

ORIGINAL ARTICLE

HEPATITIS B VIRUS GENOTYPING, DETECTION OF REVERSE TRANSCRIPTASE RESISTANCE AND IMMUNE ESCAPE MUTATIONS IN PERSONS WITH CHRONIC HEPATITIS B FROM CROATIA

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Abstract: Approximately 257 million people worldwide live with chronic hepatitis B virus (HBV) infection, which, if left untreated, can lead to liver cirrhosis or hepatocellular carcinoma. The hepatitis B virus is a DNA virus with a reverse transcriptase that has no exonuclease activity, which results in a high mutation rate. Reverse transcriptase inhibitors, which interfere with viral replication, are used to treat the infection. Mutations in the A-B reverse transcriptase interdomain can be associated with resistance to antiviral drugs, as well as immune escape. The aim of this study was to analyze HBV genotypes circulating in the Croatian population and analyze resistance as well as immune escape mutations. A selected A-B reverse transcriptase interdomain was sequenced using the Sanger method. HBV genotypes, subtypes, drug resistance as well as immune escape mutations were analyzed using the *Geno2Pheno* algorithm in 30 patients with chronic hepatitis B. Genotype A (subtype A2) was detected in 20% and genotype D (subtypes D1, D2 and D3) in 80% of viral isolates. Drug resistance mutations rtL180M and rM204V were detected only in genotype A isolates. Immune escape mutations R122K and sT131N were detected in all genotype A isolates, while mutations sD144E, sM133I, sM133L, sP120S, sQ101H and sR122K were detected in 8 genotype D isolates. Genotype distribution and the prevalence of mutations observed in this study are in accordance with data from the majority of other European countries.

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INTRODUCTION

The World Health Organization estimates that there are 257 million people in the world chronically infected with the hepatitis B virus (HBV) and 887,000 die annually as a consequence of liver-related morbidity.¹ According to the Croatian Public Health Institute, Croatia is a low prevalence country with estimated 25,000 individuals with chronic HBV infection.² Mandatory vaccination against hepatitis B for all children in 6th grade of primary school was introduced in 1999 and has been obligatory for all newborns since 2007. The incidence of HBV infection has significantly dropped since the introduction of pediatric vaccination, but chronic hepatitis B remains an important public health issue in Croatia.²

Chronic hepatitis B can, if left untreated, lead to liver cirrhosis or hepatocellular carcinoma. Antiviral treatment does not eliminate the virus from the organism completely, but rather reduces the kinetics of viral replication and lowers cccDNA levels.^{3, 4} Although not all patients with chronic hepatitis B are immediately treated, viral load, liver enzyme values and liver fibrosis progression are carefully monitored. Antiviral treatment of chronic hepatitis B is based on the use of nucleoside (lamivudine, telbivudine, entecavir and clevudine) and nucleotide (adefovir dipivoxil, tenofovir and tenofovir-alafenamide) analogues reverse transcriptase inhibitors (5). The majority of antiviral drugs target the DNA elongation step of the replication cycle, but entecavir and tenofovir can also interfere with protein priming. In addition, chronic hepatitis B can be treated with pegylated IFN- α .

According to the most recent EASL guidelines, antiviral therapy is administered to patients with HBeAg-positive or -negative chronic hepatitis B, defined by HBV DNA >2,000 IU/ml, ALT >ULN and at least moderate liver necroinflammation or fibrosis, patients with compensated or decompensated cirrhosis, with any detectable HBV DNA level and regardless of ALT levels, patients with HBV DNA >20,000 IU/ml and ALT >2xULN, patients over the age of 30 with HBeAg-positive chronic HBV infection, regardless of the severity of liver lesions and patients with HBeAg-positive or HBeAg-negative chronic HBV infection and family history of hepatocellular carcinoma or cirrhosis. The goal of the treatment is to reduce or completely stop the viral replication and keep the patient's viral load below 1000 IU/ml.⁵

HBV reverse transcriptase has no exonuclease activity leading to the high genetic variability of the virus (error rate estimated at 10^{-7} per nucleotide per day). Amino acid substitutions formed spontaneously or under selective pressure of antiviral drugs can cause resistance to reverse transcriptase inhibitors. A mutation that causes resistance to one of the reverse transcriptase inhibitors can also cause resistance to another inhibitor, e.g. cross-resistance.^{6, 7, 8} Nucleotide analogues adefovir and tenofovir exhibit no cross resistance with lamivudine, telbivudine and entecavir. Occurrence of two or more mutations in the same region increases the chances of resistance development and these mutations are called compensatory mutations.⁹ The frequency of existing reverse transcriptase inhibitors resistance mutations depends on several parameters including HBV genotype or recombinants, HBeAg serological status, ethnicity, disease progression and HIV coinfection.^{6, 10} The most frequently observed mutations associated with resistance to nucleoside/nucleotide analogues are rtL180M/rtM204(I/V) (for lamivudine, entecavir, telbivudine and clevudine) and rtA181V/rtN236T (for adefovir and tenofovir).

The A-B interdomain of the RT gene overlaps with the S gene, coding for the HBsAg "a" determinant region so mutations in that region can also lead to a lack of organism's immune response to HBV infection. These mutations are called immune-escape or secondary mutations.^{11, 12} The first immune-escape mutation was identified in Italy in 1988, in a newborn who, despite being vaccinated after birth, acquired HBV infection. The virus carried a mutation in the gene for HBsAg.¹³ S gene mutations occur as a result of host's immune system pressure, even though the mechanism has not yet been discovered.¹⁴ These mutations are changing the conformation of the HBsAg "a" determinant, so anti-HB antibodies induced by vaccine cannot recognize the viral epitope subsequently leading to the infection of the host. Mutations in the S gene can also affect the reproducibility of immunological assays for the detection of HBsAg.^{15, 16} There are ten different HBV genotypes marked A to J and over 30 subgenotypes that show characteristic

geographic distribution.¹⁷ Genotype A is common in Sub-Saharan and West Africa, North Europe, Asia (India) and North America. Genotypes B and C are usually found in Asia and the Pacific, with genotype C mainly detected in Southeast Asia. Genotype D is dominant in Africa, Europe (the Mediterranean) and India, while genotype E is limited to West Africa. Genotypes F and H are found in Central and South America, genotype G in France, Germany and USA, genotype I in Vietnam and Laos. The most recently detected genotype J was detected in the Ryukyu Islands in Japan.^{18, 19}

The aim of this study was to determine the nucleotide sequence of the A-B interdomain of the HBV reverse transcriptase gene using the Sanger sequencing method and, using the *Geno2Pheno* algorithm, to determine viral genotypes and subgenotypes as well as reverse transcriptase inhibitors resistance mutations and immune escape mutations in patients with chronic hepatitis B from Croatia.

MATERIAL AND METHODS

The study included 30 treatment-naïve patients with chronic hepatitis B receiving clinical care at the Department of Viral Hepatitis of the University Hospital for Infectious Diseases, Zagreb and the Croatian Reference Centre for Viral Hepatitis between August 2017 and October 2018 with >1,000 IU of HBV DNA/ml. Selected demographic and routine clinical data on the patients were extracted from the Department of Viral Hepatitis database. Ethical approval for this study was received from the Ethics Committee of University Hospital for Infectious Diseases "Dr. Fran Mihaljevic".

HBV DNA was extracted from serum using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). HBV reverse transcriptase A-B interdomain was amplified using the FastStart High Fidelity PCR System kit (Roche, Basel, Switzerland) and primers for the first PCR were HBV OUT F 5' TTCCTGCTGGTGGCTCCAGTT C 3' and HBV OUT R: 5' TGCTAGGAGTTCCGCGAGTATG 3' as described by Choi et al.⁶ Amplicons from the first PCR were used as a template for the second (nested) amplification step with the FastStart High Fidelity PCR System kit (Roche, Basel, Switzerland) and primers for subtype HBV IN F: 5' CTCGTGGTGGACTTCTCTC 3' and HBV IN R 5' GCAAAGCCCAAAGACCCAC 3' as described by Choi et al.⁶

Subsequent sequencing analysis of nested PCR-products was performed on the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for sequencing reaction according to the manufacturers' protocol along with primers for nested PCR. The resulting sequencing products were purified and analyzed on the Applied Biosystems capillary

electrophoresis-based genetic analyzer. Sequences were arranged in Vector NTI (Thermo Fisher Scientific), while resistance to reverse transcriptase inhibitors was detected in the *Geno2Pheno* algorithm (Genafor, Bonn, Germany). According to rules of the *Geno2Pheno* algorithm, substitutions were sorted into following resistance classes: resistant (well-characterized, clinically significant resistance-associated substitutions), reduced susceptibility (substitutions associated to resistance with insufficient evidence for the clinical outcome) and uncharacterized substitutions on scored positions. All sequences were submitted to the GenBank nucleotide sequence database with the following accession numbers: MK410095, MK410096, MK410097, MK410098, MK410099, MK410100, MK410101, MK410102, MK410103, MK410104, MK410105, MK410106, MK410107, MK410108, MK410109, MK410110, MK410111, MK410112, MK410113, MK410114, MK410115, MK410116, MK415823, MK415824, MK416163, MK416164, MK416165, MK416166, MK416167, and MK468728.

RESULTS

The study included 17 men (57%) and 13 women (43%) with chronic hepatitis B that have not been previously treated with antiviral drugs (nucleoside/nucleotide analogues). The mean age of patients was 41.5 years, and median viral load was 41,495 IU of HBV DNA/ml. None of the patients were co-infected with HIV. The patients' characteristics have been summarized in Table 1.

Genotype A (subtype A2) was detected in 20% and genotype D (subtypes D1, D2 and D3) in 80% of viral isolates. Drug resistance mutations rtL180M and rtM204V were detected only in genotype A isolates. Immune escape mutations R122K and sT131N were detected in all genotype A isolates, while mutations sD144E, sM133I, sM133L, sP120S, sQ101H and sR122K were detected in 8 genotype D isolates.

Subtyping of genotypes A and D that have been detected in the Croatian population has also been performed. Genotype A was detected in 6 patients (20%), and all sequences were shown to classify as subgenotype A2.

Genotype D was detected in 24 patients (80%), classified in three different subtypes: D1 was detected in 5 (21%) patients, D2 in 9 (37%), and D3 in 10 patients (42%). Genotype distribution was similar for both genders; 1 female patient carried genotype A (8%), 12 genotype D (92%), 2 subtype D1(17%), 4 (33%) subtype D2 (33%) and 6 subtype D3 (50%). Genotype A was detected in 5 (29%) male patients, while 12 (71%) had genotype D, 3 (25%) subtype D1, 5 (42%) subtype D2 and 4 (33%) subtype D3.

The bioinformatics algorithm *Geno2Pheno* compared sequences of A-B interdomain region for all participants with the reference sequence and detected

mutations that may cause resistance to antiviral drugs. The algorithm takes into consideration all mutations previously proven to cause resistance or reduced susceptibility to one or more reverse transcriptase inhibitors. Not a single genotype D-infected participant had resistance mutations, while two participants with A2 subtype had two mutations: rtM204V and rtL180M. The mutation rtM204V together with the mutation rtL180M leads to compensatory mutation that causes resistance to lamivudine and reduced susceptibility to entecavir.

Table 1. Patients' characteristics at enrollment

Parameter		Female (n=13)	Male (n=17)
Age (years)	mean	40	53
	SD	±17.5	±15.3
Viral load (HBV DNA IU/ml)	median	16,662	4,705
	range	1,356-9,673,492	1,050-989,000,000
Antiviral treatment		No	No
HIV co-infection		No	No

Legend: SD – standard deviation

Secondary mutations in A-B interdomain have been detected in 14 study participants (47%). Two mutations, sR122K and sT131N, which affect the serological diagnostic of HBsAg, have been detected in all participants infected with genotype A. Mutations sD144E, sM133I, sM133L, sP120S, sQ101H and sR122K have been detected in 8 participants carrying genotype D. A single secondary mutation was detected in 7 participants, while one participant had 4 different mutations detected. Detailed mutation distribution, as well as the biological and clinical relevance of the mutations, is presented in Table 2.

DISCUSSION

Chronic hepatitis B remains an important public health issue, both worldwide and in Croatia.^{1, 20} In order to slow down the progression of liver damage and prevent the development of cirrhosis or hepatocellular carcinoma, chronic hepatitis B is treated with reverse transcriptase inhibitors or pegylated interferon- α .²¹ HBV has a high mutation rate for a DNA virus, as a result of reverse transcriptase lacking exonuclease activity.¹² Mutations in the reverse transcriptase A-B interdomain gene lead to resistance to reverse transcriptase inhibitors as well as immune escape mutations.⁶

Two HBV genotypes have been detected in the Croatian population; A and D. The genotype A was present only in the form of subtype A2, while genotype D was more diverse with subtypes D1, D2 and D3.

Mutations that cause resistance to reverse transcriptase inhibitors were detected only in patients infected with

Table 2. Distribution of secondary mutations among different genotypes and subtypes

Genotype	Subtype	Mutations	Number of participants
A	A2	sR122K and sT131N - HBsAg detection	6
D	D2	sR122K - HBsAg detection	1
		sM133L - HBsAg detection, vaccine, Ig therapy	1
		sQ101H sR122K - HBsAg detection sM133I - HBsAg detection, Ig therapy sD144E - HBsAg detection, vaccine, Ig therapy	1
	D3	sP120S - HBsAg detection, vaccine	1
		sQ101H	4

subtype A2; rtM204V causes resistance to telbivudine and the compensatory mutation rtL180M causes resistance to lamivudine and reduced susceptibility to entecavir.

Detected immune escape mutations were sR122K and sT131N in subtype A2 participants and sD144E, sM133I, sM133L, sP120S, sQ101H and sR122K in genotype D participants.

National studies on HBV genotype distribution, resistance and immune escape mutations are currently not available. A regional study by Deterding et al (2008) reported that genotypes A and D were the most frequent in Eastern Europe and Croatia and reported genotype A in 8% of Croatian patients, genotype D in 80%, and mixed genotypes A and B in the remaining 12%. Our study shows a somewhat different distribution of HBV genotypes in the Croatian population, which is probably associated with the use of an accurate genotyping method (sequencing vs. reverse hybridization).²²

Genotype A is mainly found in the North of Europe and Greenland, but the results of our study confirm a geographical shift of subtype A2 from the North towards the Southeast of Europe.²³ Lazarevic et al (2007) reported genotype D subtype distribution in Serbia; D1 - 7.5%, D2 - 30.4%, D3 - 45.1% and, similarly to our study, the exclusive presence of subtype A2.²⁴

European countries other than Croatia with similar A and D genotype distribution are Estonia (A - 18.5%, D - 81%), Latvia (A - 42%, D - 57%), Lithuania (A - 41%, D - 54%), Russia (A - 6.7%, D - 93%), Romania (A - 6%, D - 67%), Serbia (A - 2.5%, D - 97%) and Italy (A - 26%, D - 73%), genotype D is exclusively found in Albania and Greece (D - 100%), while Norway (A - 89%, D - 11%) and Denmark (A - 89%, D - 11%) have the opposite distribution with genotype A being more frequent. Genotypes A and D along with B and C are detected in patients in Sweden (A - 25%, B - 5%, C - 5%, D - 50%), Czech Republic (A - 64.4%, B - 18.5%, C - 1.5%, D - 28.2%) and Hungary (A - 47%, B - 1.2%, C - 1.6%, D - 43%). Poland has individuals carrying genotype A - 75.6%, D - 33.9%, F - 3% and H - 2%; Spain A - 21.2%, D - 64.2% and F - 1.9%; United Kingdom A - 41%, B - 12%, C - 5%, D - 30% and G - 12%; Germany A - 45%, B - 4.3%, C - 7-7%, D - 26 -

42%, E - 0.8% and F - 0.8%; in Belgium A - 53%, D - 37% and G - 8% and France A - 24%, B - 7%, C - 11.5%, D - 28%, E - 11.5% and G - 16%.^{22, 25}

Subtype A2 has been detected in 48 USA states including Alaska, while genotype D was mainly limited to immigrants and was related to their country of origin.²⁶ Genotype A was found in patients from Sub-Saharan and West Africa, Asia and Australia, and genotype D in patients from Northern Africa and India.^{27, 28} Subgenotype D1 was frequently found in patients from Bulgaria, Turkey, Middle East, North Africa, Indonesia and Brazil, D2 in Serbia, Albania, Turkey, Lebanon, Brazil and West Africa, while subtype D3 was detected in patients from Serbia, West India and Indonesia.²⁸

Colagrossi et al (2018) conducted a study that included 935 chronic hepatitis B patients from 15 European countries, including Croatia, to investigate immune escape mutations, but due to overlapping of the reverse transcriptase and the S gene region, there are data on drug resistance mutations as well.²⁹ Drug resistance mutation rtM204V/I that was detected by Colagrossi et al (2018), has also been detected in our study.²⁹ In addition, the high frequency of drug resistance mutations in genotype A patients observed by Colagrossi et al (2018) corresponds to our findings as well.²⁹ Distribution of HBV genotypes A (27.3%) and D (61.3%) observed in that study is, to some extent, similar to our findings as well.²⁹ National studies on the prevalence of drug resistance mutations in our geographic region are currently not available.

A Brazilian study by Pacheco et al (2017) reported several mutations in 6% of treatment-naive patients including rtA181S, rtA194T, rtS202I, rtM204I, rtM204V and rtL180M + rtM204V. Immune escape mutations sI195M, sW196L and sG145R were detected in patients carrying rtM204V and rtL180M drug resistance mutations. The similarity between our results and Pacheco et al (2017) is probably associated with the high prevalence of drug resistance mutations in patients infected with HBV genotype A in both studies.³⁰

Fung et al (2008) found a high rate of resistance mutations in untreated patients infected with HBV genotype D; 12% of patients carried the rtM204I/V mutation and 10% of patients carried the rtL180M

mutation. Lower mutation rates have been observed in patients infected with genotype A: 4 out of 137 patients carried the rtM204V mutation while only two patients infected with genotype A carried the rtL180M + rtM204V combination of mutations. Immune escape mutations detected in this study included sR122K and sT131N.³¹ According to Bottecchia et al (2008), subtype A2-infected individuals often carry rtL217R mutation that is associated with resistance to adefovir dipivoxil, but the results of our study did not support this finding.³²

Immune escape mutations have been reported on positions 120, 123, 124, 126, 127, 129, 130, 131, 133, 134, 141, 142, 144 and 146 within “a” determinant. (33). Colagrossi et al (2018) observed that immune escape mutations can be frequently found in HBV-infected individuals, which poses a potential threat for the vaccinated individuals worldwide. A total of 22.1% of patients included in our study carried at least one immune escape mutation. The frequency of 29 mutations detected by Colagrossi et al (2018) was higher in treated compared to untreated patients due to selective pressure of reverse transcriptase inhibitors. Similarly to our results, immune escape mutations were more frequently detected in genotype D-infected individuals.²⁹ Literature data on HBV immune escape mutation frequency in the region are currently not available. Hossain and Ueda (2017) reported that selected immune escape mutations were associated with the presence of Y100S substitution, but the results of our study do not support this finding.³⁴ Liu et al (2018) showed that patients with chronic hepatitis B from South China carried sQ101H (2.3%), sT131N (11.4%) and sM133L (11.4%) mutations, which corresponds to our findings as well.³⁵

Low patient number is a limitation of our study. However, the patients have been randomly selected from patients receiving care at the largest national treatment center for viral hepatitis. Therefore, it is reasonable to assume that the data presented in this study provide a good estimate of the HBV genotype distribution, prevalence of primary drug resistance as well as distribution of immune escape mutations in Croatia. Information on the prevalence of drug resistance mutations is important for developing strategies to avoid possible treatment failure of reverse transcriptase inhibitor-based therapy. The results on primary drug resistance observed in this study confirm that the use of tenofovir as a drug with exceptionally high genetic barrier to resistance in Croatia is justified. In addition, information on immune escape mutations is important to assess the risk of possible infections of the vaccinated population as well as prevention of treatment failure with HBIg-based therapy.

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