Identification of Novel D11 Hepatitis B Surface Antigen Subgenotype in Jeddah, Kingdom of Saudi Arabia

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A B S T R A C T
Hepatitis B virus (HBV) infection remains to be a worldwide health problem. In Saudi Arabia, HBV is the most predominant type of hepatitis followed by hepatitis C and hepatitis A while little is known about the molecular epidemiology of the prevalence of HBV genotype/subgenotype particularly in Jeddah. Serum samples were collected from HBV chronic patients and subjected to HBsAg gene amplification. Sequencing and phylogenetic analysis of the entire HBsAg gene sequences revealed that 11 isolates belonged to HBV/D while 4 isolates were associated with HBV/C. Interestingly, HBV/D subgenotypes identified eight HBV/D present isolates belonged to HBV/D1 while three isolates showed a new cluster supporting by a branch with 99% bootstrap value and 4.3-5.8% nucleotide divergence over the entire HBsAg gene from other known subgenotypes D1 to D10, despite they were appearing more related to HBV/D5. The three strains of the new D subgenotype showed unique amino acid sequences consisting of Thr7non, 75Pro in the preS1 gene, 112Ile, 161Gly in the preS2 gene and 196Glu, 197Ala, 238Ser, 259Cys in the S gene. In addition to three amino acid residues in the S gene (373Ile, 374Ala and 381Thr) were specified S118S isolate. Subsequently, it have been verified that HBV/D1 is the most prevalent HBV subgenotype in Jeddah as well as we proposed a novel subgenotype designated HBV/D11. The identification of HBV/D11 novel subgenotypes in the present study suggested that further studies with a large number of subjects in previously examined and unexamined areas may lead to discovering new HBV strain genotypes and/or subgenotypes circulating in Saudi Arabia.

Key words: Hepatitis B virus, HBV surface antigen mutations, Saudi Arabian HBV, HBV/D11, HBV amino acid mutations

INTRODUCTION
Many countries in the world suffer a great financial load from the high percentage of both chronic HBV infection and approximately 400 million HBV carriers worldwide. HBV infection develops severe liver diseases, including cirrhosis and hepatocellular carcinoma (HCC) (Okamoto et al., 1988; Norder et al., 1992, 1994). Human HBV is the prototype member of the family Hepadnaviridae that contain a circular, partially double stranded DNA genome of about 3200 bp. HBV DNA contain four overlapping open reading frames for pre S/pre S/S, pre C/C, pol and X (Magnius and Norder, 1995).

Compared to most DNA viruses, HBV has high mutation rate nucleotide substitution, although this is lower than the mutation rate of RNA viruses (Okamoto et al., 1987). Previously, HBV was classified into 4 genotypes but recently it has been classified into eight genotypes designated as A-H.
This classification is based on intergenotypic divergence of at least 8% in the complete nucleotide sequence or more than 4% in the HBsAg gene. These genotypes possess different geographical distributions (Magnus and Norder, 1995; Arauz-Ruiz et al., 1997; Chu et al., 2003; Miyakawa and Mizokami, 2002). Genotype A and F are predominantly found in Northwestern Europe, American natives, Polynesia, North America and Central Africa (Kramvis et al., 2002). Genotypes B and C are found in Southeast Asia, China and Japan (Michitaka et al., 2006) and genotype D has a worldwide distribution including the Mediterranean region, the Middle East and India (Sunbul et al., 2013; Bahri et al., 2006). Genotype E was designated frequently in Africa while Genotype G was reported in France, Germany, the United States (US) and Mexico (Kato et al., 2001, 2004; Sanchez et al., 2002; Vieth et al., 2002; Chu et al., 2003). The eighth genotype named H is confined to Nicaragua, Mexico and the US (California) (Sanchez et al., 2002; Kato et al., 2004). Recently, a complex recombinant of genotypes A, C and G referred to be a new genotype (I) which was described and sequenced in Northwestern China, Vietnam and Brazil changing the genotyping of HBV into nine genotypes confirming with the stereotypes classification (Santos et al., 2010).

Subgenotypes have been illustrated in certain HBV genotypes, that is A1-A6 in HBV of genotype A (HBV/A), B1-B8 in HBV/B, C1-C16 in HBV/C and D1-D8 in HBV/D (Kramvis et al., 2002; Chekarou et al., 2010; Depamede et al., 2009, 2010).

HBV genotypes have direct correlation with the severity of liver diseases. HBV/C is associated with more severe liver diseases than HBV/B (Kramvis et al., 2008; Sugauchi et al., 2004; Banerjee et al., 2006; Huy et al., 2006; Sakamoto et al., 2006) while contrary studies reported similarity on the risk of HCC development in either HBV/B or HBV/C infection (Kao et al., 2003; Sumi et al., 2003; Yuen et al., 2003, 2009). Also, patients infected with HBV/D appear to have a higher incidence of HCC (Chan and Sung, 2006) whereas patients with either HBV/C or HBV/D have a lower response rate to treatment with IFN-a compared to those with HBV/A and HBV/B (Zollner et al., 2001). Genotype may also influence the emergence of lamivudine resistance mutations which appear to be more strongly associated with genotype A than genotype D (Wen, 2004). HBV/B has been linked to a younger HCC profile, in which cirrhosis were less commonly seen (Liu et al., 2007, 2011).

HBV surface antigen (HBsAg) is able to induce protection against HBV infection where it is related directly to B-cell epitopes and consider as the major target of neutralizing antibodies therefore can be used as vaccines (Kramvis et al., 2005). Complete HBsAg gene consists of three regions; large S, preS2 and preS1 which they share the C-terminal 226 amino acid residues (Szmuness et al., 1981; Carman, 1997). Mutant HBsAg nucleotides may cause amino acid substitutions (El Hadad et al., 2013) leading to affect the binding of specific anti-HB antibodies and detection by conventional diagnostic assays (Torresi, 2002). A correlation has been found between low antigenicity of HBV (lead to HBV reinfection) and increased incidence of HCC in Egyptian HBV chronic patients (Tian et al., 2007) and between mutations in HBsAg gene, particularly pre S regions and development of HCC was verified in HBV chronic patients (Yang et al., 2010). It is also generally known that genotype C carries a higher chance of cirrhosis and HCC (Sumi et al., 2003; Yuen et al., 2003, 2009).

Although, the prevalence of HBV infection is generally high in Asian and African countries (Lee, 1997), little is known about HBV genotypes/subgenotype sequences circulated in Saudi Arabia particularly in Jeddah province (Al-Faleh et al., 1992, 1999). In Saudi Arabia, HBV were the most predominant type accounting for 53% of the cases, followed by HCV (30%) and HAV (17%) (Alshabanat et al., 2013). Moreover, Jeddah is a main Haj (pilgrimage) entry point as well as being the largest commercial port in the country which serves as transit hub for millions of people from high-burden HBV countries such as South East Asia, Middle East and Europe. Among these people, presumably many carrying HBV that may make changes in the nucleotide sequence of the HBV genotypes in Saudi Arabia and increasing the incidence of HBV infection every year. However, up to date, there have been limited molecular studies about the prevalence of HBV genotypes/subgenotypes and the genetic characteristics of HBV in Saudi Arabia particularly in Jeddah. Therefore, the present study was conducted to further identify and phylogenetically characterize the various HBV isolates circulating in Jeddah.

MATERIALS AND METHODS

Sample collection: Serum samples were collected from 23 HBV chronic Saudi patients (9 female and 14 male, mean ages 32.7 years) and were randomly selected as they became available from different hospitals in Jeddah; all samples were HBsAg positive with no Co infection with either HIV or HCV. The samples were divided into aliquots and stored at -70°C until used.

Informed consent was obtained from all participants who were included as the subjects of the present study. This study conforms to the Saudi Arabian Health Ethics Regulation.

HBV-DNA isolation and illustration of HBsAg genotype and subgenotypes: HBV DNA was extracted from 23 patient’s sera by using Mini Elute viral extraction Kit (QIAGEN, Inc, Valencia, CA) according to the manufacturer’s instructions. Extracted HBV-DNA samples were stored at -70°C until used (McElhinney et al., 2011). The presence of HBV DNA was determined by nested PCR using Hot start Taq plus PCR Master Mix Kit (QIAGEN, Inc, Valencia, CA). The 1st round PCR amplification reaction was performed according to the manufacturer’s instructions using 50 pmol of each SBFO10 (GGGTCAACATATCTTGG) and SBRO20
(CCCACCTTAGAGTCCAAGG) primers. The thermal cycling conditions were performed with an initial 5 min of preheating at 95°C, followed by 35 cycles of denaturing for 30 sec at 95°C, annealing for 30 sec at 52°C and an elongation step for 1 min at 72°C, with a final extension period of 10 min at 72°C. Nested PCR was performed using the SBFJ30 (GAACAAGAGCTACCCGATGGG) and SBRJ40 (CAAGAGCAAAAAGAAAAATTGG) primers and PCR products were obtained from the first round amplification as templates. The 2nd round of amplification was performed with an initial 5 min preheating at 95°C, followed by 35 cycles 95°C of denaturing for 30 sec at 95°C, annealing for 30 sec at 55°C and an elongation for 1 min at 72°C, with a final extension period of 10 min at 72°C. All PCR contamination precautions were observed and negative controls using sera of HBsAg gene, complete sequences obtained from HBV isolated from chronic Saudi patients was submitted to the DDBJ/EMBL/GeneBank database and identified by their accession number and the country of origin were used to investigate the subgenotype of the 11 present isolates. All 82 isolates were grouped into clusters represented the nine subgenotypes of HBV/D (D1, D2, D3, D4, D5, D6, D7, D8 and D10). Eight HBV/D isolates (S18014, S140014, S20014, S140914, S02E12, S2312E, S07E and S10612E) (72%) were exclusively observed to be closest (72%) reported distance, average±0.032 and three isolates (S028, S118S and S318S) demonstrated nucleotide distance average±0.072 (Fig. 2, Table 1).

Whole HBsAg sequence references of 82 HBV/D subgenotypes strains retrieved from DDBJ/EMBL/GeneBank database and identified by their accession number and the country of origin were used to investigate the subgenotype of the 11 present isolates. All 82 isolates were grouped into clusters represented the nine subgenotypes of HBV/D (D1, D2, D3, D4, D5, D6, D7, D8 and D10). Eight HBV/D isolates (S18014, S140014, S20014, S140914, S02E12, S2312E, S07E and S10612E) (72%) were exclusively observed to be closest to reported HBV/D1 strains with identities of 97.5-99%, arguing that these eight HBV/D isolates belong to subgenotype D1. As for three isolates (S028, S118S and S318S), they were shown a specific cluster belong to subtype Da, despite they segregated into a specific cluster related to D5 with identities of 94-96% (Fig. 2). Whether they interpret a novel subgenotype of HBV/D11 or belonging to HBV/D5 awaits further analysis was needed, including sequence determination of the entire virus genome.

**Detection of the HBsAg gene using nested PCR:** Nested PCR confirmed the presence of HBV-DNA in 15 samples (65.21%) out of 23 positive HBsAg samples while 8 samples (34.78%) were verified the absences of HBV DNA even after the 2nd cycle of PCR. All positive PCR products were at expected size approximately 1.2 kb which include nearly the entire preS1/preS2 and S regions (Fig. 1).

**Results**

**Amino acid sequence analysis:** Protein-coding regions of HBsAg (pre S1, Pre S2 and large S) gene were translated into amino acid sequences using the standard and universal genetic codes, respectively and was compared to surface antigen of other HBsAg strains which were retrieved from DDBJ/EMBL/GeneBank database (Tallo et al., 2008).

**Nucleotide sequence accession number:** HBsAg gene, complete sequences obtained from HBV isolated from chronic Saudi patients was submitted to the DDBJ/EMBL/GeneBank database and identified by their accession number KP191641-KP191650 for the 1.2 kb partial sequences.

**phylogenetic analysis:** Purified PCR products were sequenced in both directions using Big Dye Terminator cycle sequencing kit. The ABI Prism genetic analyzer 310 was used for electrophoresis and data collection. All isolates sequences were assembled using SeqMan II software (DNAStar Inc., Madison, Wisconsin) and multiple alignments with reference sequences of HBsAg genotypes/subgenotypes (A-I) were confirmed using CLUSTAL W and MEGA 5.2.2 software. Phylogenetic trees were constructed using the Tamura-Nei model of evolutionary distance and the topology was evaluated by bootstrap analysis (1,000 replicates) using the Neighbor Joining Method (Saitou and Nei, 1987; Tamura and Nei, 1993).

**Phylogenetic analysis:** Phylogenetic analysis of 1.2 kb genomic regions, corresponding to the complete HBsAg gene was performed in order to analyze the nucleotide heterogeneity of the present isolates and references HBV genotypes (A-I). The tree yielded nine distinct clusters comparable to HBV genotypes (A-I) where 4 (27%) isolates (S4S214, S5S014, S34014, S04S) out of the 15 isolates clustered with HBV/C with nucleotide distance average±0.036 while 11 (73%) isolates showed a rapport to HBV/D. The present isolates that belonged to HBV/D illustrated a variation in the nucleotide distance identity where eight isolates (S18014, S140014, S20014, S140914, S02E12, S2312E, S07E and S10612E) (72%) were exclusively observed to be closest to reported HBV/D1 strains with identities of 97.5-99%, arguing that these eight HBV/D isolates belong to subgenotype D1. As for three isolates (S028, S118S and S318S), they were shown a specific cluster belong to subtype Da, despite they segregated into a specific cluster related to D5 with identities of 94-96% (Fig. 2). Whether they interpret a novel subgenotype of HBV/D11 or belonging to HBV/D5 awaits further analysis was needed, including sequence determination of the entire virus genome.

**Comparison in between amino acid residues of novel HBV/D11 and other HBV/D subgenotypes:** Comparison of the deduced amino acid sequences among the complete ORF of HBsAg gene of HBV/D11 confirmed specific amino acid
Table 1: Pair wise distances between entire nucleotide sequence of HBsAg gene of genotypes A-I (AY233279_HBV/a, D00331_HBV/B, X75664_HBV/E, X75663_HBV/F, AB656515_HBV/G, AY090457_HBV/H, AB231908_HBV/I, X01587_HBV/C and X80924_HBV/D) and the 15 present isolates generated by MEGA5.05 software. Values represent the mean distances within each genotype and the present isolates.

<table>
<thead>
<tr>
<th>HBV genotypes and present isolates</th>
<th>HBV (A)</th>
<th>HBV (B)</th>
<th>HBV (E)</th>
<th>HBV (F)</th>
<th>HBV (G)</th>
<th>HBV (H)</th>
<th>HBV (I)</th>
<th>HBV (C)</th>
<th>HBV (D)</th>
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Bold figures represents the internucleotide distance between the eight new isolates and genotype HBV/D (the lowest distance), "Values represents the inter nucleotide distance between the four new isolates and genotype HBV/C (the lowest distance), " Astericks represents the inter nucleotide distance equations (0)
Fig. 2: Phylogenetic tree constructed by the neighbor-joining method (NJ), based on the 1.2 kb full length of HBsAg reference sequences retrieved from GeneBank database and represented all HBV genotypes (A-I) and HBV/D subgenotypes. HBV/D subgenotypes (D1-D10) represented by different colored branches and indicated with the accession No., followed by the country of isolation. In addition to 15 Saudi HBV/D isolates whose HBsAg sequences were determined in the present study (indicated with a violet closed circle). Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 resampling.
Table 2: A comparison of the amino acid residues encoded by PreS1/preS2/S open reading frame of HBV/D subgenotypes and the novel subgenotype isolates (D11)

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<th>D1 n = 23</th>
<th>D2 n = 43</th>
<th>D3 n = 7</th>
<th>D4 n = 6</th>
<th>D5 n = 2</th>
<th>D6 n = 4</th>
<th>D7 n = 3</th>
<th>D8 n = 3</th>
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|                                   | Pro       | Pro       | Pro       | 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Importance of illustrating the difference in HBsAg nucleotide sequence of the present isolates urges further study of the differences in the sequence of amino acids. Sense mutation of nucleotides may lead to changes in the amino acid sequence either by substitution, insertion or deletion (Weinberger et al., 2000), otherwise establishment of nonsense mutations does not cause any change in the amino acid sequence (Rodriguez-Frias et al., 1999; Thuy et al., 2005). Sense mutations can lead to the creation of escape mutants which can alter group-specific antigenicity (Kohno et al., 1996; Miyake et al., 1996; Kfoury Baz et al., 2011). It can disrupt the antigenicity of HBsAg by modifying amino acids directly involved in expression of the antigen (Rodriguez-Frias et al., 1999). Existence of HBV quasi-species (Schatzl et al., 1997) has facilitated the development of mutants with specific ability to escape antibody detection and neutralization. These mutants may lead to reinfection, because it replicates through an RNA intermediate synthesized by reverse transcriptase of viral genomes (Kreutz, 2002; Ohishi et al., 2004) and quasi-species are generated (Torresi, 2002; Liu et al., 2002; Hsu et al., 2004). This resulted in the production of viral mutants during naturally occurring infections (Chong-Jin et al., 1999). Alterations of the structure of HBsAg can disrupt the binding ability of polyclonal antibodies to it, because they contain several epitopes for T or B cells. The S mutants emerge during chronic HBV infections, often in patients treated with interferon and may represent the way by which the virus overcomes host immune responses (Roznovsky et al., 2000; Seddighi-Tonekaboni et al., 2001; Wakil et al., 2002). As shown in Table 2, the present amino acids HBV/D11 isolates to sure that subgenotype should harbor nucleotide and amino acid motifs which are specific to novel subgenotype. S028, S318S and S118S (D11) isolates harbored 7, 7, 10 amino acid residues, respectively in the preS1/preS2/ S gene that is unique to the respective subgenotype (Table 2). In addition to the presence of amino acid residue deletion Thr7 in the preS1 region that characterized the three novels isolates. Our result may explain one of the main reasons of why In Saudi Arabia, the incidence of viral Hepatitis is decreasing in both HAV, HCV, except for HBV that showed minimal increase. Of hepatitis A, B and C. HBV were the most predominant type, accounting for (53%) of the cases, followed by Hepatitis C virus (HCV) (30%) and HAV (17%) (Alshabanat et al., 2013). This study was highlighted the sequence of HBsAg genes isolated from HBV chronic Saudi patients. The sequence results obtained from isolates verified that the predominant HBV genotypes is HBV/D followed by HBV/C. This result in agreement with those demonstrated that HBV/D were predominant in the Middle East (Saudy et al., 2003; Norder et al., 2004; Zekri et al., 2007; El Hadad et al., 2013). In addition, present study revealed the presence of multiple subgenotypes of HBV within genotypes D with the predominant distribution of seemingly indigenous subgenotype D1 as well as a new novel subgenotype, tentatively designated D11 in Jeddah which meet the proposed rules for classification (Mulyanto et al., 2011; Norder et al., 2004). The identification of HBV isolates of novel sub genotypes in the present study suggested that further studies with a large number of subjects in previously examined and unexamined areas would lead to discovery of HBV strains genotypes even novel subgenotypes circulating in Saudi Arabia.

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REFERENCES


