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Epidemiological, Virological and Clinical Characteristics of Hepatitis B Virus Genotypes in Chronically Infected Persons in Slovenia

Nina Kmet Lunacek,[1,*] Mario Poljak,[2] Jelka Meglic-Volkar,[1] Mojca Rajter,[1] Janja Prah,[1] Gorazd Lesnicar,[3] Tanja Selic Kurincic,[3] Zvonko Baklan,[4] Tatajana Remec,[5] Emil Pal,[6] Petra Markocic,[2] Maja Lunar,[2] and Mojca Maticic[1]

[1]Clinic for Infectious Diseases and Febrile Illnesses, University Medical Centre Ljubljana, Slovenia
[2]Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia
[3]Department of Infectious Diseases and Febrile Conditions, General Hospital Celje, Slovenia
[4]Department of Infectious Diseases and Febrile Illnesses, University Clinical Centre Maribor, Slovenia
[5]General Hospital Novo mesto, Slovenia
[6]General Hospital Murska Sobota, Slovenia

[*]*Corresponding author*: Nina Kmet Lunacek, Clinic for Infectious Diseases and Febrile Illnesses, University Medical Centre Ljubljana, Japljeva 2, 1000 Ljubljana, Slovenia. Tel: +386-40817921, Fax: +386-1 5222456, E-mail: dr.ninika@gmail.com

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Abstract

Background: Hepatitis B virus (HBV) genotypes have been shown to have virological, clinical, and therapeutic implications. Knowledge about HBV genotype distribution in Slovenia is scarce. This study was the first to determine various characteristics of patients with chronic HBV infection with regard to HBV genotypes at the national level.

Methods: HBV genotype determination was performed on randomly selected patients out of 1,729 patients from all Slovenian regions who tested positive for HBV surface antigen (HBsAg) at the national reference laboratory for viral hepatitis between January 1997 and December 2010. Demographic, epidemiological, virological, and clinical data were extracted from the medical records and statistically analyzed with regard to HBV genotypes.

Results: A total of 186 HBsAg positive patients with the mean age of 40.1 years were identified from whom, 65.1% were male. 157 (84.4%) cases presented with genotype D, 23 (12.4%) with genotype A, and 6 (3.2%) with other HBV genotypes. Sexual transmission was more significantly associated with lower odds for HBV genotype D infection compared to blood-related risk factors (P = 0.023). Genotype A was significantly more common in men who had sex with men (P = 0.043). Compared to females with genotype D, genotype A positive women presented unknown risk factors more significantly (P = 0.002).

Conclusions: HBV genotype D is the most prevalent genotype in Slovenia. However, future changes might be expected due to recent massive immigrations to Europe. Routine HBV genotyping is recommended in patients with certain risk factors prior to initiation of hepatitis B treatment.

Keywords: Hepatitis B Virus, Genotypes, Risk Factors, Clinical Characteristics

1. Background

With an estimated 250 million of infected persons worldwide, hepatitis B virus (HBV) infection presents a global public health problem. Despite the existence of effective vaccine and treatment, chronic hepatitis B (CHB) remains the leading cause of chronic liver disease and associated mortality (1, 2). HBV prevalence varies with region, between 0.01% in Northern Europe and 22.4% in some African areas (3). In some European countries, the HBV prevalence is higher, with up to 10.4% reported from Kyrgyzstan (3).

HBV genotypes are effective on clinical outcome, seroconversion rates of HBV e antigen (HBeAg), mutations in the (pre)core promoter regions, and response to therapy (4). The prevalence of the HBV genotypes varies geographically: genotype A prevails in Northern Europe, North America, India, and Africa, while genotypes B and C are more frequent in Southeast Asia, and genotype D in Southern Europe, the Middle East, and India (4).

In Slovenia, a central European country with 2 million people, the HBV prevalence has been estimated at below 1% (5). However, it is believed that the prevalence is underestimated due to under-reporting (6).

2. Objectives

The aim of this national study was to determine epidemiological, virological, and clinical characteristics of individuals with chronic HBV infection in Slovenia with regard to HBV genotypes in order to optimize the national

diagnostic and treatment guidelines following European association for the study of the liver (EASL) recommendations (7).

3. Methods

3.1. Patients

The database of 1,729 HBsAg-positive patients from all regions of Slovenia who tested positive for HBsAg at the reference laboratory for molecular microbiology and diagnostics of hepatitis and HIV/AIDS, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana between January 1997 and December 2010 was reviewed. HBV genotyping had already been performed on 127 cases. From the remaining, 450 presented with available medical data. From these, every fifth patient was selected for further genotyping following a systematic random sampling approach, yielding another 90 patients. Genotyping was successfully performed on 59 cases; in the remaining 31 cases, genotyping failed: in 24 due to undetectable HBV DNA or viral load < 100 IU/mL and in 7 due to inadequate quantity of sample.

Available epidemiological and clinical characteristics were reviewed from the medical documentation.

3.2. Serological and Molecular Methods

In all samples, HBsAg and HBeAg were determined using the ARCHITECT immunoassay analyzer (Abbott, Weisbaden, Germany) and HBV DNA viral load using the real-time PCR based test Abbott RealTime HBV Test (Abbott). Viral load was categorized according to the clinical implications into three ranges: $\leq$ 2,000 IU/mL, 2,000 - 20,000 IU/mL, and $\geq$ 20,000 IU/mL (7).

For determination of HBV genotypes, a two-stage procedure was followed. In the first step, real-time PCR was employed (8). The assay differentiates reliably between genotypes A and D in a single reaction, since probes are labeled with different fluorescein dyes (FAM for genotype A and YAK for genotype D). To determine HBV genotype in non-A-non-D positive samples, a portion of the HBV polymerase gene was sequenced using primers reported previously (9), and HBV genotypes determined using NCBI genotyping tool. The dual infection sequences were determined using real-time PCR method and confirmed by sequencing. The obtained sequences were analyzed using an online tool geno2pheno (10). The sequences determined in this study were deposited in the GenBank (accession numbers KY613598-KY613603).

3.3. Statistical Methods

Association between patients' characteristics and HBV genotype was tested using univariate and multiple logistic regressions, likelihood ratio, or the Mann-Whitney U test. No correction for multiple comparisons was made. Significance tests were two-sided. P values $\leq$ 0.05 were considered statistically significant. Statistical analysis was performed using R program 3.1.1 and SPSS 23.0.

The study was approved by the national medical ethics committee of the Republic of Slovenia on May 4th 2015 (consent number: 6/04/15).

4. Results

186 patients met the inclusion criteria. The baseline characteristics of the enrolled patients are presented in Table 1.

Half of the studied patients reported no known risk factors for acquiring infection. Among the remaining, blood-related factors were the most common causes (41.9%). Patients with unknown risk factors were significantly older than patients with blood-related and sexual behavior-related risk factors (P = 0.001 and P = 0.009, respectively).

The majority (70.1%) of patients were HBeAg-negative (Table 1).

Genotype D was present in 157/186 (84.4%) and non-D in 28/186 (15.1%); one patient (0.5%) had mixed AD genotype (Table 1). 146/173 (84.4 %) Slovenians presented with genotype D, of the remaining (7 Bosnians, 2 Romanians, 2 Albanians, 1 Bulgarian, and 1 Chinese), 11 presented with genotype D, one (Chinese) with genotype B, and one (Bosnian) with mixed genotype AD.

The association between HBV genotypes and epidemiological, virological, and clinical characteristics of the included patients is presented in Table 2. Compared to the patients with blood-related risk factors, patients with risky sexual behavior had lower odds for HBV genotype D (OR = 0.14 [95 % CI, 0.02; 0.75]; P = 0.023). Genotype A had a statistically significant association with men who had sex with men (MSM) and bisexual men (P = 0.043).

Statistical analysis performed separately for males and females showed a significant association between HBV genotypes and risk factors; but no association between genotypes and age, alanine aminotransferase (ALT) levels, and viral load was observed. The risk factor was unknown for more genotype A infected females compared to genotype D infection cases (P = 0.002).

Multiple logistic regression with age, HBeAg status, viral load, risk factors for HBV infection, and ALT level as predictors and HBV genotype as the dependent variable showed that HBeAg-positive patients and those with sexual

Table 1. Baseline Demographic, Epidemiological, Virological, and Clinical Characteristics of the Included Patients (N = 186)

	Patients (N = 186)
Gender	
Male	121 (65.1)
Age, y	40.1 ± 14.4
Risk factors-general (n = 155)	
Unknown	78 (50.3)
Blood-related	65 (41.9)
Blood- and sexual- behavior related	5 (3.2)
Sexual-behavior related	7 (4.5)
Risk factors-specified (n = 155)	
Unknown	78 (50.3)
HBV in family	50 (32.5)
Blood transfusion	10 (6.4)
Sexual behavior	10 (6.4)
Healthcare worker	5 (3.2)
Hemodialysis	4 (2.6)
Tattooing	3 (1.9)
Incident	2 (1.3)
Intravenous drug use	1 (0.6)
Man having sex with man	1 (0.6)
Bisexual male	1 (0.6)
HBV-positive cohabitant	1 (0.6)
Surgical procedure in the past	1 (0.6)
HBeAg (n = 144) Negative	101 (70.1)
Genotype (n = 186) D	157 (84.4)
Other	28 (15.1)
A	23 (12.4)
B	2 (1.1)
C	2 (1.1)
F	1 (0.5)
AD	1 (0.5)
Viral load, IU/mL (n = 126)	
< 2,000	44 (34.6)
2,000 - 20,000	13 (10.2)
> 20,000	70 (55.1)
AST, IU/L (n = 137)	
> 0.52, UNL	79 (57.7)
ALT, IU/L (n = 137)	
> 0.56, UNL	94 (68.6)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; SD, standard deviation; UNL, upper normal limit.
[a] Values are expressed as mean ± SD or No. (%).

behavior-related risk factors have lower odds for HBV genotype D infection than for HBV non-D genotype (Table 3). The regression analysis was performed although Agresti's rule of thumb concerning ten events for independent variable had been violated (11).

5. Discussion

Only scarce data are available on the characteristics of HBV infected patients in Slovenia. A 2.5% prevalence for HBsAg positive persons injecting drugs on substitution treatment was reported (12), which ranks Slovenia as a country with the lowest rate among European countries (13). In HIV-positive Slovenian patients, HBsAg was determined in 3.5% and 3.9% (14, 15), which are lower compared to other European countries (16).

So far, no study on HBV genotype distribution has been performed in Slovenia. According to our results, genotype D is the most prevalent one, followed by genotype A. The genotype D prevalence of 84.4% positions Slovenia alongside other countries of the Balkan region, such as Serbia (82%; 85%), Montenegro (80.2%), and Croatia (80%) (17-20).

The results highlighted out the association of HBV genotypes with the risk factors for acquiring the infection; patients with risky sexual behavior, especially MSM and bisexual men, were significantly more often infected with genotype A. VanHoudt et al. also reported genotype A as the most prevalent genotype in MSM population in Europe (21). Similar reports came from Japan (22).

In a patient originating from Bosnia-Herzegovina, a dual infection with genotypes A and D was determined. The prevalence of co-infections with two HBV genotypes in Europe has been poorly documented. Sporadic reports showed up to 27% prevalence of mixed genotype infections, predominantly AD (20, 23). Sequencing is less sensitive than other methods detecting mixed genotypes, as it detects mainly the predominant genotype in mixtures, leading to potential underestimation (24). In highly prevalent genotype D regions, a single-step PCR method can be used to distinguish HBV genotypes D from non-D (25).

HBeAg-positive patients were less likely to be infected with genotype D, and genotype D was more frequently present in patients with HBeAg-negative status. Our finding reflects the natural course of HBV infection characteristic in the Mediterranean region, where genotype D and precore mutations prevail and consequently, HBeAg-negative prevalence is higher (26). A 70.1% prevalence of HBeAg-negative infection in Slovenia is in accordance with other reports from Europe, where it varies from 70% - 100% (27).

Females infected with genotype D were significantly younger and had a higher viral load regardless the risk factor for infection compared to other genotypes. Similarly, Tran et al. reported very high viral loads in HBV genotype D infected women with $\leq$ 44 years compared to those infected with HBV of the non-D genotype (28).

Interestingly, no significant differences regarding the viral load between genotypes D and A were seen when the whole population was examined. Lindh et al. reported

Table 2. Association Between Hepatitis B Virus Genotypes and Demographic, Epidemiological, Virological, and Clinical Characteristics of the Included Patients (N = 185)[a]

	Genotype Non-D (N = 28)	Genotype D (N = 157)	OR (95 % CI)	P Value	Genotype A (N = 23)	P Value[b]
Female gender	11 (39.3)	54 (34.4)	0.81 (0.35; 1.85)	0.617	8 (34.8)	0.971[c]
< 30 years of age	20 (71.4)	118 (75.2)	1. 12 (0.49; 2.97)	0.676	18 (78.3)	0.744[c]
Risk factors						0.022[c]
BR	6 (25)	59 (45.4)	1		3 (15)	
SR	3 (12.5)	4 (3.1)	0.14 (0.02; 0.75)	0.023	3 (15)	
BR & SR	1 (4.2)	4 (3.1)	0.41 (0.04; 4.25)	0.453	1 (5)	
Unknown	14 (58.3)	63 (48.5)	0.46 (0.16; 1.27)	0.133	13 (65)	
HBeAg-positive	9 (40.9)	34 (28.1)	0.56 (0.22; 1.44)	0.228	6 (31.6)	0.757[c]
		Males (N = 120)				
	N = 17 (14.1%)	N = 103 (85.8%)			N = 15 (12.5%)	
Risk factors	n = 15	n = 84			n = 13	0.041[c]
BR	4 (26.7)	38 (45.2)	1		3 (15.4)	
SR	3 (20)	2 (2.3)	0.07 (0.01; 0.55)	0.012	3 (15.4)	
BR & SR	1 (6.6)	2 (2.3)	0.21 (0.02; 2.87)	0.242	1 (7.7)	
Unknown	7 (46.7)	42 (50)	0.63 (0.17; 2.33)	0.49	6 (46.2)	
MSM	1 (6.7)	0		0.05[c]	1 (7.7)	0.043[c]
Bisexual men	1 (6.7)	0		0.05[c]	1 (7.7)	0.043[c]
HBeAg-positive	7/13 (53.8)	23/81 (28.4)		0.077[c]	6/12 (50)	0.144[c]
Viral load, IU/mL	n = 15	n = 67		0.980[c]	n = 13	0.856[c]
< 2,000	4 (26.7)	17 (25.4)			4 (30.8)	
2,000 - 20,000	2 (13.3)	8 (11.9)			1 (7.7)	
> 20,000	9 (60.0)	42 (62.7)			8 (61.5)	
		Females (N = 65)				
	N = 11 (16.9%)	N = 54 (83.1%)			N = 8 (12.3%)	
Risk factors	n = 9	n = 46			n = 7	0.020[c]
BR	2 (22.2)	21 (45.7)	1		0	
SR or BR & SR	0	4 (8.6)	-	-	0	
Unknown	7 (77.8)	21 (45.7)	0.29 (0.05; 1.54)	0.145	7 (100)	
Unknown risk f.	7 (77.8)	21 (45.7)		0.07[c]	7 (100)	0.002[c]
HBV in family	2 (22.2)	11 (23.9)		0.913[c]	0	0.06[c]
HBeAg positive	2/9 (22.2)	11/40 (27.5)			0/7 (0)	0.043[c]
Viral load, IU/mL	n = 8	n = 36		0.492[c]	n = 6	0.237[c]
< 2,000	5 (62.5)	18 (50)			5 (83.3)	
2,000 - 20,000	0	3 (8.3)			0	
> 20,000	3 (37.5)	15 (41.7)			1 (16.7)	

Abbreviations: BR, blood-related risk factors, CI, confidence interval; HBV, hepatitis B virus; MSM, men having sex with men; OR, odds ratio; SR, sexual behavior-related risk factors.

[a] Values are expressed as No. (%).

[b] Comparison between HBV genotypes D and A.

[c] Likelihood ratio test.

that HBeAg-positive patients with genotype D had higher viral load than those infected with other genotypes (29). Oommen et al. indicated viral load was higher in children infected with genotype D in comparison with genotype A (30).

Our study has several limitations. First, selection bias cannot be excluded. Due to financial limitations, only 90 additional randomly chosen patients could be genotyped. Besides, some statistical analyses were performed in smaller subpopulations due to lack of data. The strength of the study lies in its nationwide analysis of HBV genotypes in Slovenian patients.

In conclusion, the results suggest that genotype D is the most prevalent genotype in Slovenia that indicates the need to change the national guidelines for the management of CHB in males with history of high risk sexual behavior, especially in MSM. As CHB treatment response differs per HBV genotype (7), this risk group should be routinely genotyped before the treatment initiation.

Table 3. Association Between Hepatitis B Virus Genotype D and Epidemiological, Virological, and Clinical Characteristics of the Included Patients (Results of Multiple Logistic Regression; Reference Category: Non-D Genotypes) (N = 109)

	OR (95 % CI)	P Value
Age, y	0.99 (0.95; 1.03)	0.543
Risk factors		
BR		
SR	0.11 (0.02; 0.87)	0.036
BR & SR	0.54 (0.05; 6.18)	0.623
Unknown	0.46 (0.13; 1.69)	0.244
HBeAg-positive	0.23 (0.05; 0.98)	0.047
Viral load, IU/mL		
< 2,000		
2,000 - 20,000	1.4 (0.14; 13.87)	0.773
> 20,000	1.4 (0.33; 5.86)	0.649
ALT > 0.56 IU/L	2.14 (0.57; 7.97)	0.258

Abbreviations: ALT, alanine aminotransferase; BR, blood-related risk factors; SR, sexual-related risk factors.

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