CORRELATION BETWEEN HBSAG QUANTIFICATION AND HBV GENOTYPESIN CHRONIC HBV INFECTIONS

[1]QABASNEAMAHHADI, [1]MOHAMMED IMAD AL-DEEN MUSTAFA, [2]HUI YEE CHEE, [3]KHAIRULAZHAR JAAFAR

[1]Dept. of Basic Medical Sciences, [3]Dept. of Gastroenterology, Faculty of Medicine, IIUM, Kuantan, Malaysia
[2]Dept. of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, UPM, Kuala Lumpur, Malaysia
E-mail: qabasnima@yahoo.com , miamustafa@yahoo.com, cheehy@upm.edu.my, Dr.drkhai72@yahoo.com

Abstract- Accumulated data has been proposed that viral genotypes and sub genotypesplay an important role in clinical outcome and antiviral monitoring of chronic HBV infection. Genotyping and quantitating of HBV DNA levels molecularly is relatively expensive. On the other side, serum HBsAg quantification has been recently standardized by automated quantitative assays leading to revived interest in the clinical utilization of this marker for diagnosis using sensitive and reliable commercial assays. Several studies have referred to the kinetics of HBsAg to predict response to antiviral therapy. Thus, a cheaper laboratory test as a surrogate diagnostic marker might simplify patients' management. Objectives: to evaluate whether quantitative HBsAg levels correlate with HBV genotypes in Malaysian CHB patients. Patients and Methods: In this cross-sectional study, fifty serum samples from patients with hepatitis B were used, however those tested positive for antibodies to HCV and HDV were excluded. The serum HBsAg level was quantified by Elecsysassay. In addition, HBV DNA load was measured by real-time polymerase chain reaction whereas identification of HBV genotypes was done by direct sequencing. Results: Of 50 patients, 31 were males (44%) and 19 females (27%); the mean age was37 ± 12 years. Forty-two (42) patients had HBsAg level >100 IU/mL.Eightypercent (40) were HBeAg-negative while 20% (10) were positive. Genotyping showed that 22 patients had genotype C (44%), and 18 patients were of genotype B (36%). Applying Mann-Whitney test, HBs Agtiters correlated differentially with HBV genotypes C& B. ($p<0.05$). By Non-parametric T test, there was no significant correlation between HBsAg levels and HBV DNA levels ($p<0.05$).Conclusions: the statistically significant association between levels of HBsAg and HBV genotypes was observed especially for genotype C. Hence, HBsAg level could be a useful serological marker to predict the genotypes B and C of HBV and thus possibly anticipate the response to treatment with antiviral drugs.

Keywords- Hepatitis B genotypes, chronic hepatitis b virus infection, HBsAg quantitation

I. INTRODUCTION

Globally, nearly one third of the world's population has serological evidence of past or present infection with the hepatitis B virus (HBV), with an expected 350-400 million people who are surface HBV antigen (HBsAg) carriers. Thus, HBV infection is one of the most important infectious diseases worldwide that about one million persons die from HBV-related causes annually. Many diagnostic factors are considered when determining HBVtreatment such as quantitative levels of HBV DNA, ALT levels, genotypes and histological finding. Recently, there has been increased interest in quantify the serum levels of HBsAg as an alternate marker to predict HBsAg loss and monitor anti-HBV therapy. During the natural history of HBV infection, the loss of serum HBsAg is generally associated with their seroconversion to anti-HBs, the hallmark of a successful immunological response to HBV infection. It is hypothesized that it can be a useful tool for managing patients as well.

HBV can be classified into eight genotypes and four major serotypes from A to H, based on an intra-group nucleotide divergence of up to 4.2% of the S-genome sequences or in >8% of the entire genome sequences. These genotypes arise during replication as a result of nucleotide mis-incorporations, in the absence of any proofreading capacity by the viral polymerase. Data from several investigators have been shown the effect of HBV genotypes on clinical outcomes in chronic HBV patients, including disease severity, HCC development, and response to therapy, disease chronicity, transplantation outcomes, and occult infection. Active viral infection and HBV genotypes can be detected by quantifying and typing of HBV DNA, using molecular-based assay which relatively are expensive. Considering the distribution of HBV, particularly in developing countries, a cheaper laboratory test that can be used as a surrogate marker for the molecular detection of HBV DNA might make our management more practical.

Many studies showed a correlation of HBV genotypes with HBeAg clearance, liver damage, and the response to treatment, but with HBsAg is not clarified well. Also, several retrospective and case-control studies have been reported that patients who infected with HBV- genotype C have more severe liver disease outcomes (cirrhosis and HCC) than those patients who are infected with HBV- genotype B In addition, There have been reports about differing therapeutic responses with nucleos(t)ide analogs and interferon α with respect to different genotypes. Furthermore, some genotypes, such as B and C, may have a greater risk for the development of hepatocellular carcinomas. Nevertheless, in the clinical setting in contrast to hepatitis C, the diagnosis of HBV genotypes is not part of the clinical

routinewhereas, DNA level is the routine assay for monitoring the infection. Serum HBsAg concentration was related to HBV DNA replication level; nevertheless, it is still not practicable touse HBsAg concentration to monitor HBV replication levels.However, the aim of the present study is to investigate the possible relationships between serum levels of HBV DNA and HBsAg, and how HBV genotype could impactthis relationship.

II. MATERIAL & METHODS

2.1 Subjects:
50 consecutive CHB patients (31 males and 19 females, age 18 to 70 years with the mean age was 37 ± 12 years) from Gastroenterology Department at Hospital TengkuAmpuanAfzan (HTAA) in Pahang, Malaysia were recruited as study group, Informed consent of individuals was obtained prior to their enrolment in the study.

All patients were sero-negative for hepatitis C virus, delta virus and human immunodeficiency viruses. The patients were positive for HBsAg for more than six months, and have clinical features of chronic HBV infection according to the hospital records and clinician report.

2.2 Serological and biochemical assays
The serum HBeAg, anti-HBe, HBsAg, and anti-HBs status of the subjects were checked by commercial third –generation ELISA (MONOLISA® Bio-Rad) conducted as routine assays to follow up the status of the patients at screening laboratories in HTAA. Serum as partate transfer aselactate dehydrogenase and alanine transferase were tested by routine automated techniques.

2.3 Quantification of serum HBsAg assay
Elecsys assay was used in serum HBsAg quantification (Roche Diagnostics, Germany) following the manufacturer's protocol for HBsAg II assay. If the results of cut off index (c.o.i) are between 1 and 1000, the final result is the c..o..i X 400, if c.o.i> 1000, the sample is retested at a 1:8000 dilution and the final result is calculated as c.o.i X 8000. While, if the c..o..i is < 1 the sample is retested undiluted. This method was validated by others and a very strong correlation was found between this method and the Architect HBsAg quantitative assay (Abbot).

2.4 HBV DNA Detection and genotyping
2.4.1 DNA Extraction
HBV DNA was extracted from 200 ul of each serum sample with chronic HBV infection using QIAampMinElute Virus Spin Kit (Qiagen, USA) owing to the manufacturer's protocol.

2.4.2 Polymerase Chain Reaction for HBV DNA detection
PCR reactions were carried out in a thermal cycler (Bio-Rad, USA). The Nested PCR condition was done to amplify the target of s small region using outer and inner primers (table1). The first round (30 cycles) of amplification was performed in Initial Denaturation 95 °C for 5 min ,Denaturation 94 °C for 30 sec, annealing 50 °C for 30 sec,Extension 72° C40 sec, and Final Extension 72° C for 8 min with 5 µl of extracted DNA by using an outer sense primer and an outer antisense primer specific to the surface gene of HBV (600 bp),whereas, the second round of PCR(35 cycles) was carried out with inner sense primer and inner antisense primer(340bp) Initial Denaturation 95 °C for 5 min ,Denaturation 94 °C for30 sec,Annealing 46.9°C for30 sec, Extension72° C 40 sec, and Final Extension 72° C for10 min. Amplified products were subjected to electrophoresis in 2% agarose gel and evaluated under UV transillumination

Table 1: oligonucleotide primers used to amplify the hepatitis BS small gene using nested PCR

Primers	Sequence (5'-3')	Products size(bp)
F1(Forward outer primer)	TAGGACCCCTGCTCGTGTTA	600
R1(Reverse outer primer)	ATGTTGTACAGACTTGGCCC	600
F2(Forward inner primer)	CTCGTGGTGGACTTCTCTCA	340
R2(Reverse inner primer)	AATTTCCGTCCGAAGGTTTT	340

2.4.3 Direct sequencing
Direct Sequencing was done for selected samples of the patients who diagnosed with positive samples of HBV to read the nucleotide sequence of the target gene (S small region). The sequencing assay was achieved in both directions of the primers (forward and reverse primers) used the same amplification primers mentioned in table 1. The PCR products were commercially sequenced (First base company, Malaysia) using employing ABI PRISM® automated dye-terminator system.

2.4.4 HBV Genotyping and phylogenetic analysis
Sequences were assembled using Bioedit version 7.2.0 to reorganize the sequences like remove vague gaps and aligned sites. The molecular evolutionary analysis was carried out MEGA v.5.2 software package.

2.4.5 Real-time PCR for DNA quantitation

HBV DNA levels were quantified by in house real-time PCR (RT-PCR) using(Rotor-Gene™ 6000, Qiagen) instrument. The specific primers (table 2) for each genotype were designed based on s small region of envelope HBV genome. The reaction was carried out using a commercial SYBR Green developed method using SensiFAST SYBR No-ROX Kit (Bioline, USA). It has been developed for fast, highly reproducible real-time PCR. The master mix (2x) containing all the components necessary for real-time PCR, including the SYBR® Green I dye, dNTPs, HotStarTaq polymerase.

The reaction was under thermo cycling conditions consisted from Initial Denaturation for 5min at 95 °C, followed by 40 cycles of 95°C for 10 second ,60° C for 30 second and 72°C for 20 second.

Table 2: oligonucleotide primers used in Real time PCR to amplify the hepatitis B S gene using PCR

Primers	Sequence (5'-3')	Products size(bp)
FC1(Forward primer)	GATTGGGACCTCAACCCACA	182
RC1(Reverse primer)	CCTAACTGCCGATTGGTGGA	182
FB2(Forward primer)	CATGCAAAACCTGCACGACT	203
RB2(Reverse primer)	GGAAAGCCCTACGAACCACT	203

III. RESULTS

A total of 50 from HBs Ag positive individuals with meanage 37.5 ±12 years were included31(44%) men and19 (27%) women. Of the total, 42 they had HBsAg level >100 IU/mL(figure 1)which confirmed that they are detected as chronic carrier. Our data showed that eighty percent (n=40) were HBeAg-negative while 20% (n=10) were positive. The Mean value of HBsAg titer in CHB with HBeAg positive and negative patients (figure 2) were 14,955 and 8,128 IU/mL, respectively; while mean HBV-DNA in HBeAg-positive patients and HBeAg-negative patientswere 60,000 and 143,977 copies/mL, respectively.

It was found from our study that the common HBV genotypes in chronic hepatitis Malaysian patients were genotype C in percentage 44% (n = 22), and genotype B in percentage 36% (n = 18), and 10 unknown because no DNA detection in these samples. By Applying Mann-Whitney test, it was been found HBsAg titers correlated significantly with HBV genotypes C ($p < 0.05$), while there was weakly correlation showed with genotype B. Moreover, using Non-parametric correlation (Spearman's test), there was no significant correlation between HBsAg levels and HBV DNA levels ($r=0.291$, $p= 0.07$) ($p<0.05$).

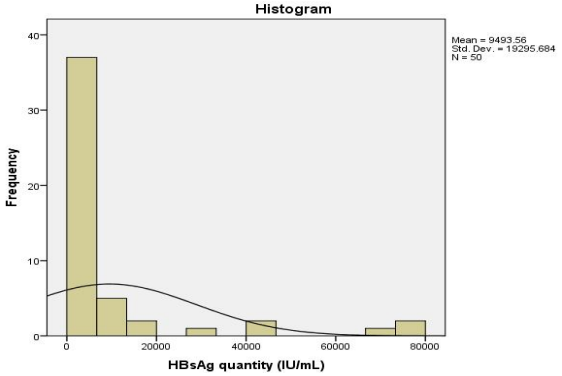

Figure1. Illustrates the HBsAg titer distribution in CHB patients

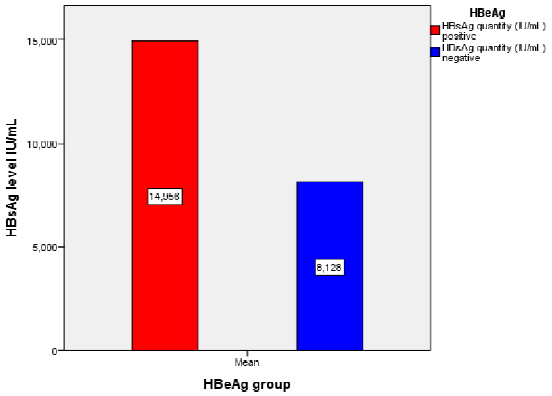

Figure 2: Illustrates the HBsAg titer distribution in HBeAg positive and negative groups in CHB patients

IV. DISCUSSION

Recent attention has motivated on the use of HBsAg quantification assay for the evaluation and management of HBV-infected patients. The serum HBsAg titers different in the two phases of HBeAg – ve or +ve HBV infection suggest that quantitative HBsAg testing might be a good diagnostic tool along with the quantitation of HBV DNA. Recently, HBV antigens have been offered as biomarkers for assessment the treatment response, as well as clinical surrogates for HBV cccDNA levels, the intranuclear HBV reservoir responsible for persistence. Clinical interpretation of HBsAg and HBeAg titers might therefore be refined by considering the phase of disease, as well as quasispecies diversity. This study results showed no significant correlation between quantitative HBsAg and HBeAg groups of CHB patients. Therefore our results were concordance with that reported by some researchers who confirmed that there was no significant correlation between HBsAg and HBeAg and HBV-DNA content before and during interferon therapy. While, different results in HBeAg-positive and negative CHB have been showed by Thompson et al, when they found that HBsAg had strong correlation with HBV-DNA ($r = 0.69$; $p < 0.01$), while in HBeAg-negative CHB group

HBsAg had weak correlation with HBV-DNA (r = 0.28; p = 0.01).Measuring serum HBV-DNA is the gold standard for monitoring viral load, but it is relatively expensive and not yet readily available in some areas. Hence, there is a definite need of a tool which is economical, reliable, and easy to perform. HBsAg quantitation is a recent serological marker being evaluated.

The variation between HBV DNA levels and HBsAg titers patients is thought to be related to the fact that serum HBsAg levels depend mainly on the translation of specific messenger RNAs for the "s" gene generated from the covalently closed circular DNA (cccDNA) in addition to integration of HBV DNA within the host genome, and these levels decline during transition from the active to the inactive phase. Altogether, HBsAg levels may reflect the "transcriptionally" active cccDNA rather than its absolute amount or HBV DNA-integrated sequences. Based on the results of study which suggested that Quantitative HBsAg has significant correlation with HBV-DNA in CHB patients and can consider quantitative HBsAg as asurrogate marker to HBV-DNA. Our data, to some extent, differ from those reported in some earlier studies that showed the positive correlation between HBsAg titers and DNA viral load, while our finding proposed that there was weak or no related relationship between them. The same study also suggested that immune control of HBV replication does not necessarily impair HBsAg production, possibly due to HBV integration into the host genome. The possible explanations of the apparent "disconnect" between HBsAg titers and HBV DNA levels may be due to many reasons. First, the regulation of viral replication may have resulted in an altered ratio of HBV virion to subviral HBsAg particles. Second, the highly dynamic interaction between HBV and the host immunity during chronic hepatitis B may result in some developing reactivation of HBV replication and vice-versa.

Finally, it may be explained by differences in HBsAg synthesis, which is distinct from the HBV DNA replication pathway, under the influence of different immune control mechanisms. Similarly, Jaroszweicz et al., found in a cohort mainly infected with HBV genotype-A and genotype-D that HBsAg productionwas well preserved in selected HBeAg – vepatients with low HBV replication.

Our results from genotyping assays used in this study demonstrated that there are the common genotypes in CHB Malaysian patients were genotype B & C. our results confirmed the previous studies happened in Malaysia. Interestingly, our investigation appeared that significant correlation HBsAg titers production during chronic phase with HBV genotypes C & B, especially for type C more than B. This relationship may be related to the efficiency of the HBV DNA template to produce HBsAg that could be differently affected by HBV genotype diversity. However, our data to some extent differed from earlier studies that demonstrated that there is no impact of HBV genotype on HBsAg quantitation or on the ratio of HBsAg/HBV DNA.

On another side, many studies have recently submitted the significant correlation between HBV genotype with the clinical features of HBV infection and the response to antiviral treatment. In fact, with regard to genotypes B and C, which are prevalent in Asia, genotype C has been shown to be more frequently found in severe liver disease and in hepatocellular carcinoma and it presents a lower response rate to interferon alfa therapy, while genotype B is associated with faster HbeAg/antiHBeseroconversion. Therefore these different genotypes, probably characterized by a different natural history and a different response to therapy, could require a different clinical and therapeutic approach.

In conclusion, the statistically significant association between levels of HBsAg and HBV genotypes were observed especially for genotype C. Hence, HBsAg level could be a useful serological marker to predict the genotypes B and C of HBV and thus possibly anticipate the monitoring and response to treatment with antiviral drugs. HBsAg titers are appropriate for the monitoring of patient response to antiviral therapy. Some highly cost-effective assays in genotyping and HBV DNA determination, such as PCR and colorimetric assays are applicable in resource-constrained countries. To ensure a better use of HBsAg quantitation during therapy, studies are needed to establish clinically relevant thresholds according to HBV genotype, HBeAg status, HBsAg/HBV DNA ratio and clinical stage of HBV persistent infection.

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