e-05[a]
Resistant	1 (0.6%)	1 (1.2%)	0 (0.0%)	0 (0.0%)	1.000[a]
ETV-response					
Sensitive	142 (89.9%)	78 (97.5%)	31 (72.1%)[b]	33 (94.3%)	6.68e-05[a]
Intermediate/reduced susceptibility	13 (8.2%)	2 (2.5%)	10 (23.3%)[b]	1 (2.9%)	3.13e-05[a]
Resistant	3 (1.9%)	0 (0.0%)	2 (4.7%)	1 (2.9%)	0.118[a]
TFV-response					
Sensitive	157 (99.4%)	79 (98.8%)	43 (100.0%)	35 (100.0%)	1.000[a]
Intermediate/reduced susceptibility	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1.000[a]
Resistant	1 (0.6%)	1 (1.2%)	0 (0.0%)	0 (0.0%)	1.000[a]
Any resistance substitution					
Sensitive	140 (88.6%)	76 (95.0%)	31 (72.1%)[b]	33 (94.3%)	6.97e-04[a]
Intermediate/reduced susceptibility	5 (3.2%)	0 (0.0%)	5 (11.6%)[a]	0 (0.0%)	0.0017[a]
Resistant	13 (8.2%)	4 (5.0%)	7 (16.3%)[a]	2 (5.7%)	0.0489[a]
HBsAg substitutions					
s118K	1 (0.6%)	0 (0.0%)	1 (2.3%)	0 (0.0%)	0.494[a]
s120T	1 (0.6%)	0 (0.0%)	1 (2.3%)	0 (0.0%)	0.494[a]
s126I	1 (0.6%)	0 (0.0%)	1 (2.3%)	0 (0.0%)	0.494[a]
s128V	1 (0.6%)	0 (0.0%)	1 (2.3%)	0 (0.0%)	0.494[a]
s129H	8 (5.1%)	0 (0.0%)	8 (18.6%)	0 (0.0%)	2.10e-05[a]
s144E	2 (1.3%)	0 (0.0%)	0 (0.0%)	2 (5.7%)	0.048[a]
s145R	1 (0.6%)	0 (0.0%)	1 (2.3%)	0 (0.0%)	0.494[a]

(continued)

Table 2. *(continued)*

Mutation	Total, *n*=158	HBV-alone, *n*=80	HIV/HBV, *n*=43	HIV status not reported, *n*=35	*p*-value
Escape mutants					
without escape	143 (90.5%)	80 (100.0%)	30 (69.8%)	33 (94.3%)	
with escape	15 (9.5%)	0 (0.0%)	13 (30.2%)[a]	2 (5.7%)	1.40e-05[a]
HBV genotypes					
A2	17 (10.8%)	11 (13.8%)	6 (14.0%)[c]	0 (0.0%)	0.033[a]
D	3 (1.9%)	2 (2.5%)	1 (2.3%)	0 (0.0%)	1.000[a]
E	1 (0.6%)	0 (0.0%)	0 (0.0%)	1 (2.9%)	0.222[a]
F1b	11 (7.0%)	4 (5.0%)	1 (2.3%)	6 (17.1%)[c]	0.032[a]
G	18 (11.4%)	0 (0.0%)	6 (14.0%)[a]	12 (34.3%)	9.60e-08[a]
H	108 (68.4%)	63 (78.8%)[d]	29 (67.4%)	16 (45.7%)	0.002[a]
HBV recombinants					
without evidence	150 (94.9%)	77 (96.2%)	39 (90.7%)	34 (97.1%)	
with evidence	8 (5.1%)	3 (3.8%)	4 (9.3%)	1 (2.9%)	0.392[a]
Recombination pattern					
A/G	4 (2.5%)	1 (1.2%)	3 (7.0%)	0 (0.0%)	0.118[a]
A/D	1 (0.6%)	1 (1.2%)	0 (0.0%)	0 (0.0%)	1.000[a]
H/F	1 (0.6%)	0 (0.0%)	1 (2.3%)	0 (0.0%)	0.494[a]
H/G	2 (1.3%)	1 (1.2%)	0 (0.0%)	1 (2.9%)	0.468[a]
without recombination	150 (94.9%)	77 (96.2%)	39 (90.7%)	34 (97.1%)	0.392[a]

[a]Pearson's Chi-squared test or Fisher's exact test. *The substitutions 180M and 180M+204M+173L were considered as mutations associated with intermediate/reduced susceptibility. [a]HIV/HBV vs. HBV-alone, [b]HIV/HBV vs. Other groups, [c]HIV/HBV vs. HIV status not reported group, [d]HBV-alone vs. HIV status not reported group. 3TC, lamivudine; ADV, adefovir; ETV, entecavir; HBsAg, hepatitis B surface antigen; LdT, telbivudine; TFV, tenofovir.

(0.9%, 1/108), s129H (7.4%, 8/108), s144E (0.9%, 1/108), and s145R (0.9%, 1/108) were more frequent in genotype H. Particularly, s144E (*n*=1) and s120T (*n*=1) mutations were detected in genotypes F1b and G, respectively.

Discussion

This study found evidence of the circulation of HBV genotypes H, G, A2, F1b, D, and E in Mexico. This genotypic distribution was different compared to other regions of the world.[1,5] The high prevalence of genotype H is explained because it is endemic to the Mexican indigenous populations and is widely distributed in all high-risk populations in the country.[10–12] Most of the HBV genotypes were present in both HIV/HBV coinfected and HBV monoinfected individuals, except for genotype G. The high prevalence of this genotype in HIV/HBV coinfected individuals may have resulted from a combination of factors, including a weakened immune system and high prevalence of risk factors. HBV genotype G is often detected in MSM and in patients with HIV.[11] This genotype has a unique insertion of 36 nucleotides[24] that may confer a genetic predisposition for HBV anal-genital transmission among the MSM group.[25] However, further study is needed to fully understand the mechanisms behind genotype G infection in this high-risk group. On the other hand, genotypes A2 and D were probably introduced during the Spanish conquest, as a study has reported that the genotypes A2 and D4 found in Mexico have a genetic relationship with European and Caribbean strains.[11] Here, we also identified the subgenotype F1b, common in South American countries.[26,27] In 2017, subgenotype F1b was firstly reported in Mexico.[11] Particularly, three cases were identified in Mixtecos, a native population from Oaxaca.[11] Also, Fragoso-Fonseca *et al*.[28] confirmed the presence of four subgenotype F1b complete genomes.[28] The data suggest that the distribution of subgenotype F1b may be greater than expected in Mexico. Finally, one case of African genotype E was found. This genotype is mainly reported in Senegal, Nigeria, Cameroon, Congo, and Angola.[29] According to the information available in the GenBank, genotype E (KT192626.1) was probably introduced to Mexico in 2011 by an African immigrant with chronic hepatitis B.[30] Also, the Blast tool reported a 99.56% identity with a genotype E variant 1 found in South Africa.[29,30] The data indicate that current human migratory movements may introduce new HBV genotypes to Mexico, particularly in the most vulnerable groups.

Overall, we found a prevalence of 5.1% of HBV strains with evidence of recombination in Mexico, mainly in those HIV/HBV coinfected. Recombinant HBV infections may be due to the high prevalence of HBV genotype mixtures in patients with HIV.[31] Previously, was reported that HIV patients begin their HBV infection at 21 years, and they can be coinfected with more than one HBV genotype around 37 years of age.[17] The long-term presence of different HBV genotypes in a single patient may increase the risk of recombinant strains with new clinical properties.[32] Herein, the most common recombinant HBV strain was A/G, consistent with other studies.[32–34] Unlike elsewhere, we also found recombinant strains with the endemic genotype H, such as H/F and H/G.

To date, two studies have reported the frequency of HBV resistance in Mexico. Alvarado-Esquivel *et al*.,[35] in 2005, using a line probe assay, reported 2.6% of antiviral resist-

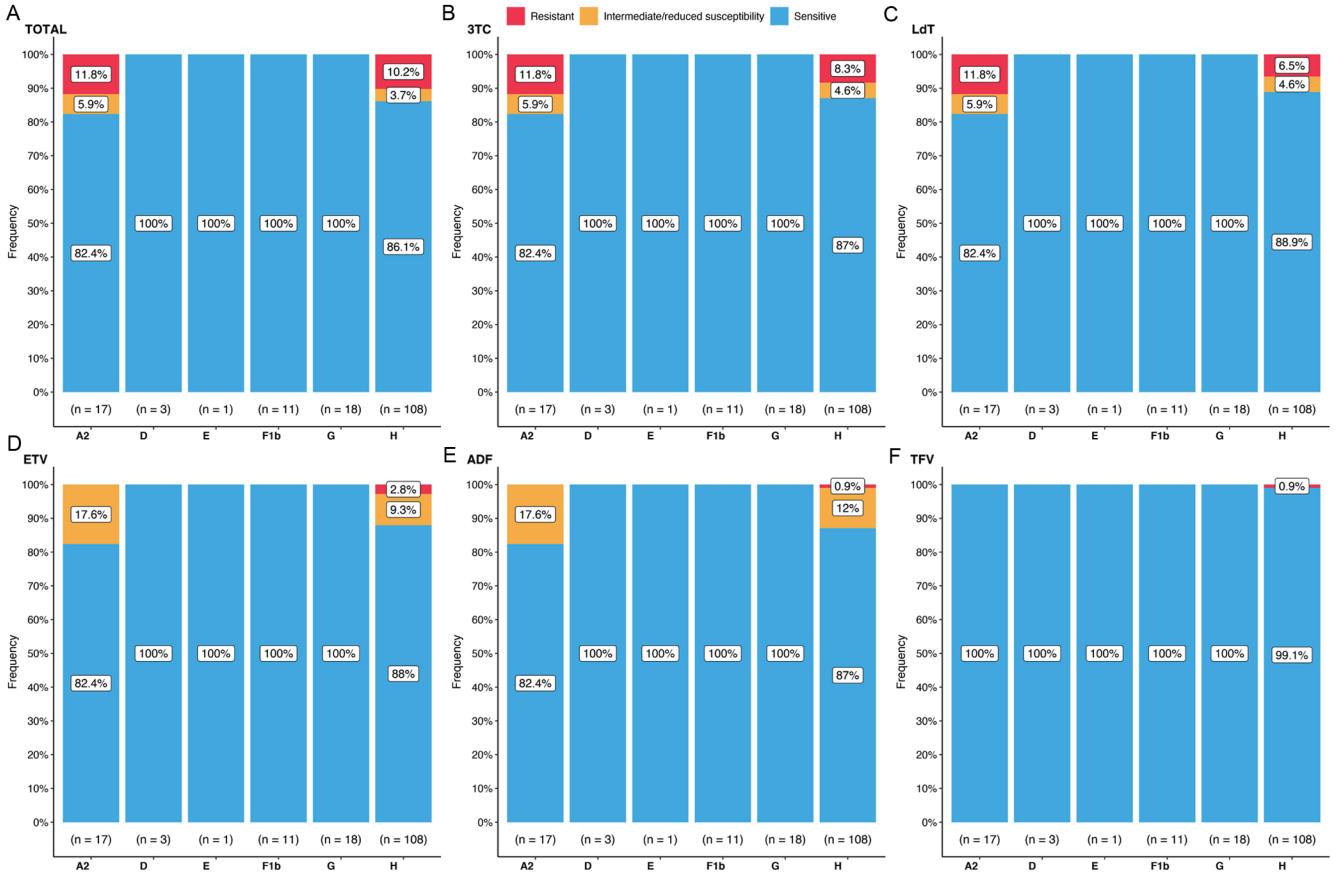

Fig. 2. Frequency of resistance and intermediate/reduced susceptibility mutations by HBV genotype in Mexico. 3TC, lamivudine; ADF, adefovir; ETV, entecavir; LdT, telbivudine; TFV, tenofovir.

ances.[35] Ten years later, using DNA sequencing, Fernandez-Galindo *et al*.[36] found a prevalence of 18.2%.[36] In this study, we found an intermediate frequency of 8.2% compared with previous studies. Our study showed that in an HBV population with high exposure to antivirals, such as patients with HIV, the prevalence of resistance mutations was significantly increased compared with a population with low exposure to treatment, such as HBV monoinfected individuals (16.3% vs. 5.0%, p=0.0489). The finding is consistent with what has been reported in other HIV cohorts.[37–39] Worldwide, the HBV drug resistance rate differs among countries. The highest frequencies have been detected in Italy (72.1%),[40] and a rate of 42.8% has been reported in ethnic regions of China.[41] Chevaliez *et al*.[42] found a resistance frequency of 25.0% in France. In North America and Brazil, frequencies of 21.0% and 1.6% were reported, respectively.[22,43] The heterogeneous distribution of HBV drug-resistance mutations could be explained by factors such as the use of different detection methods (line probe assay, Sanger DNA sequencing, or next generation sequencing), the number of samples analyzed, accessibility of antiviral in each region, the period during which the studies were conducted, and even the distribution of HBV genotypes.[35–43] Here, we showed that HBV resistance mutations were common in genotypes H and A2. As mentioned before, the prevalence of genotype H was expected as it is predominant in Mexico. Regarding subgenotype A2, previous studies have shown its strong association with the presence of resistance mutations. A study by Kobayashi *et al*.[44] in 2006 found that during the first four years of 3TC therapy, patients infected with genotype A had more YMDD mutants than those with Asian genotypes B or C. In Spain, the HBV subgenotype A2 was related to a lower response to ADF.[45] Similar results were found in Argentina, where eight of 13 patients with ADF or 3TC resistance mutations were infected with subgenotype A2.[46] This evidence suggests that patients infected with HBV genotype H and subgenotype A2 should be monitored carefully to prevent the development of HBV resistance mutations during therapy.

On the other hand, the results of this study may have important implications for HBV treatment in Mexico. First, the most common resistance pattern was rt180M+rt204V, which is associated with resistance to 3TC and LdT, suggests that these drugs may be losing their effectiveness in Mexican patients with HBV, particularly in those who have been treated at length with 3TC. Some patients develop resistance during 12 years of treatment.[47] Although it is well known that patients treated with 3TC can develop between 6.4% and 15% of 3TC resistance mutations in the first year of treatment.[48] Factors that increase the likelihood of developing resistance, include age, duration of infection, high HBV viral load, high serum ALT, high body mass index, and HBV genotype.[3,5,49]

In addition, exposure to hepatitis C Virus or HIV can increase the risk of resistance, particularly in HIV patients where 3TC (combined with other antivirals) is used as first-line therapy.[50] Second, we found a low frequency of rt181V and 194T, which confer resistance to ADF and TDF, respec-

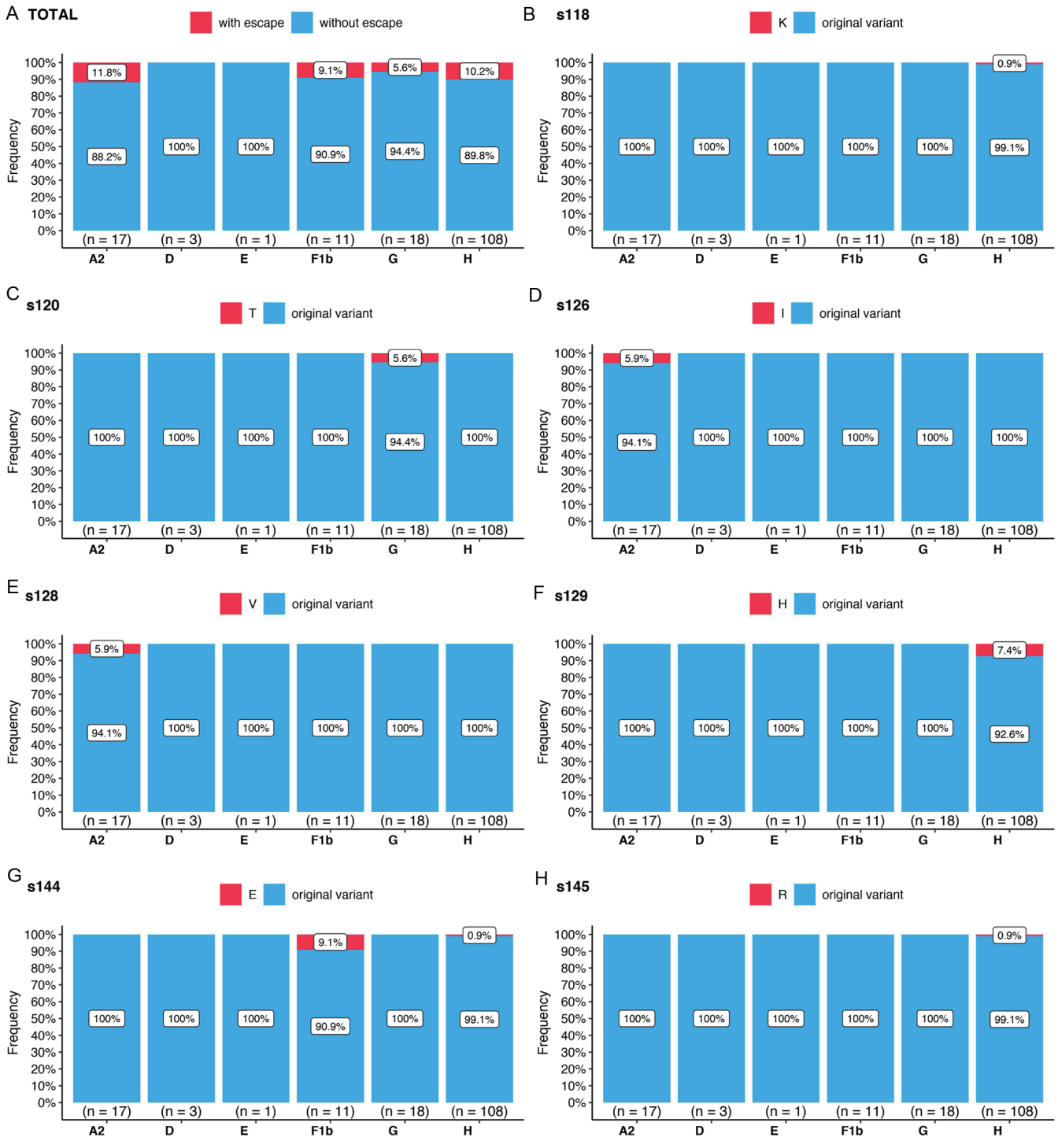

Fig. 3. Frequency of escape mutations in HBV genotypes circulating in Mexico. The numbers indicate the mutation position on the surface gene open reading frame. E, glutamic acid; H, histidine; I, isoleucine; K, lysine; R, arginine; T, threonine; V, valine.

tively. This result may be because the antivirals have a high genetic barrier to resistance,[51] suggesting that ADF and TDF may still be effective in treating patients with HBV in Mexico, at least for now. Finally, 1.9% of the HBV sequences analyzed had the combinations rt180M+rt204V+rt173L or rt180M+rt204V+rt202G that confer multidrug resistance to 3TC, LdT, and ETV.[3] This prevalence was lower than that reported in Bangladesh (3.5%).[52] The highest prevalence of multidrug resistance has been found in Korea, at 34.2%.[53] A study in China reported a 25% incidence of multidrug resistance in patients treated with 3TC and ADF.[54] The emergence of multidrug resistance is a serious problem since people who

present it can develop hepatocellular carcinoma, despite receiving rescue therapy with tenofovir alafenamide (TAF).[55] With new DNA sequencing technologies, some authors have proposed that multiresistant HBV mutations might be more common than expected, and their number could increase.[56,57] The development of multidrug resistance in HBV is a process that begins with classic mutations, and then rare variants appear.[5] This could explain why multidrug resistance mutations frequently emerge in chronic HBV patients who have received sequential monotherapy.[20,58] This study provides the first evidence of HBV multidrug resistance in Mexico and highlights the need for routine surveillance of drug-resistant mutations in this region.

Furthermore, this study confirms the presence of escape mutations in HBV genotypes circulating in Mexico, as mentioned above (A2, H, F1b, and G). Overall, escape mutations were detected in 9.5% of the HBV sequences. The most common variant was s129H, particularly in genotype H. At a global level, other studies have reported escape mutations of 29.6%,[59] 22.1%,[60] 10.7%,[61] 8.3%,[62] and 7.5%.[63] Based on these reports, our prevalence would be intermediate (9.5%). Several reasons might explain this, such as the predominance of HBV genotype H in Mexico, sample size, or the type of population analyzed. Previous studies have reported the highest escape mutation rates in individuals treated with analog nucleos(t)ides.[59,60] In 2008, Sheldon and Soriano proposed that HBV escape mutations could be induced by antiviral therapy, as mutations in the RT domain of polymerase affect the open reading frame (ORF) of HBsAg.[9] This would explain the higher prevalence of escape mutations in HIV/HBV coinfected individuals compared to the HBV-alone group. HBV escape mutations can alter the structure of the surface antigen, affecting the body's ability to mount an effective immune response.[6,64]

The mutation capacity of the HBsAg gene is one of the most important mechanisms HBV has developed to escape treatment and simultaneously evade the host's immune response.[6–9] In some cases, these mutant antigens may also decrease the sensitivity of ELISA-based methods, making HBV infection more difficult to diagnose.[7,8] Escape mutations could partly explain the high prevalence of OBI in patients with HIV and native populations from Mexico. Further studies in other regions of Mexico are necessary to confirm this hypothesis.

The study has several limitations to be considered when interpreting the results. First, the study was cross-sectional, meaning it only provides a snapshot of HBV genotypes, recombination, and resistance/escape mutations at a specific time. Second, the sample size was small, and the prior treatment presence in the HIV group can act as a confounding factor, which limits the extrapolation of the results to other high-risk groups or the general population. Third, to maximize the sample size, only the RT domain of the polymerase gene was used, while mutations in other regions of the HBV genome that could affect viral replication were not analyzed. Despite these limitations, the study provides valuable information about the molecular epidemiology of hepatitis B infection. It is important to note that this study is the largest, showing the distribution of resistance and escape mutations across different HBV genotypes in Mexico. In our region, this study is one of the first to report the prevalence of recombinant HBV genotypes, and the identification of multidrug resistance mutations highlights the need for continued surveillance of HBV mutations in Mexico.

In conclusion, resistance and escape mutations and recombinant hepatitis B genotypes were predominant in HIV/HBV coinfected compared with HBV monoinfected patients. In our region, HBV genotypes A2 and H were the most susceptible to presenting both resistance and escape mutations, suggesting that individuals with these genotypes should be carefully monitored. Finally, this study provides the first evidence of multidrug resistance to 3TC, LdT, and ETV in three HBV sequences from Mexico.

Acknowledgments

We thank Ali Sebastian Meza-Vazquez, Mariela Guadalupe Macedo-Montero, and Rosa Magaly Gomez-Gutierrez for their technical support. AJ-A was supported by a CONACYT Doctoral Scholarship 2013–2017 to perform this work.

Funding

This research was funded in part by Consejo Nacional de Ciencia y Tecnologia (CONACYT), grant number PN-2017-5254 to AP, FOSSIS-S0008-2010-1-08-139085 to SR, and the Programa de Apoyo a la Incorporación de NPTC. No. UDG-PTC-1439 to AJ-A.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceptualization (AP, SR), methodology (AJ-A, AP, SR, JRRP, MSG-G), validation (MSG-G), formal analysis (AJ-A, AP, SR), investigation (AP, AJ-A, SR), data curation (AJ-A), writing, original draft preparation (AJ-A), writing, review, and editing (SR, AP, JRRP). All authors credit authorship by contributing with intellectual content, revised and approved the final version of the manuscript.

Ethical statement

The Institutional Review Board of the Health Sciences Center from the University of Guadalajara approved the study (CI-07218). This research was conducted following the ethical principles of the 2013 Declaration of Helsinki.

Data sharing statement

No additional data are available.

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