HEPATITIS D VIRUS IN BULGARIA: VIROLOGY, EPIDEMIOLOGY AND PATHOGENESIS IN CHRONIC HBV CARRIERS WITH LIVER DYSFUNCTION

Denitsa Tsaneva-Damyanova[1], Zhivka Stoykova[1,2], Irina Ivanova[3], Tsvetelina Kostadinova[4], Liliya Ivanova[1]

[1]Department of Microbiology and Virology, Faculty of Medicine, Medical University of Varna
[2]Laboratory of Clinical Virology, St. Marina University Hospital
[3]Second Department of Internal Diseases, Faculty of Medicine, Medical University of Varna
[4]TRS Medical Laboratory Assistant, Medical College, Medical University of Varna

ABSTRACT

INRODUCTION: Hepatitis D (HDV) is the most interesting and unique among animal viruses. It causes viral hepatitis D only in individuals already infected with HBV (hepatitis B). This dual infection leads to the most aggressive hepatic dysfunction of all human viral hepatitis.

AIM: This study was made to outline the hepatitis D virus among patients with chronic liver disorders in northeastern Bulgaria, in the sight of virus epidemiology, pathogenicity and viral genotype.

MATERIALS AND METHODS: This is a retrospective study conducted between 2013-2019 at St. Marina University Hospital,Varna, Bulgaria. We have analyzed 418 serum samples from 391 patients with chronic liver disease using ELISA, PCR and HDV sequencing and genotyping.

RESULTS AND DISCUSSION: From 391 patients with chronic liver abnormalities, 16.6% (95% CI: 15.9% - 23.8%, n=65) had an etiological association with HDV in ELISA. We found HDV RNA positive results in 63 out of all 65 anti-HDV Ab (antibody) positive patients (96.9%). Twenty-four of them, or 38.1% (95% CI: 26.1% - 51.2%, n=24), were on antiviral HBV/HDV therapy. For five of them, or 20.8% (95% CI: 7.1% - 42.2%, n=5), HDV genotype I was found.

CONCLUSION: HDV infection has still many mysteries to discover - in terms of pathogenesis, clinical outcome in chronic HBV/HDV-infected individuals, as well as genotype variations and their role in avoiding immune elimination of the virus. All these unanswered questions pose a great challenge to the scientific thought and efforts of humankind to reduce and gradually eliminate viral hepatitis D.

Keywords: *HBV, HDV, HDV RNA, HDV genotype*

Address for correspondence:
Denitsa Tsaneva-Damyanova
Faculty of Medicine
Medical University of Varna
55 Marin Drinov St
9002 Varna
e-mail: dr.tsaneva@gmail.com

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INTRODUCTION

Hepatitis D virus (HDV) infection is still a serious global problem affecting 15-20 million individuals worldwide (1). While hepatitis B (HBV) is a DNA virus coding for several proteins, HDV is a circular single strand negative-sense ribonucleic acid (RNA), group V, genus: Deltavirus, that codes for a single protein-HDV antigen (HDAg). The nucleocapsid is

made up of two forms - HDAg-L and HDAg-S. They are the only proteins encoded by HDV. HDV RNA contains 6 ORFs (open reading frames), 3 of the genomic chain and 3 of the anti-sense chain (2). The virus causes viral hepatitis D only in individuals already infected with HBV. This dual infection leads to the most aggressive hepatic dysfunction of all types of human viral hepatitis, causing fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Patients with HBV and HDV chronic infection have a twofold higher risk of developing cirrhosis, a threefold higher risk of developing HCC and a twofold increased mortality rate compared with HBV monoinfected individuals (3). HDV infection remains one of the main causes of death from viral hepatitis and an indication of liver transplantation. The severity of liver disease caused by HBV/HDV is thought to be associated with the HDV genotype and viral loads (4). There are eight different HDV genotypes worldwide. Up to now, the inverse correlation between HDV and HBV viremia has not been fully elucidated (5). There are multiple routes of transmission of HDV, predominantly parenteral route. The same type of spreading mechanisms determines the possibility of simultaneous HBV/HDV infection (co-infection), as well as HBV/HDV superinfection in chronic HBV carriers. Susceptibility to hepatitis D virus is typical for all age groups, but the disease primarily affects children and adolescents (6). Endemic zones for HDV include the countries of the Mediterranean basin, Russia, Romania, Southern Italy, Central Africa, as well as Western Brazil (7). The pathological changes during hepatitis D are restricted to the liver and this is the only organ in which HDV virus replicates (8). Most researchers believe that HDV genomic replication does not have a cytopathic effect on hepatocytes. Thus, both types of specific immunity - humoral and cellular - can be involved in the pathogenesis of HDV infection (9). Induction of INF-γ, TNF-α, IL-2, IL-10 in hepatocytes to suppress non-cytolytically HBV replication has been demonstrated in the liver of transgenic mice (10). HLA antigens (human leukocyte antigen) A1, DR3 and B18, from MHC I (major histocompatibility complex-I), are associated with more severe HDV infection outcomes (11). By means of dysregulation of the kappa B (NF-κB) nuclear factor, which is associated with inflammation and carcinogenesis processes, hepatocellular carcinoma can also develop (1). Despite the various widely discussed mechanisms, the pathogenesis of HDV-induced hepatic impairment has not yet been fully defined. In Bulgaria HDV is not so fully investigated (only by a few small inquiries) and there is not enough data for viral epidemiology, pathogenesis and especially for circulating HDV genotypes, a fact that underlines the relevance and importance of our project.

AIM

This study was made to outline hepatitis D virus distribution among patients with chronic liver disorders in northeastern Bulgaria, in the sight of virus epidemiology, pathogenicity and HDV genotype.

MATERIALS AND METHODS

This is a retrospective study conducted between 2013-2019 at St. Marina University Hospital, Varna, Bulgaria and in the hospital's laboratories of Clinical Virology and Clinical Immunology. We have analyzed 1056 serum samples in order to determine the relative share of patients with chronic HBV and HDV infection in northeastern Bulgaria. In 418 serum samples from 391 patients with chronic liver disease we have detected HDV using ELISA (enzyme-linked immunosorbent assay), PCR (polymerase chain reaction - real-time PCR and nested PCR) and HDV sequencing and genotyping of five patients (n=5). In our study the genotype assignment was based on the analysis of the sequences that corresponded to nucleotides between 906 and 1256. Analysis of the sequences encoding the C-terminal half of the delta antigen of HDV genome of five individuals was made via direct sequencing of isolates. We have analyzed the sequences via BLAST analysis in NCBI (National Center for Biothechnology and Information).

Ethics Statement

The current study and the corresponding results reported has been conducted in an ethical and responsible manner, and is in full compliance with all relevant codes of experimentation and legislation, coordinated and approved by the Commission for Scientific Ethics at the Medical University of Varna (Protocol/Decision No. 61, from 30.03.17), in compliance with the requirements of article 28 of the Health Law. All of the participants provided written informed consent for the use of their blood and blood components (such as sera and plasma).

Statistical Analysis

Statistical analyses were performed using SPSS v.23. Quantitative variables were expressed as mean ±SD or median (range) as appropriate and the qualitative variables were reported as a number, relative proportion (%) and confidence intervals (95% CI). Data were analyzed by t-test, Pearson's χ^2 test, Spearman's rank correlation (r). Two-sided p-values <0.05 were considered statistically significant. Figures and tables were made using Microsoft Office Pack 2010.

RESULTS

Our retrospective study in Varna and northeastern part of Bulgaria of 1056 serum samples with clinical and laboratory data on liver disease (21 - 86 years), mean age 51.6 y. (SD±14.37), showed an ethological relationship with HBV (HBsAg carrier, HBV monoinfection) in 23.6% (116/492) of the tested patients.

Of all patients tested for HDV (n=391) with proven liver disease, 16.6% (95% CI: 15.9% - 23.8%, n=65) had an etiological association with HDV in ELISA. The distribution of the HDV tested individuals as per their age is presented on Table 1. The higher prevalence of hepatitis D in younger and middle-aged patients 29-48 y. indicates that dual infection is predominantly acquired at a younger age and mainly in people with risky behaviour. A statistically significant difference was found between the anti-HDV Ab seropositivity of individuals in the 29-38 age group and that in the other age groups (Pearson's χ^2=4.48, p=0.03 <0.5).

From 65 registered anti-HDV positive patients, we found HDV RNA positive results in 63 of them in PCR - 96.9% (95% CI: 89.3% - 89.6%). A higher prevalence of hepatitis D was found in patients aged 29-38 years. From them, males - 63.1% (95% CI: 50.2% - 74.7%, n=41), females - 36.9% (95% CI: 25.3% - 49.8%, n=24), between 24 and 68 years of age, mean age 47.36 y. (SD±10.93). Pearson's χ^2 analysis found no statistically significant differences between gender and anti-HDV Ab positive results (X^2=1.4, p=0.22, p>0.05).

Twenty-four of these 63 PCR HDV RNA positive patients or 38.1% (95% CI: 26.1% - 51.2%, n = 24) from 28 to 68 years of age (mean age 44.45±SD 12.17) were on antiviral HBV/HDV therapy. The distribution of HBV/HDV positive patients (n=24) enrolled in antiviral therapy in this study based on laboratory, histologically and clinical ground is presented on Fig. 1. Clinical and laboratory assessment was made according to Child-Turcotte-Pugh (CTP score for cirrhosis mortality) criteria. According to the CTP classification, in stage A liver cirrhosis were 33.3% (95% CI: 15.6% - 55.3%, n=8), in stage B - 12.5% (95% CI: 2.7% - 32.4%, n=3), and in stage C - 16.7% (95% CI: 4.7% - 37.4%, n=4). In 37.5% (95% CI: 18.8% - 59.4%, n=9) of the patients, chronic hepatitis was reported progressing to cirrhosis. A total of 62.5% of the pa-

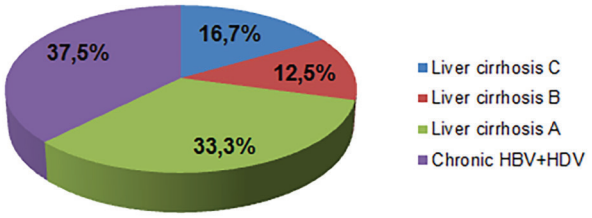

Fig. 1. Stage and severity of chronic liver disease in patients with dual HBV/HDV infection

Table 1. Distribution of the HDV tested individuals as per their age

Age groups (years)	HDV tested	Anti-HDV Ab positive	
		HDV (positive)	Relative share %, (95%CI)
18 – 28 y.	32	2	6.3% (0.8% – 20.8%)
29 – 38 y.	67	17	25.4% (15.5% – 37.5%)
39 – 48 y.	68	14	20.6% (11.7% – 32.1%)
49 – 58 y.	117	20	17.1% (10.8% – 25.2%)
59 – 68 y.	82	11	13.4% (6.9% – 22.7%)
above 68 y.	25	1	4.0% (0.1% – 20.4%)
Total	391	65	16.6% (13.1% – 20.7%)

tients (95% CI: 40.6% - 81.2%, n=15) were with already diagnosed liver cirrhosis.

These 24 patients were classified into A, B and C groups, based on the average values of bilirubin, albumin and prothrombin time (international normalized ratio (INR)). Ascites and encephalopathy were also marked according to their severity. Liver cirrhosis belongs to diseases for which patients' survival is a matter of principle. There are prognostic models for estimating the probability of death in a certain time interval. They are based on an assessment of the functional "reserve" of the liver. Among a series of new prognostic systems, the Model for End Stage Liver Disease (MELD) system is offered as the most promising alternative to the CTP criteria in patients with cirrhosis or complicated liver disease. Summary data for our 24 patients, based on MELD score, are presented in Table 2. Patients with worsening prognosis, according to the MELD system, either had decompensated liver disease (n=7, as per our data), or

Table 2. Clinical and laboratory data for the tested HBV/HDV positive patients (n=24), as per MELD prognostic system, for assessment of liver function and patient's survival

№	Name	Age	Bil. mol/L	Alb. g/L	INR sec.	Ascites	Enchephalopathy
1	**R.A.B**	**28**	**64.0**	**28.0**	**2.34**	**Yes**	**Yes Ex. letalis (HDV-genotype I)**
2	S.D.A	57	31.0	42.0	1.28	No	No
3	G.A.S	35	34.0	38.0	1.34	No	No
4	M.S.S	37	60.0	28.0	1.82	No	No
5	K.A.A	52	15.0	44.0	1.0	No	No
6	V.B.S	47	23.0	36.0	1.10	No	No
7	J.F.B	50	24.0	34.0	1.15	No	No
8	H.B.G	63	22.0	32.0	1.17	No	No
9	T.S.R	33	8.0	46.0	1.03	No	No
10	D.A.K	33	39.0	34.0	1.25	Yes	No
11	G.E.D	60	8.0	44.0	1.04	No	No
12	R.I.P	35	7.0	47.0	1.06	No	No
13	K.M.H	36	7.0	47.0	1.06	No	No
14	N.N.A	29	17.0	44.0	2.93	No	No
15	A.S.S	68	12.0	41.0	1.32	No	No
16	H.S.K	59	31.0	47.0	1.04	No	No
17	**M.K.A**	**36**	**52.0**	**44.0**	**1.23**	**Yes**	**Yes Ex. letalis (HDV-genotype I)**
18	**S.A.M**	**61**	**490.0**	**32.0**	**1.25**	**Yes**	**Yes Ex. letalis (HDV, genotype I)**
19	O.A.M	38	8.0.	45.0	0.99	No	No
20	**V.K.S**	**56**	**45.0**	**30.0**	**1.48**	**Yes**	**Yes Ex. letalis (HDV, genotype I)**
21	H.V.M	45	71.0	20.0	1.76	Yes	No
22	G.S.V	38	6.0.	37.0	1.27	No	No
23	**M.A.E**	**37**	**70.0**	**23.0**	**0.98**	**Yes**	**Yes Ex. letalis (HDV, genotype I)**
24	M.H.S	41	19.0	31.0	0.97	Yes	No

had already died (n=5). The results of the Child-Turcotte-Pugh system, as well as MELD, are the two liver systems that are mainly used to assess the severity of the disease in patients with liver cirrhosis.

All of the five dead patients - 20.8% (95% CI: 7.1% - 42.2%, n=5), with chronic dual HBV/HDV viral infection (3 women and 2 men; mean age 45,8 years; range 28-61 years) were genetically analyzed via direct sequencing of the HDV RNA amplicons. The genotype assignment was based on the analysis of the sequences that corresponded to nucleotides between 906 and 1256. Analysis of the sequences encoding the C-terminal half of the delta antigen of HDV genome of five individuals was made via direct sequencing of isolates. We have analyzed the sequences via BLAST analysis in NCBI. Comparison of the obtained in our survey HDV determined that all of the tested individuals were closely related to HDV genotype I, circulating in our neighboring countries (Greece, Turkey), as well as in Europe (Table 2). Nucleotide similarity among the five isolated sequences ranged from 89% to 96% (mean 93%). The disease pattern associated with infection by HDV genotype I appears to be highly variable. These five patients were at different stagec of liver dysfunction – 40.0% (95% CI: 0.5% - 71.6%, n=2) with chronic hepatitis, stage A - 20.0% (95% CI: 0.5% - 71.6%, n=1), stage B - 20.0% (95% CI: 0.5% - 71.6%, n=1), and stage C – 20.0% (95% CI: 0.5% - 71.6%, n=1) of liver cirrhosis.

Viral HBV DNA and HDV RNA loads tests were performed every 3-6 months during treatment (M3, M6, M9, M12), at the end of the treatment course, and at 6 months after the end of treatment. HBV DNA among different patients was in the range 1.9×10^2 – 7.0×10^6 copies/mL. HDV RNA viremia, as per our results, was 2.5×10^2 - 1.3×10^7copies/mL. Our data also showed no correlation between cirrhosis and disease stage and amount of HDV RNA viremia (rs=0.38, p=0.54, p>0.05). HDV RNA and HBV DNA showed a medium scaled inverse correlation, i.e. higher HDV levels are usually associated with lower HBV viremia.

DISCUSSION

Hepatitis B vaccination programs have led to the trend of a gradual decrease in the incidence of hepatitis B worldwide. As per our results HBV was found in 23.6% of the tested patients and compared to previous studies (HBV/HDV) in patients with chronic liver disease in northeastern Bulgaria - 30.4% (12) and 26.5% (13). This percentage is decreasing.

The prevalence of hepatitis D according to various studies in patients with chronic liver disease shows variability. Various studies support a significantly higher prevalence of concomitant HBV/HDV infection in patients aged 20-39 years, with an overall prevalence of 41.9% (14). The results we have obtained show an incidence of anti-HDV Ab in 16.6% of HBV-infected serum samples from patients with chronic liver disease. In other European countries (Romania), concomitant HBV/HDV infection is detected in 20.4% of patients with chronic hepatitis B. The prevalence of HDV in Europe (France, Germany, Italy, the United Kingdom) is between 8-12% of all of the HBsAg positive subjects tested (15). In a large prospective study in Greece among 4 673 patients with chronic hepatitis B, 2137 individuals were tested for HDV and HBV/HDV positive results were obtained in 4.2% of them. A significantly higher prevalence was found among emigrants (7.5%), compared to the local Greek population (2.8%) (16). In Turkey, serological data for anti-HDV positivity were found in 27.5% of patients with chronic hepatitis (17). According to another study in the Mediterranean basin, 14.8% of the asymptomatic HBsAg (+) patients are infected with HDV, with liver cirrhosis being a common complication (18). In 2013 a study was conducted in North Carolina (USA) among 499 patients with chronic HBV infection and 40 of them (8%) were with dual HBV/ HDV infection (19).

Monitoring the spread of HBV/HDV infection is important because of the socioeconomic significance of these infections and the severe complications for the patients. The relative risk of developing fibrosis, cirrhosis and HCC, is 25 to 35 times higher than that of individuals with HBV monoinfection (20).

We found HDV RNA positive results in 96.9% of all anti-HDV Ab (+) patients which is close to the results reported by EASL (2017), according to which 87% of anti-HDV positive serum samples also hepatitis D nucleic acid positive (21).

Phylogenetic reconstructions based on HDV-encoding sequences showed an ancient division from African HDV sequences - not the previously known

three, but eight different HDV genotypes (22). Isolates of HDV genotype 1 are found in all parts of the world and lead to asymptomatic liver disease to fulminant hepatitis. HDV genotype-1 is found in Europe and North America. All of our five patients with exitus letalis, sequenced for HDV, were from genotype 1 (23). HDV genotype 2 is found mostly in Asia, including Japan, Taiwan, and recently - in Yakutia (Russia). Some viral sequences from the Taiwan and Okinawa Islands have been identified as genotype 2 subtype, called genotype lib (24). HDV genotype 3 is isolated only in the northern parts of South America (Peru, Venezuela, Colombia) and is associated with the most severe and aggressive forms of hepatitis D. New data from a study of HDV-positive patients with genotype 3 HDV infection showed a correlation between serum HDV RNA levels and serum liver enzymes levels (25). Genotype 4 occurs in Japan and Taiwan and has heterogeneous pathogenesis. Genotype 4 isolated from Okinawa, Japan is associated with a faster progression to cirrhosis compared to the predominant genotype 4 in Taiwan (26). Genotypes 5 to 8 occur in patients from Africa who have migrated to Northern Europe (27). All genotypes of HDV refer to one viral serotype.

CONCLUSION

HBV and HDV, continue to be a serious health and social problem despite the widespread specific prophylaxis against HBV and the numerous strategies and options for therapy. HDV infection has still many mysteries to discover in terms of pathogenesis, clinical outcome in chronic HBV/HDV infected individuals, as well as genotype variations and their role in avoiding immune elimination of the virus. All these unanswered questions pose a great challenge to the scientific thought and efforts of humankind to reduce and gradually eliminate viral hepatitis D.

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