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Evaluation of Serum Levels of HBV-DNA Concentration and HBSAG Titers of Hepatitis B Virus-Infected Subjects at NAUTH Nnewi

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Abstract

Hepatitis B is an infectious disease of great public health importance. Nigeria is one of the countries with the highest incidence of Hepatitis B Virus (HBV) infection worldwide. However, the accessibility and affordability of HBV DNA quantification (viral load) assay which is the key laboratory test for therapy initiation, and monitoring is a challenge to HBV management. The study was done to evaluate serum levels of HBV-DNA concentration and HBsAg titers of hepatitis b virus-infected subjects at NAUTH Nnewi. Cross sectional study design was used with a total of 264 subjects comprising of 88 HBsAg seropositive treatment naïve subjects, 88 HBsAg seropositive subjects on antiviral therapy as case subjects and 88 age-matched apparently healthy HBsAg seronegative individuals were recruited as control subjects. Hepatitis BVirus DNA assay was performed using real time PCR technique, ELISA technique was used for Hepatitis B surface antigen quantification, Hepatitis Bcore Antibody Immunoglobulin M and Hepatitis D Virus Immunoglobulin G assay. Immunochromatography was used for HBV Panel, Hepatitis C Virus assay, Human Immunodeficiency Virus testing. HBsAg quantification showed strong positive correlation with HBV DNA viral load both in treatment and non-treatment groups ($r = 0.673$; $p < 0.001$). The non-treatment group has higher viral load (M=805.50IU/ml) compared with treatment group (M = 65.50IU/ml) ($p<0.001$).

Keywords: HBV-DNA concentration, HBsAg Titers, hepatitis B virus subjects.

INTRODUCTION

Hepatitis B is an infectious disease of great public health importance caused by hepatitis B virus (HBV). It is an enveloped DNA virus that infects the liver and causes hepatocellular necrosis and inflammation [1]. This virus belongs to the family hepadnaviridae and genius orthohepatodnavirus and it is the only hepadnavirus that causes infection in humans [2]. Hepatitis B virus is one of several viruses known to cause viral hepatitis and continues to be the major cause of viral hepatitis in the developing and underdeveloped world. In addition to causing chronic liver disease and cirrhosis, it has a formidable track record of being linked to primary hepatocellular carcinoma. It is estimated that HBV and HCV is the root cause of about 80% of all hepatocellular carcinomas (HCC) by promoting cirrhosis which significantly reduced the life expectancy of the infected patients [3].

About 257 million people are chronically infected annually and about 2 in 3 people with Hepatitis B do not know they are infected [4]. Recent statistics indicate that not less than 23 million Nigerians are estimated to be infected with the HBV, making Nigeria one of the countries with the highest incidence of HBV infection in the world [5]. A national study done in Nigeria in 2016 shows a prevalence rate of 12.4 percent [6]. This worldwide burden of hepatitis B mandates accurate and timely diagnosis of patients infected with HBV and the use of treatment strategies derived from evidence-based guidelines. Most hepatitis B patients are asymptomatic in the early stage as specific clinical symptoms often occur at advanced disease stages, which are usually irreversible. Hence, the prognosis of the infection to liver disease is very crucial. The presence of derangement in specific laboratory analytes at the early stage of infection may signal a risk of fibrosis, cirrhosis and ultimately HCC.

The diagnosis of HBV is not only imperative but also complex because of different viral antigens, which bring about varying serological profiles in different stages of the disease [7]. The ability of HBV to induce chronic hepatic inflammation gives rise to these intricate serological profiles. Serological markers are used routinely as diagnostic and prognostic indicators of acute and chronic HBV infection [8].

Among all the several clinical diagnostic tests which have been developed for the detection of HBV infections, the serum HBV DNA level is a key factor affecting the initiation of antiviral therapy and evaluation of its efficacy [9]. Evaluation of the relationship between the serum HBV DNA levels and hepatic pathology is a current hotspot in the diagnosis and treatment of CHB [10]. Quantification of the HBsAg levels has received renewed attention because of its diagnostic potential in predicting the response to antiviral treatment and identifying the infection status of an individual [11]. Determination of the circulating levels of HBsAg could provide crucial information that could complement the measurement of HBV DNA. Studies of HBV infections conducted under various clinical settings have suggested that serum HBsAg could be used as a combinative or substitutive marker of HBV DNA levels [12].

Hepatitis B Virus DNA quantification has been in use for diagnosis and monitoring of patients who are being treated for chronic hepatitis B (CHB). This diagnostic method is molecular-based and expensive; thus, less complex and cheaper laboratory tests as surrogate diagnostic markers might simplify hepatitis B management [3]. Quantification of hepatitis B surface antigen (HBsAg) by automated chemiluminescent micro-particle immunoassay has been proposed to be a surrogate marker [13].

The study was done to evaluate serum levels of HBV-DNA concentration and HBsAg titers of hepatitis b virus-infected subjects at NAUTH Nnewi.

MATERIALS AND METHODS

Study Area

The study was carried out at the Gastroenterology unit of Nnamdi Azikiwe University Teaching Hospital (NAUTH).

Study Design

Cross sectional study design was used in the study. A total of 264 subjects were recruited which comprised of 88 HBsAg seropositive treatment naïve subjects, 88 HBsAg seropositive subjects on antiviral therapy (Tenofovir 300mg daily/Entecavir 0.5mg daily or Pegylated interferons 180µg weekly) as case subjects and 88 age-matched apparently healthy HBsAg seronegative individuals were recruited as control subjects.

Sample Size Determination

Power analysis for a one-way ANOVA with three groups was conducted in G Power to determine a sufficient sample size using an alpha of 0.05, a power of 0.96, and a medium effect size(f=0.25). Based on the aforementioned assumptions, the total sample size is 264, with 88 subjects per group [14].

Sampling Technique

Purposive sampling technique was employed in selecting the participants based on the inclusion criteria. Patients that gave their consent who also met the selection criteria were recruited as they come to the clinic until the sample size was completed.

Sample Collection

After obtaining informed consent, 10ml of venous blood was collected from the fore arm of each subjects using a disposable syringe; 7ml was dispensed to a sterile K_2-EDTA vacutainer (1.2mg/ml concentration) for hepatitis B DNA Viral load, retroviral screening (RVS) and anti HCV screening while 3ml was added to a sterile plain container for HBsAg quantitation, HDV IgG assay. The plasma and serum samples were separated into sterile plastic containers and preserved at –86°C.

Ethical Consideration

Ethical Approval

Ethical approval was sought and obtained from the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital Nnewi before the commencement of this study (Reference: NAUTH/ CS /66 /VOL.10/187/2017/096).

Inclusion Criteria

Participants included in this study were hepatitis B seropositive subjects attending Gastroenterology Clinic of NAUTH, Nnewi and apparently healthy HBsAg seronegative individuals (NAUTH staff, students and others) who gave their consent.

Exclusion Criteria

Those patients who were co-infected with HIV, HDV and HCV including HBsAg seronegative subjects who have received HBV vaccination were excluded from the study.

Laboratory Diagnosis

HBV DNA Viral Load using CobasAmpliprep/ Taqman Real Time PCR Technique (Roche Molecular System Inc, USAas described by Iregbu and Nwajiobi-Princewill [1]

Procedure

The frozen plasma was placed at room temperature until completely thawed before use. The High Positive, Low Positive and Negative controls were removed from 2-8°C storage and brought to room

temperature before use. All reagent cassettes were removed from 2-8°C storage and loaded immediately onto the COBAS AmpliPrep Instrument and allowed to equilibrate to ambient temperature on the instrument for at least 30 minutes before the first specimen was processed. The appropriate number of reagent cassette racks, sample racks with Input S-tubes, SPU racks, K-tip racks, K-tube racks and K-carriers on K-carrier racks was loaded onto the respective rack positions of the COBAS AmpliPrep Instrument. Sample rack was prepared by attaching a barcode label clip to each sample rack position where a specimen (S-tube) was to be placed. The specific barcode label clips for the controls were attached to each sample rack position where the controls (S-tube) were to be placed. One Input S-tube was placed into each position containing a barcode label clip.

The Amplilink software was used to create specimen orders for each specimen and control in the Orders window Sample folder and HBV test definition file was selected and saved. The specimen and controls were vortexed for 5 seconds and 650 µL added to the S-tubes. The tubes were then inserted in the sample rack and loaded to the Ampliprep. The Amplilink software was used to start the COBAS Ampliprep. At the completion of the COBAS TaqMan Analyzer run, the Results Report was validated and printed.

HBsAg Quantification by ELISA Technique (Fortress Diagnostics Limited United Kingdom as described by Bhatnagar *et al.*, [15]

Procedure

The wells were marked and 20µl of Specimen Diluents were added into each well except the blank. Then 100µl of Positive control, Negative control and Specimen were added into their respective wells except the blank. The plate was covered with plate cover and incubated for 60mins at 37^{o}C. At the end of the incubation, the plate cover was removed and discarded, 50µl HRP-Conjugate were added into each well except the blank and mixed by tapping the plate gently. The plate was then covered and incubated at 37^{o}C. At the end of the second incubation, each well was washed times with diluted buffer allowing microwells to soak for 30-60 secs each time. After the final washing cycle, the plate was turned down onto blotting paper and tapped to remove any remnant. After the washing, 50µl of Chromogen A and 50µl of Chromogen B solutions were added into each well including the blank incubated in the dark at 37^{o}C for 30 mins. After the incubation, 50µl of stop solution was added into each well using multichannel pipette and mixed gently. The blank was then used to calibrate the plate reader and the absorbance was read at 450nm.

Anti HBcAb IgM quantitation by ELISA (Diagnostics Automation Inc. Woodland Hills, California as described by Kesslar *et al.*, [16]

Procedure

All reagents and samples were brought to room temperature before use. The number of wells needed for the assay were removed from storage and the remaining were kept back at 4^{o}C.Three wells were marked as negative controls, two wells, positive controls and one blank. Then, 100µl of samples and controls were added into their respective wells and Blankwell was set without any solution. Adhesive strips were used to cover the wells and incubated for 30 minutes at 37°C. At the end of the incubation, the strips were removed, and each well was washed five times with diluted buffer allowing the microwells to soak for 60secs at each washing. After the final washing cycle, the plate was turned down onto a blotting paper and tapped to remove any remaining liquid. Then, 100µl of HRP-Conjugate reagent was added into each well except the blank covered and incubated for 30mins at 37°C. The plate cover was then removed and the aspiration/wash process was repeated for five times, 50µl each of Substrate A and B were added to each well, incubated for 15mins at 37°C avoiding light. Multichannel pipette was used to add 50µl of stop solution into each well and mixed gently. The blank well was used to calibrate the plate reader and the absorbance was read at 450nm. The cut-off for each batch was calculated using the mean optical densities of the negative control in accordance with the manufacturer's instruction. This cut-off value was then used to calculate the activity index for each sample by dividing the mean OD of each sample with the cut-off value. Samples with the activity index values higher or equal to those of positive control were considered positive, while those with values below were reported as negative (Index value > 1.10 was reported as positive).

HDV IgG ELISA by Diagnostics Automation Inc. Woodland Hills, California (as described by Lai, 1995) [17]

Procedure

The reagents and samples were brought to room temperature and votexed before use. The number of wells needed for the assay was removed from storage. Then 100µlof specimen diluents were added to into each well except the blank. Three wells were marked as negative controls, two wells, positive controls and one blank. Also, 10µl of positive, negative controls and samples were added into their respective wells except the Blankwell covered and incubated for 30 minutes at 37°C. At the end of the incubation, the strips were removed, and each well was washed five times with diluted buffer allowing the microwells to soak for 60secs at each washing. After the final washing cycle, the plate was turned down onto a blotting paper and tapped to remove any remaining

liquid, 100µl of HRP-Conjugate reagent was then added into each well except the blank covered and incubated for 30mins at 37°C. The plate cover was then removed, and the aspiration/wash process was repeated for five times, 50µl of Chromogen A and Chromogen B were added to each well, incubated for 15mins at 37°C in the dark. Multichannel pipette was used to add 50µl of stop solution into each well and mixed gently. Intensive yellow colour developed in Positive control and HDV IgG positive sample wells. The blank well was used to calibrate the plate reader and the absorbance was read at 450nm.

Anti-Hepatitis C Virus (HCV) Serological Assay (Chembio Diagnostic Systems, Inc. Medford, Newyork as described by CDC, 2003 [18]

Procedure

The test kit was brought to room temperature, removed from the foil and placed on a flat surface. It was labeled with the specimen's identification number, and approximately 50µl of the serum sample was applied onto the sample pad. The test was allowed to run for 15 minutes after which the result was read.

Human Immunodefiency Virus (HIV) Screening by Serial Algorithm

Determine HIV 1/2 Assay (Abbort Rapid Diagnostics CA, USA as described by Delaney and Branson, 2011 [19]

Test Procedure

The protective foil was removed, and the test device was kept on a flat surface. The device was labeled with the specimen's identification number and 50µl of the serum sample was applied to the sample pad. The result was read after 15 minutes.

Unigold HIV 1/2 Rapid Test (Trinity Biotech PLC, Ireland as described by CDC, 2001 [20]

Procedure

The test device was removed from the protective wrapper and kept on a flat surface. On the sample port, 60µl of serum sample was added also, two drops of wash buffer reagent. Result was read after 10 minutes.

Chembio Stat-Pak HIV 1/2 Assay (Chembio Diagnostic Systems, Inc. Medford, Newyork as described by Kenealy *et al.*, 1987 [21]

Test Procedure

The test kit was removed from its pouch and placed on a flat surface. The test device was labeled with specimen's identification number. The 5µl sample loop was touched on the specimen allowing the opening of the loop to be filled. The sample loop was then held vertically to touch the centre of the SAMPLE (S) well of the device to dispense approximately 5µl of sample onto the sample pad. The Running Buffer bottle was held vertically and three drops (approximately 105µl) of the Buffer was added slowly. The test result was read after 10 minutes of adding the Running Buffer.

STATISTICAL ANALYSIS

Data obtained were analyzed using Statistical Package for Social Sciences (SPSS) version 20) software. Data were expressed as mean ± SD and median. The significance of differences in mean values among groups were analyzed using one-way Anova for normally distributed variables, while Kruskall Wallis was used to analyze the significant differences in median values among different groups for variables not normally distributed. Mann-Whitney was also used to analyse significant differences between groups. Spearman's correlation coefficient was used to assess the levels of relationship between two variables. Regression analysis and receiver Operators curve were also used appropriately. The level of significance was considered at $p < 0.05$.

RESULTS

Median serum levels of HBV-DNA concentration and HBsAg Titers of the hepatitis B subjects

Table-1 shows that the median serum HBV DNA level observed in the whole cohort (n = 176) was 422.50 IU/mL, whereas the median serum HBsAg titer was 1350.50 IU/mL. There was no significant difference in the median HBV DNA level in the male group compared with the female group patients; 475 IU/mL) versus 337 IU/mL); P = 0.523. A similar pattern was observed for the HBsAg titer whose median values did not show significant gender differences.

Correlation between serum HBsAg titers and HBV-DNAin: (a) the whole hepatitis B patient cohort (b) drug treatment group (c) the treatment naïve group

Pearson's linear regression analysis indicated a significant positive correlation between HBsAg and HBV DNA among all patients at r = 0.627, P< 0.001 as shown in Figure-1a. Similarly, there were significantly positive correlations between HBsAg and HBV DNA for both the treatment and non treatment groups, (Fig-1b, r = 0.565, P< 0.001 and Fig-1c, r = 0.673, P< 0.001).

Table-1: Median serum levels of HBV-DNA concentration and HBsAg Titers of the hepatitis B subjects

Variable	Whole Cohort (n = 176)	Males (n = 100)	Females (n = 76)	P Value
HBVDNA (IU/mL)	422.50	475.0	337.0	0.523
HbsAg (IU/mL)	1350.50	1355.0	1343.50	0.239

Key: HBV DNA = Hepatitis B Viral DNA Quantification, HBsAg = Hepatitis B surface antigen quantification.

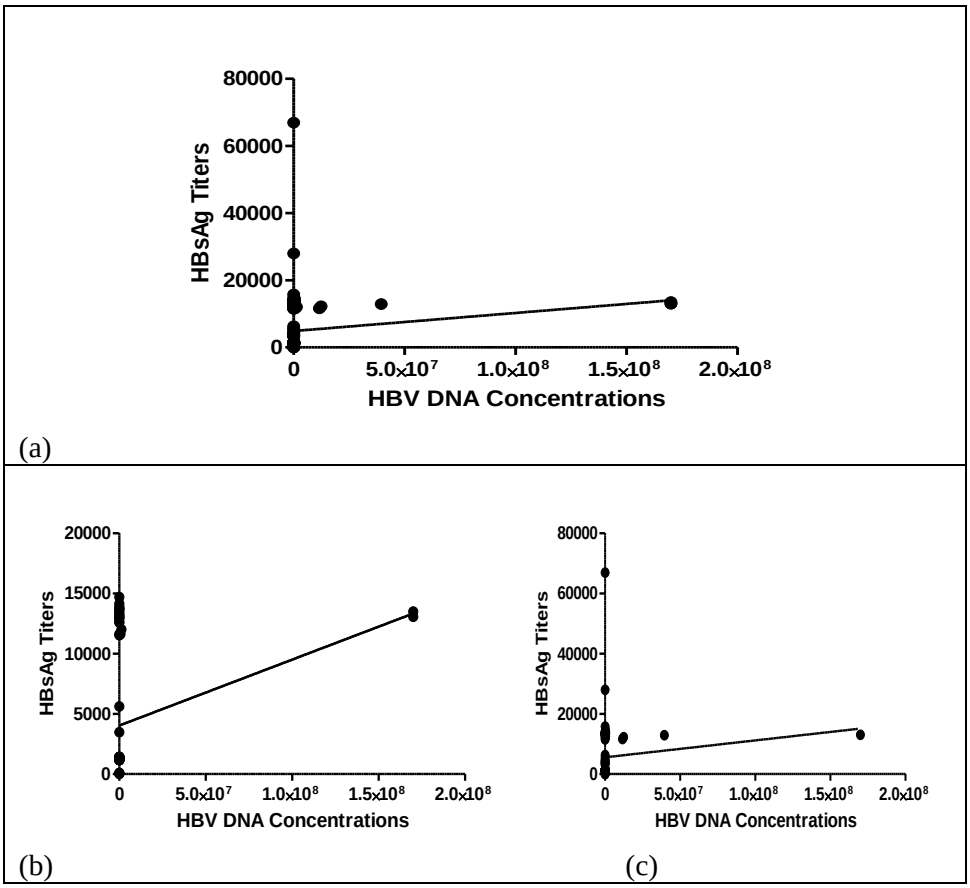

Fig-1: Correlation between serum HBsAg titers and HBV-DNA levels in: (a) the whole hepatitis B patient cohort (n = 176); (b) drug treatment group (n = 88) (c) the treatment naïve group (n = 88)

DISCUSSION

Quantification of HBsAg indirectly reflects the number of infected hepatocytes and is known to change over the natural course of chronic HBV infection [22]. Also, during antiviral therapy, it could be used to differentiate true inactive carriers from patients in remission who are likely to progress to cirrhosis [23]. The HBsAg quantification in this study, showed a strong positive correlation with HBV viral load both in treatment and non treatment groups. Kim *et al.*, 2011 [24] also reported a high significant correlation (r=0.657, P<0.001). On the contrary, Ganji *et al.*, [25] reported negative correlation between HBsAg and HBV viral load among chronic HBV infected individuals. The variations could be due to differences in disease stage, HBeAg status and HBV genotypes involved.

The above finding also agrees with Kim *et al.*, [24] and supports the fact that hepatitis B surface antigen (HBsAg) is an important diagnostic marker, generally detectable in patients with acute and chronic infection; positive testing indicates high HBV replication in the liver, elevated blood HBV titers and greater infectivity to others. Level of HBsAg correlates with covalently closed circular DNA (cccDNA) level in the liver and reflects the amount of cccDNA inside hepatocytes. Furthermore, it correlates with the transcriptional activity of cccDNA and is considered a surrogate marker of infected cells. Although this study demonstrates a relationship between serum HBsAg titers and HBV DNA levels in the whole cohort of HBsAg positive patients and in its clinical subgroups, the area under the curve (0.537; p = 0.002) showed that HBsAg quantitation indicated a very poor performance in discriminating or distinguishing between normal and abnormal viral loads in hepatitis B subjects, when compared with the serum HBV-DNA test. This suggests that HBsAg quantitation may not be an effective alternative to serum HBV-DNA quantification in hepatitis B virus infected patients. This finding is similar to the report of Mathia *et al.*, [26]. HBVDNA, is the template for gene transcription and replication and level is the most important and most direct etiological evidence for HBV.

The sex distribution of the subjects in this study showed that there were more males 100(57.2%) males and 76(42.8%) females. This shows that HBV infection is more prevalent in males than females and the finding is in keeping with the findings of Yewande *et al.*, [27] which reported males are 2.8 times more likely to get infected with HBV compared to their female counterparts [7]. This could be due to the fact that males are more prone to risk factors and behaviors sexual intercourse, injection drug use, barbing et ce tera which predispose them to HBV infection. Regarding

the age distribution, highest frequency of HBsAg seropositivity was found among the younger age group 21-30 followed by 31-40 group for both males and females. Also another work done by Gheorghe *et al*., [28] gave a similar report. Considering the modes of transmission of HBV, the high sexual activity of individuals within these age brackets might explain this. The age group <20 had the least frequency both among males and females (3.9 and 3.9) respectively. The >50 age group were the second to the least of the subjects (9% females and 12% for females). This finding is in agreement with that of Yewande *et al*., [27]. The children (<20) and the aged (>50) have less exposure to risky behaviours than the middle aged (21-30, 31-40).

CONCLUSION

This study has revealed that HBsAg quantification has strong correlation with HBV viral load but might not be efficient in clinical practice as a predictor of serum HBV viral load due to its poor performance characteristics in identifying high positive viral load.

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