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Quantitative Hepatitis B Surface Antigen as Biomarker for the Evaluation of Liver Fibrosis in Chronic Hepatitis B Patients

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Abstract

Background The hepatitis B virus (HBV), are the primary causative agents of blood borne infectious disease. Cirrhosis and hepatocellular cancer are potential complications of chronic hepatitis. The quantitative HB surface antigen (qHBsAg) assessment can provide clinicians with more precise information. This can be especially useful for predicting treatment response, sustained virological response, disease progression, and other clinical situations, and laboratory-developed methods are not standardized. Persisting the HBV is caused by intrahepatic covalently closed circular DNA (cccDNA).

Objective To find out the relation between the degree of liver fibrosis and the levels of HBsAg and HBeAg and the usefulness of these two markers in prognosis of chronic hepatitis.

Methods Eighty-nine patients with chronic HBV infection took part in a cross-sectional study Gastrointestinal Tract Hospital in Medical City in Baghdad during a period from the first of September 2022 to the end of January 2023. Those patients were >18 years old with and without prior or current anti-HBV therapy and those patients were subjected to fibroscan to monitor liver fibrosis. qHBsAg and HBV (DNA) were taken from the medical records of those who recruited according to the specialist decision. Measuring the blood levels of HBsAg by using quantitative enzyme-linked immunosorbent assay (ELISA). Determining the HBeAG status by qualitative ELISA.

Results Fibroscan results showed that 47% had no fibrosis and the remaining 53% had different ranges of fibrosis, qHBsAg was directly correlated non significantly associated with DNA, age (P >0.05). Regarding HbeAg status (94.4% were negative, and 5.6% were positive). HBeAg was found to be associated with the staging of fibrosis (P <0.01); about 1 (2.4% F0 was HBeAg positive), 0 (0% F1) HBeAg positive), 1 (9.1% F2 HBeAg positive), 3 (27.3% F3 HBeAg positive), and, 0 (0% F4 HBeAg is positive).

Conclusion qHBsAg can be used as a biomarker for liver fibrosis and hepatitis B. HBeAg may be unrelated to the liver fibrosis to determine its clinical implications. The age was non-significantly associated with qHBsAg. qHBsAg levels are a reliable indicator of the immune system's ability to control HBV.

Keywords Chronic HBV, Fibroscan, HBsAg, HBeAg

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List of abbreviations: cccDNA = Covalently closed circular DNA, CHB = Chronic hepatitis B, ELISA = Enzyme-linked Immunosorbent assay, HBeAg = Hepatitis B e antigen, HBsAg = Hepatitis B surface antigen, HBV = Hepatitis B virus, LSM = Liver stiffness measurement, MVB = Multivesicular body, NTCP = Sodium taurocholate co-transporting peptide, qHBsAg = quantitative Hepatitis B surface antigen, rcDNA = Relaxed circular DNA

Introduction

Around 2.57 billion people are infected with the hepatitis B virus (HBV), which results in about 750000 deaths every year. Over 250 million of them have a chronic

infection, which raises the chance of getting HBV-related liver diseases like cirrhosis and hepatocellular carcinoma [1]. The HBV enveloped DNA virus is a member of the hepadnavirus family. The virion's 3.2 kb relaxed circular (rc) DNA genome is double-stranded (ds). In order to create an episomal closed circular DNA (cccDNA), the viral rcDNA infects a hepatocyte by binding to the hepatocyte-specific receptor sodium taurocholate co-transporting polypeptide (NTCP). It looks like mini chromosome. The 2.4/2.1-kb surface mRNAs, 3.5-kb pre-core (pc) and pre-genomic (pg) RNA, cccDNA uses the host RNA polymerase II to create five overlapping 3' end mRNAs, including the 0.7-kb X mRNA. Reverse transcription of pgRNA, which is catalyzed by viral polymerase, is protein-primed and used by HBV for replicating its DNA genome in the cytoplasmic nucleocapsid, the produced rcDNA-containing nucleocapsid is encapsulated by viral surface proteins and released through the cell's multivesicular body (MVB) secretory pathway to create a progeny virion. Besides the virions that contain DNA, the endoplasmic reticulum (ER) lumen also contains a large number of nucleocapsid-free subviral particles. These particles are secreted through the endogenous secretory route and aggregate outside the cell as hepatitis B surface antigen (HBsAg) [2,3].

Serum HBsAg level functions as an additional marker to viral load for predicting HBV related complications in patients with low viral loads. Low viral loads and high blood HBsAg levels are associated with increased risks of cirrhosis and hepatocellular carcinoma [4]. Besides showing intrahepatic HBV activity, the serological marker HBsAg can also predict the efficacy of antiviral drugs used to determine chronic HBV infection [5]. Since then, the qualitative status of the HBsAg has been used to assess the HBV infection status. For those with a persistent HBV infection, this marker was not used for monitoring progressing diseases. Clinical circumstances include disease progression, treatment response, and sustained virologocal response, prediction may all benefit from the improved clinical insight offered by quantitative HBsAg (qHBsAg) analysis. When HBsAg is eliminated, either or because of treatment, it is believed that an HBV functional cure has occurred [6].

There is currently no commercially available quantitative procedure for HBeAg, and laboratory-developed methods are not standardized. Fibroscan is unable to correctly differentiate between adjacent fibrosis phases and has a significant overlap in liver stiffness measurement (LSM) to intermediate stage [7]. Interpreting patients with ascites and elevated body mass index (BMI) should be done with caution and in the entire clinical scenario because of the possibility for unpredictability. The Fibroscan uses ultrasonic technology, therefore it can be impacted by several factors. For instance, the shear wave's velocity is susceptible to the effects of fluid and adipose tissue. Ascites, thickening of the intercostal walls, hepatic obstruction, and elevated portal venous pressure the accuracy of the fibroscan can be impacted by inexperienced operators, obesity, liver obstruction, and heart failure [8,9].

Methods

Subjects and data collection

This cross-sectional study was conducted during a period from the first of September 2022 to the end of January 2023. Eighty-nine patients with chronic hepatitis B infection (CHB) in Gastrointestinal Tract Hospital Hospital in Medical City Complex, Baghdad were taken part in the study (52 males and 37 females) with an age range (18 to 80 years).

Fibro scan staging F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), F4 (advanced fibrosis). The patients were classified into these stages by a specialist doctor.

Relevant data, including qualitative HBsAg, HBV (DNA) was taken from the medical records of those who recruited according to the specialist decision; some of these information, such as age, sex, duration of antiviral therapy, and

duration of the disease, was provided by direct patient interviews.

Inclusion criteria

Patients with CHB infection, their age >18 years old, with or without prior or current anti-HBV therapy and those patients were subjected to fibroscan to monitor liver fibrosis.

Exclusion criteria

Patients with other chronic diseases such as hepatitis C virus, or patients on immunosuppressive or immune-modulating therapy for any reason.

Specimens' collection

CHB patients were attended to GIT hospital for their routine investigation. The patients were classified into fibro scan stages F0, F1, F2, F3, F4 by a specialist doctor. Five ml of whole blood was drawn from them. The sample was divided into three parts, the first part was three ml, which was injected into the EDTA tube for molecular purposes, it was centrifuged for 10 min at 1000 g. One ml of resultant plasma transferred into sterile Eppendorf tube. The second and third part was one ml injected into each serum separator tube for serology. The blood was left at room temperature for about two hours. Then, it was centrifuged for 10 min at 1000 g.

One ml of resultant serum from each serum separator tube was transferred into other sterile Eppendorf tubes. The resultant plasma was stored in -70°C, and the resultant serum was stored at -20°C for later use in enzyme-linked immune sorbent assay (ELISA).

Ethical approval

This study was approved by the Institutional Review Board (IRB) of the College of Medicine, Al-Nahrain University (number 202207178 in 8/7/2022, and conducted in the Department of Microbiology of this college.

Quantification of HBsAg

By using kit for HBsAg Catalogue No. YLA3739HU, Biont, China uses ELISA based on the Biotin double antibody sandwich technology, during reaction precoated HBsAg mono clonal Ab capture the Ag in the sample. Then when added HBsAg antibodies labeled with Biotin unite with streptavidin-horse reddish peroxidase (HRP), which forms immune complex. Unbound enzymes were removed after incubation and washing. When Substrate A, B was added the solution was turned blue and changed into yellow with the effect of the acid. The shades of solution and the concentration of HBSAg were positively correlated. This assay has high sensitivity and excellent specificity for detection of HBsAg assay range: 5-2000 ng/L; lower limit of detection: 2.64 ng/L.

Reagents preparation

- All reagents were brought to room temperature (18-25°C) before use. When the kit was not used up in one assay, only the necessary strips and reagents were taken out for present experiment.
- Wash Buffer: 20 mL of Concentrated Wash Buffer were diluted with 580 mL of deionized or distilled water to prepare 600 mL of Wash Buffer.
- Standard working solution: shown in table (1).

Assay procedure for QHBsAg

- The standard was diluted by small tubes first, then the volume of 50 µl were pipetted from each tube to micro plate well, standard solutions was diluted as shown in table (1).
- Sample injection included Blank well like Chromogen solution A and B, and stop solution only was added. Then Standard solution well was 50 µl standard and streptavidin-HRP 50 µl was added. After that sample well to be tested included 40 µl sample and then 10 µl HBsAg antibodies was added, 50 µl streptavidin-HRP, and then cover it.

- Preparation of washing solution: The concentrated washing buffer was diluted with distilled water (30 times for 96 tests).
- Washing the seal plate membrane was removed carefully, then drain the liquid automatically by ELISA washer this procedure repeated five times and the plate blotted.
- Color development determine by 50 µl chromogenic solution A firstly was added to each well and then 50 µl chromogen solution B to each well. Shacked gently to mix them up. Incubated for 10 min at 37°C away from light for color development.
- Stop reaction included 50 µl Stop Solution was added to each well to stop the reaction (the blue color changed into yellow immediately at that moment).
- Assay included blank well was taken as zero, the absorbance (OD) of each well one by one measured under 450 nm wavelength, which carried out with in the 10 min after having added the stop solution.
- According to standards' concentrations and the corresponding OD values, calculated by special software employed too as well. Software tools were applied to obtain the results from the data (BIO RAD system).

This assay has high sensitivity and excellent specificity for detection of HBsAg. The assay range: 5-2000 ng/L; lower limit of detection: 2.64 ng/L.

Table 1. Preparation of standard dilutions

Standard concentration	Standard number	Standard working solution
1200 ng/L	Standard No.5	120 µl original standerd+120 µl standard dilutions
600 ng/L	Standard No.4	120 µl Standard No. 5+120 µl standard dilutions
300 ng/L	Standard No.3	120 µl Standard No. 4+120 µl standard dilutions
150 ng/L	Standard No.2	120 µl Standard No. 3+120 µl standard dilutions
75 ng/L	Standard No.1	120 µl Standard No. 2+120 µl standard dilutions

Determination of Hepatitis B e antigen status

Hepatitis B e antigen (HBeAg) status was determined by ELISA kit (HBeAg ELISA system, Catalogue No. EL13-1416 Monocent, USA, (qualitative method) sandwich technology. Sensitivity and specificity were 99.5% and 99.4%, respectively.

Principle of the test

The solid phase is coated with specific anti HBe monoclonal antibody (Ab) that in the 1st incubation captures the Ag, which present in the sample, after washing, in the 2nd incubation, a second mono clonal Ab labeled with peroxidase (HRP) binds the Ag captured on the solid phase by means of a second binding site the Ag, in the 3rd incubation the enzyme bound generates a color by acting on the chromogen/substrate solution that can decided by micro titer ELISA reader. The intensity of the color is proportional to the concentration of Ag in the sample.

Reagents preparation

- Wash Buffer: 50 mL of Concentrated Wash Buffer was diluted with 1200 mL of deionized or distilled water to prepare 1250 mL of Wash Buffer
- Conjugate preparation: concentrated conjugate was diluted 1:20 with conjugate diluent.

Note: the diluted conjugate is stable for 1 week at 2-8°C when stored in sterile disposable container.

- Chromogen/ Substrate: about 5 min before use. One volume of chromogen with 1 volume of substrate was mixed in a disposable plastic container.
  Note: This solution stable for 4 hr at room temperature protected from light

Assay procedure of HBeAg

- A1 well was left empty for blank operations and then 100 µl of HBeAg added as a negative control, 100 µl of HBeAg positive control, and 100 µl of the sample.
- Preparation of washing solution: The concentrated washing buffer was diluted with distilled water (25 times for 96T).
- Washing the plate sealer peeled out carefully, then the liquid drained automatically by ELISA washer this procedure repeated five times and the Plate blotted.
- One hundred µl of diluted conjugate added in all wells but A1, the seal plate membrane covered and incubated strips for 60 min at 37°C. The plate sealer was peeled out carefully, then the liquid drained automatically by ELISA washer this procedure repeated five times and the plate blotted.
- One hundred µl chromogen/ substrate solution was added into all wells. A1 includad strips incubated for 20 minutes at room temperature, all the wells A1 included.
- The enzymatic reaction was stopped by adding 100 µl of Stop Solution to all the wells A1 included.
- The plate was read at 450 nm wavelength, and 620-630 nm the instrument blanked on A1 well.
- The cut-off value was calculated through the following formula (cut-off = NCmean+0.100) Samples with an OD value lower than cut-off were classified as negative for HBeAg, and samples with an OD value higher than cut-off were classified as positive for HBeAg (cut-off=0.119)

Statistical analysis

The statistical analysis system program includes chi-square, t-test, and, Pearson correlation analysis, that were used to analyze the data of this study. Entry of data into Excel systems and the tests were achieved by statistical package for social sciences (SPSS) version 20 (2020). P values equal to or less than 0.05 were considered statistically significant.

Results

Eighty-nine patients with CHB infection were collected from GIT hospital in medical city in Baghdad (52 male and 37 female) with a range age years (range, 18 to 80 years had undergone fibro scan staging 42 (47.2% F0, 20 (22.5% F1), 11 (12.4% F2),11(12.4% F3) and 5 (5.6% F4).

Correlation analysis of different indicators of HBV infected patients

It was found that HBsAg was directly correlated non significantly associated with HBV(DNA), age (P>0.05) as shown in table (3).

Table 3. Correlations among HBsAg, DNA, and age

		HBSAg(Q) ng/L	Age (yr)
HBVDNA (copy n./mL)	r	0.010	0.014
	P	0.930	0.897
qHBsAg ng/L	r		0.151
	P		0.158

Relationship between qHBsAg and severity of fibrosis and staging of fibrosis

The mean±SD of qHBsAg in patients with severe-advanced fibrosis (No. 16) was 150.4±51.8 ng/L, and in patients with mild-moderate fibrosis (No. 31) was 94.8±41.0 ng/L, while in those with no fibrosis (No. 42) was 86.2±41.6 ng/L, the difference was significant (P <0.001) (Tables 4).

Likewise in staging of fibrosis by fibroscan, there was significant difference qHBsAg level according to the stage (P <0.001) where the highest mean±SD of qHBsAg found in patients with F3 (severe fibrosis) (No. 11) 157.2±46.5 ng/L, whereas the lowest level was found in patients with F1 (mild fibrosis) (No. 20) 80.9±31.6 ng/L, however, those with F0 (no fibrosis) (No. 42) the level of qHBsAg was 86.2±41.6 ng/L (Table 5).

Association of HBeAg status with staging of fibrosis

Regarding HbeAg status, 94.4% were negative, and 5.6% was positive as shown in table (6) and figure (1).

Table 4. Comparison of qHBsAg level according to the severity of fibrosis

HBsAg ng/L	N	Mean	Std. Deviation	P value*
No fibrosis	42	86.2	41.6	
Mild-moderate (F1-F2)	31	94.8	41.0	0.001
Severe-advanced (F3-F4)	16	150.4	51.8	
Total	89	100.7	49.0	

* P value obtained by ANOVA test

Table 5. Comparison of qHBsAg level according to the staging of fibrosis

qHBsAg ng/L	N	Mean	Std. Deviation	P value*
F0	42	86.2	41.6	
F1	20	80.9	31.6	
F2	11	120.0	45.5	0.001
F3	11	157.2	46.5	
F4	5	135.3	65.1	
Total	89	100.7	49.0	

* P value obtained by ANOVA test

Table 6. Frequency of HBeAg in CHB patients

HBe Ag	Frequency	Percentage
Negative	84	94.4
Positive	5	5.6
Total	89	100.0

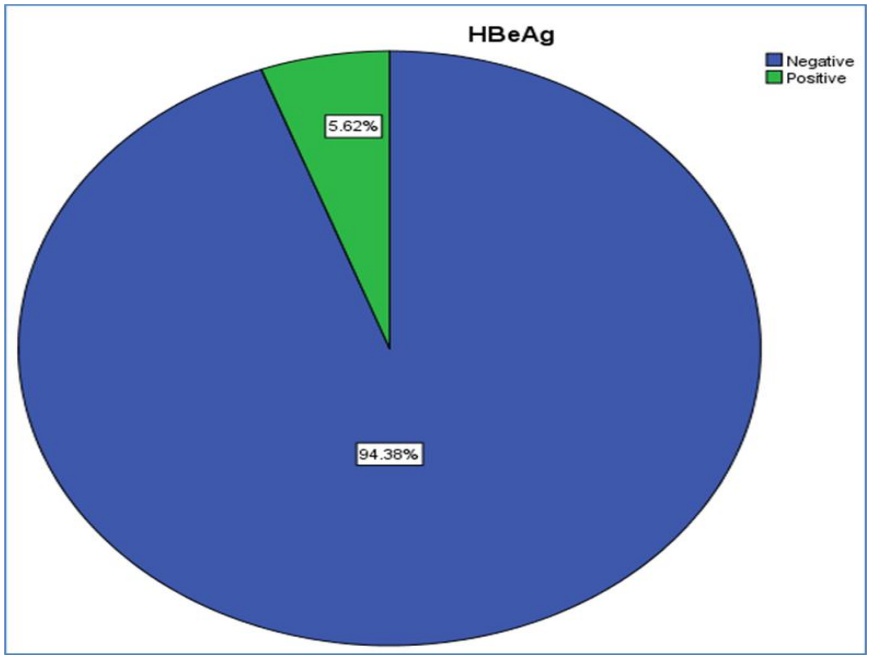

Figure 1. HBeAg in chronic hepatitis B patients

HbeAg was found to be significantly associated with staging of fibrosis (P ≤0.01); as in F0, 1 (2.4%) was HbeAg positive, in F1, 0 (0%) HbeAg positive, in F2, 1 (9.1%) HbeAg positive, in F3, 3 (27.3%) HbeAg positive, 0 in F4, 0 (0%) HbeAg positive, as shown in table (7).

Table 7. Association between HBeAg status and staging of fibrosis

Staging (F0-F4)		HBeAg		Total
		Negative	Positive	
F0	Number of patients	41	1	42
	% within staging (F0-F4)	97.6%	2.4%	100.0%
	% within HBeAg	48.8%	20.0%	47.2%
	% of Total	46.1%	1.1%	47.2%
F1	Number of patients	20	0	20
	% within staging (F0-F4)	100.0%	0.0%	100.0%
	% within HBeAg	23.8%	0.0%	22.5%
	% of Total	22.5%	0.0%	22.5%
F2	Number of patients	10	1	11
	% within staging (F0-F4)	90.9%	9.1%	100.0%
	% within HBeAg	11.9%	20.0%	12.4%
	% of Total	11.2%	1.1%	12.4%
F3	Number of patients	8	3	11
	% within staging (F0-F4)	72.7%	27.3%	100.0%
	% within HBeAg	9.5%	60.0%	12.4%
	% of Total	9.0%	3.4%	12.4%
F4	Number of patients	5	0	5
	% within staging (F0-F4)	100.0%	0.0%	100.0%
	% within HBeAg	6.0%	0.0%	5.6%
	% of Total	5.6%	0.0%	5.6%
Total	Number of patients	84	5	89
	% within staging (F0-F4)	94.4%	5.6%	100.0%
	% within HBeAg	100.0%	100.0%	100.0%
	% of Total	94.4%	5.6%	100.0%

Discussion

In this study, it was found HBsAg was directly correlated, non-significantly associated with DNA (P >0.05) These findings disagree with a previous study by Pollicino et al. who suggested that surface Ag levels do not always correlate with viral replication activity but may serve as a consistent source of viral RNA and proteins [(10)]. The results of a current study indicate that the majority of HBsAg in this study comes from cccDNA, which serves as the template for preS1 RNA and preS2/S RNA.

The association between the mean serum level of qHBsAg and the severity of fibrosis was found to be significant (P <0.001). This means that as the qHBsAg level increases, the severity of fibrosis also increases. The main goal of treatment is to halt the progression of severe liver disease, such as cirrhosis, liver failure, and HCC. This will result in less liver damage and a lower likelihood of developing severe disease outcomes [(11)]. It has been found that the results of a current study do not align with an earlier study conducted by Tatar et al. investigated the correlation between qHBSAg and fibrosis stage in treatment-naive patients is still a topic of debate. According to the majority of qHBsAg from integrated DNA, integrated HBV DNA serves as a source of HBsAg expression [(12)]. It is possible that the majority of DNA in this study was from cccDNA, which could explain this difference.

It has been found that qHBsAg indicates a response of T cells and B cells specific to HBV. While the levels of HBsAg have been reported to not affect the specific immune cell

composition, higher levels of qHBsAg have been linked to the phenotypes of exhaust CD4+ T cells and malfunctioning B cells [13]. According to these findings, the level of immunological control of the HBV in patients with CHB is determined by the qHBsAg. The findings of the current study align with previous research conducted by Cornberg et al. that mention the ultimate objective of treating chronic HBV infection is to achieve a functional cure. A sustained reduction in qHBsAg level signifies a functional cure for CHB patients. Although it is the preferred treatment target, it is rarely attained in CHB patients [14].

In 2021, Brakenhoff et al. conducted a study on patients with HBV infection who were treated with pegylated interferon (peg-IFN) with or without nucleoside analogues (NA); the study found that qHBsAg can be used to predict treatment response in patients who are positive or negative for HBeAg. The study also observed that patients with low pre-treatment qHBsAg levels and a significant decline in HBsAg levels during early peg-IFN treatment had higher rates of sustained virologic response (SVR) and HBsAg loss [15].

A present study agrees with a study by Le Bert et al. that suggested there is no significant association between HBsAg levels and age. Le Bert et al. were the first to suggest that the T-cell response, specific to the surface antigen, is not solely dependent on the concentration of HBsAg at a specific time. Instead, it is the duration of infection and exposure to the antigen that is connected to this response. However, Le Bert et al. demonstrated in their study that ageing did not cause a decline in T cells specific for HBV core and HBV polymerase [16].

Aliabadi et al. [17] reported a negative correlation between HBsAg levels and age, which in turn negatively affected the HBV-specific T cell response, especially the pol CD4+ response.

In the current study, qHBeAg was shown to be significantly associated with the staging of fibrosis (P ≤0.01); In the current study, qHBeAg was shown to be significantly associated with the staging of fibrosis (P ≤0.01). The current study disagrees with earlier findings that HBeAg was a risk factor for positive pgRNA because pre-C mRNA, translated from HBV cccDNA, was used for producing HBeAg, cccDNA, which serves as the source of PgRNA. The two forms of 3.5 kb RNA were thought to modify a specific ratio; in other words, patients in the active phase of the disease had high loads of HBV cccDNA as well as high levels of HBV viral expression in the livers [18-20].

In conclusions;

- qHBsAg reflects on cccDNA, and can be used as a biomarker for liver fibrosis which is a non-invasive, cost-effective method.
- qHBsAg is a reliable biomarker to predict severity of fibrosis but it is not reliable to predict staging of fibrosis
- qHBeAg is may be unrelated to the liver fibrosis More research and development are necessary to create an assay for HBeAg to determine its clinical implications.
- Age was non-significantly associated with qHBsAg
- qHBsAg levels are a reliable indicator of the immune system's ability to control HBV.

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Author contribution

Dr. Abdulamir: participated in its design and coordination. Al-Hamadani: contributed to the acquisition and interpretation of data, sampling, and doing all laboratory tests and writing manuscript.

Conflict of interest

There is no conflict of interest.

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