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CORRELATION BETWEEN HBSAG QUANTITATIVE ASSAY RESULTS AND HBV-DNA LEVELS IN CHRONIC HEPATITIS B PATIENTS: A SINGLE-CENTER EXPERIENCE

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Summary

Background. Quantifying hepatitis B virus (HBV) DNA is the main laboratory test for diagnosis and monitoring of patients with chronic HBV infection (CHB). Such assays are molecular-based and often expensive for health systems worldwide. Measure of HBV surface antigen (HBsAg) levels by automated chemiluminescent immunoassay (CLIA) has been proposed as a surrogate and cost-effective marker that may simplify HBV management. In this study, we determined whether quantitative HBsAg levels correlate with HBV-DNA levels in a cohort of CHB patients.

Material and methods. This cross-sectional study was performed in the Laboratory of Virology, "St. Marina" University Hospital, Varna, Bulgaria, from January 2022 to December 2024. In total, 315 serum samples from CHB patients were obtained, and HBsAg, HBV-DNA and HBV early antigen (HBeAg)/anti-HBe levels were measured.

Results. The Spearman's rank correlation between the HBsAg and HBV-DNA in the entire

sample was 0.51 with a *p*-value <0.001. In the HBeAg-negative patients, the correlation coefficient was 0.49, and in HBeAg-positive – 0.49 (both *p*-values <0.001).

Conclusions. HBsAg levels, measured by chemiluminescence assay, moderately correlated with HBV-DNA levels in CHB patients from Varna, Bulgaria. The correlation's strength was not related to the individuals' HBeAg status.

Keywords: real-time polymerase chain reaction, hepatitis B surface antigen, viral load, immunoassay, hepatitis B

Introduction

The hepatitis B virus (HBV) is a small DNA virus belonging to the *Hepadnaviridae* family*, genus Orthohepadnavirus.* With an estimated 254 million infected individuals worldwide, HBV infection remains a global burden despite the long-term existence of a highly effective hepatitis B vaccine and relatively effective treatment for chronic cases [1].

Infection by HBV establishes five distinctive clinical forms: asymptomatic, acute, chronic, fulminant and occult hepatitis B infection [2]. Infected individuals unable to clear the virus six months after the infection develop a life-long persistent disease known as chronic hepatitis B (CHB). CHB is a major health challenge worldwide and the principal cause of chronic liver disease leading to one million deaths each year due to complications such as end-stage liver disease and primary hepatocellular carcinoma [1,3].

HBsAg (HBV surface antigen) is the main protein of the viral envelope and the first serological marker for HBV infection. Qualitative HBsAg assays are widely used to screen for the infection and have guided HBV diagnosis for many years. The frequency of HBsAg carriage across the European region varies widely but generally increases from North to South and from West to East, varying between 0.1% in Scandinavian countries and >7% in Balkan

countries [4]. In Bulgaria, hepatitis B is intermediate-endemic with a trend of decreasing HBsAg carrier rates due to the introduction of the universal vaccine for newborns in August 1991. HBsAg positivity of 3.9% was detected in 2010-2011 for the general population but with a significant difference among age groups – the HBsAg prevalence rate in individuals ≤19 years of age (targeted by vaccination) was significantly lower compared to the rate in adults ≥20 years of age – 1% versus 4.8%, respectively [5]. In 2018-2019, HBsAg positivity was 5.1% in a sample of predominantly adult individuals (>30 years and not vaccinated) [6].

CHB is laboratory confirmed when positive HBsAg and/or HBeAg (HBV early antigen) are detected for more than six months and/or HBV-DNA (viral nucleic acid) is present in the serum [7]. Quantitative levels of serum HBV-DNA, ALT/AST levels and histological findings are the three factors to consider when determining HBV treatment and prognosis. HBV-DNA measurement is the recommended tool for monitoring viral replication in chronic cases, but such assays, although increasingly accessible, are time-consuming and often expensive for health systems worldwide. Furthermore, in some patients, there are significant fluctuations in the viral loads regardless of the clinical representation. Recently, the quantification of HBsAg was proposed to be a useful and more cost-effective substitutional marker instead of the molecular detection of HBV-DNA. Early studies have found a good correlation between the two markers, but a core group report from 2011 which summarized the available controversial information recommended that "serum HBsAg quantification is different but complementary to that of serum HBV-DNA, and HBsAg quantification should not be used as a substitute for HBV-DNA measures" [8]. It seems that HBsAg is a more indirect marker for the control of the virus in chronic cases and strongly depends on the HBeAg status of the patient and the presence of cirrhosis [9,10]. The HBV genotypes that are present in different geographic areas may also play a role in the correlation between the serum levels of HBsAg and HBV-DNA [8]. Such studies, although with uncertain practical benefits, do not exist for the Bulgarian cohort of

CHB-infected individuals. Both laboratory tests are available and are performed in many settings, but the correlation between them is still unknown.

Aim of the work

The present study is the first attempt to our knowledge to assess the correlation between the quantitative measure of HBsAg and HBV-DNA in the sera of chronic CHB patients for more practical and economically sound management of HBV in Bulgaria.

Material and methods

We retrospectively investigated the records of CHB patients hospitalized at "St. Marina" University Hospital, Varna, Bulgaria, referred for a routine virological follow-up between January 2022 and December 2024. The following inclusion criteria were set: diagnosis of CHB, registered records for HBsAg quantitative assay, HBV-DNA quantitative PCR and quantitative HBeAg/anti-HBe test at the same time-point. Disease stage, treatment regimen, duration of treatment, disease outcome and socio-demographic parameters were not considered for inclusion in the study. The above-mentioned laboratory tests were part of the standard diagnostic plan for CHB patients monitored in the hospital. Written informed consent was obtained from patients upon admission. No additional patient intervention (laboratory visits or sampling) was performed. In this respect, the conducted retrospective research is not related to either human or animals use.

The initial screening showed 315 records in the laboratory system with the four tests. Additional screening for repeated records showed that 21 patients had been tested more than once during the study period. In such a situation (when the same patient was tested more than

once), only the first result was included in the analysis. Of the 294 patients left, 253 patients had at least one positive result for HBV-DNA or/and HBsAg quantification, and 41 patients with both HBsAg and HBV-DNA negative results were excluded from further analysis. The final sample for analysis consisted of 99 females (39%) and 154 males (61%), all of Bulgarian nationality. The mean age of females was 54.7±13.2 years, and for men – 55.2±12.8 years.

The serum samples were collected and stored at -20°C before DNA extraction and real-time quantitative PCR analysis. DNA was extracted from 400 µl serum using a Viral Nucleic Acid Extraction Kit (Sacace Biotechnologies S.r.l., Italy). All PCR reactions were performed with Taq-man Real-Time Quantitative PCR for HBV-DNA detection (Sacace Biotechnologies S.r.l., Italy) in the presence of internal control to identify possible inhibition of the PCR reaction. The amplification was performed with a PCR instrument from Sacace Biotechnologies S.r.l., Italy in a final volume of 50 µl. The quality of each PCR assay was confirmed by valid positive and negative controls. Results were reported as IU/ml.

The same serum samples (without freezing) were tested in the serological analyses within 24 hours of collection. Commercial CLIA (chemiluminescent immunoassay) kits (Dia Sorin, Italy) were used for quantitative HBsAg detection (in IU/ml) according to the standard instructions of the manufacturer. Commercial ELISA kits (Dia Pro, Italy) were used for semiquantitative HBeAg/anti-HBe detection according to the standard instructions of the manufacturer. For simplification of the analysis, data for HBeAg/anti-HBe was presented as positive or negative, and the results from the grey zone (12 in total) were considered as negative.

The results obtained were processed with R project software (https://www.r-project.org) to calculate the medians and means of variables, IQR (interquartile range), the relative proportions and Spearman's correlation coefficients. We used the Wilcoxon rank-sum or Kruskal-Wallis test to measure the significance of the association of HBV-DNA and HBsAg

with other parameters and considered $p<0.05$ as statistically significant.

Results

Table 1 summarizes the characteristics of the study population, as well as the quantitative levels of HBsAg and HBV-DNA among the different patient groups. There was no statistically significant difference in HBsAg or HBV-DNA levels between genders. The serum's quantity of HBsAg in patients under 30 years was several times higher than in older patients, and there was a well-defined trend of a significant decrease of HBsAg with age. The same was not valid for the level of HBV-DNA: although with a statistically significant difference among the groups, the viral load did not show a clear tendency of increase or decrease with age. Both variables were significantly higher in HBeAg-positive patients when compared to HBeAg-negative individuals. In anti-HBe-positive patients, only HBsAg was significantly lower than in negative patients, while HBV-DNA did not significantly differ.

Table 1. Quantitative HBsAg and HBV-DNA in different patient groups

Patient groups	HBsAg median (IQR)	*p*-value	HBV-DNA median (IQR)	*p*-value
Gender				
Males (n=154)	540 (33-3,700)	0.33*	521 (17-19,600)	0.78*
Females (n=99)	720 (82-3,900)		893 (34-31,250)	
Age				
<30 years (n=10)	5700 (290-20,000)	0.0002#	410 (13-4,160)	0.02#
30-60 years (n=154)	1050 (86-4,300)		1385 (53-41,800)	
>60 years (n=89)	110 (11-1,800)		264 (2-4,170)	
HBeAg				
Negative (n=226)	460 (43-3,000)	0.0004*	562 (17-10,400)	0.004*
Positive (n=27)	4400 (660-19,000)		19600 (139.5-23,350,000)	
Anti-HBe				
Negative (n=49)	3000 (120-9,200)	0.004*	340 (9-691,000)	0.55*
Positive (n=204)	385 (42.5-2,800)		649 (33.5-19,750)	

Notes: *p-value from Wilcoxon rank-sum test; #p-value from Kruskal-Wallis test; IQR (interquartile range).

Both HBsAg and HBV-DNA were not normally distributed within the study population, with medians of 570 (IQR=50-3,800) and 577 (IQR=23-29,500), respectively. The Spearman's rank correlation between HBsAg and HBV-DNA in the whole sample was 0.51 with a *p*-value <0.001. After stratification based on the HBeAg status of the patient, the HBeAg negative group showed a correlation coefficient of 0.49 ($p<0.001$), and HBeAg positive – also 0.49 ($p=0.001$).

In addition, an HBsAg level of <1,000 IU/mL was accepted as a cut-off value to predict a lower risk for the development of hepatocellular carcinoma, especially in the predominant HBeAg-negative study population [11]. The medians of the levels of HBV-DNA in individuals with HBsAg levels below and above 1,000 IU/mL differed approximately 8-fold, 128 vs. 8,545 ($p<0.001$) (Table 2). The HBeAg status did not seem to significantly affect the HBV-DNA serum levels in the two groups – in patients with HBsAg below 1,000 IU/mL, the levels of HBA-DNA were almost the same in HBeAg-negative and -positive individuals; in patients with HBsAg above 1,000 IU/mL, there was a difference between the serum levels of viral load, though without statistical significance.

Table 2. HBV-DNA in individuals with low and high HBsAg levels

HBsAg	Age	Males/females	HBV-DNA median (IQR)	*p*-value
<1,000 IU/ml				
Total	57.6±12.5	86/53 (ratio 1.6)	128 (3-1,075)	0.95*
HBeAg negative	-	-	130.5 (3-1,075)	
HBeAg positive	-	-	128 (12.5-9,500,075.5)	
>1,000 IU/ml				
Total	51.8±12.8	46/68 (ratio 1.5)	8545 (322-1,600,000)	0.06*
HBeAg negative	-	-	6035 (164-1,000,000)	
HBeAg positive	-	-	421500 (760.5-68,850,000)	

Notes: **p*-value from Wilcoxon rank-sum test.

Discussion

The present study analyzes the correlation between the quantity of HBsAg and HBV-DNA in the serum of individuals with CHB. We measured an overall correlation of 0.51 between the two markers, which was similar to that of other studies [9,10,12]. In contrast to most of the previous analyses, the relatively high positive correlation found in our study was not dependent on the HBeAg status of the patients. The lack of dependence could result from the significant disproportion between the number of HBeAg-negative and HBeAg-positive individuals (almost 10-fold) included in the study. This is not surprising as currently, most of the infected individuals, especially in Europe (including Bulgaria), are HBeAg-negative HBV carriers, and compared to HBeAg-positive cases, their number is 7-9 times more frequent [13]. Among all genotypes described for HBV, HBeAg negativity was most prevalent in individuals with genotype D because of the frequent pre-core mutations associated with this genotype [13]. Although we are unaware of the genotypes in our sample (genotype testing is not routinely performed as it is not required for the treatment strategy), a previous study confirmed genotype D as being the most prevalent in Bulgaria [14]. In addition, in our country, there is a very high prevalence of pre-core mutations regardless of HBV genotype [15].

HBV-DNA is still the sole virological marker accepted unambiguously by all guidelines to monitor the progression of CHB individuals. However, the existing positive correlation raises the question of the utility of quantitative HBsAg testing. Currently, quantification of HBsAg is recognized as a useful additional marker in HBeAg-negative patients and in those treated with interferon-based regimens [16]. For HBeAg-positive patients, serum levels of HBsAg are considered the major predictor of the failure to respond to peginterferon [17]. The latest EASL (European Association for the Study of the Liver) guidelines recommended that all individuals treated with pegylated interferon should be tested periodically for both serum HBV-DNA and

HBsAg levels. The 2017 WHO guidelines accept that "quantification of HBsAg is a potential alternative marker of viraemia and to monitor response to antiviral treatment" but emphasize that most HBV antivirals inhibit DNA replication and do not affect HBsAg levels [18]. The latest WHO guidelines also recommend quantification of HBsAg (if available) to be included in the management plan for CHB patients and place its clinical significance in the research and knowledge gaps currently associated with HBV [19]. In Bulgaria, the National Health Insurance Fund requires quantification of HBsAg and HBV-DNA for each patient starting either interferon treatment or nucleotide/nucleoside analogues. Both analyses are also obligatory during the monitoring of the treatment response.

It is clear that a correlation coefficient of 0.51 could not motivate an imperative substitution of HBV-DNA testing with quantitative HBsAg measurement, but the dynamics of both markers are worth a discussion. The data obtained showed a more visible and predicted trend in the levels of HBsAg compared to the fluctuating dynamics of HBV-DNA – the serum HBsAg decreased as expected with age and with the seroconversion of HBeAg to anti-HBe, and what is more, this decrease is logical and statistically significant. This was not the case for the viral load – the highest median of HBV-DNA was observed in middle-aged patients, and anti-HBe-positive patients had higher HBV-DNA than negative patients. Fluctuations in HBV-DNA are present not only at the population level (as in our study) but were also reported in individual patients [20,21].

Recently, a combined single-point quantification of HBsAg and HBV-DNA levels has been proposed as a very effective marker to discriminate between inactive carriage and active disease [22]. The same study defined single-point levels of HBsAg <1,000 IU/mL and HBV-DNA ≤2,000 IU/mL as adequately sensitive and specific to replace a prolonged follow-up. In this context, our study showed a significant difference in the viral loads of individuals with HBsAg serum levels below and above 1,000 IU/mL. This may be the best direction to follow

in the actual management of HBV – using both tests to help researchers learn as much as possible from the specifics behind each individual case, as well as to optimize the number of required assays.

To our knowledge, this study is the first of its nature in Bulgaria, and it fills a significant gap in the available information. However, the study has some limitations, among which the major are its retrospective nature and the heterogeneity of the sample. We evaluated a mixed population of patients on different treatment regimens and at different time points of their treatment. In this way, we were unable to stratify or analyze the data for specific treatment groups. Additionally, only patients from one hospital were included, and this makes the study not representative of the whole country. The current study focusses only on the virological aspects of the analyzed samples. Crucial patient information was not included in the analysis (disease stage, type of treatment, degree of liver fibrosis, etc.), and we are unable to link our results with demographic and/or clinical variables. This could impact the conclusions, as important information for factors such as clinical presentation and infection outcome are missing.

Conclusions

HBsAg levels, measured by chemiluminescence assay, had a moderate correlation with HBV-DNA levels in CHB patients from Varna, Bulgaria. The correlation's strength was not related to the HBeAg status of the individuals.

Disclosures and acknowledgements

The authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval was not sought for this retrospective analysis of existing medical records, as it did not involve any new data collection or interventions.

Artificial intelligence (AI) was not used in the creation of the manuscript.

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