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ORIGINAL ARTICLE

Sequence analysis of sub-genotype D hepatitis B surface antigens isolated from Jeddah, Saudi Arabia



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Population studies

Abstract Little is known about the prevalence of HBV genotypes/sub-genotypes in Jeddah province, although the hepatitis B virus (HBV) was identified as the most predominant type of hepatitis in Saudi Arabia. To characterize HBV genotypes/sub-genotypes, serum samples from 15 patients with chronic HBV were collected and subjected to *HBsAg* gene amplification and sequence analysis. Phylogenetic analysis of the *HBsAg* gene sequences revealed that 11 (48%) isolates belonged to HBV/D while 4 (18%) were associated with HBV/C. Notably, a HBV/D sub-genotype phylogenetic tree identified that eight current isolates (72%) belonged to HBV/D1, whereas three isolates (28%) appeared to be more closely related to HBV/D5, although they formed a novel cluster supported by a branch with 99% bootstrap value. Isolates belonging to D1 were grouped in one branch and seemed to be more closely related to various strains isolated from different countries. For further determination of whether the three current isolates belonged to HBV/D5 or represented a novel sub-genotype, HBV/DA, whole HBV genome sequences would be required. In the present study, we verified that HBV/D1 is the most prevalent HBV sub-genotype in Jeddah, and identified novel variant mutations suggesting that an additional sub-genotype designated HBV/DA should be proposed. Overall, the results of the present *HBsAg* sequence analyses provide us with insights regarding the nucleotide differences between the present *HBsAg*/D isolates identified in the populace of

Abbreviations: HBV, hepatitis B virus; HAV, hepatitis A virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; P, HBV polymerase gene; C/pre C, HBV core/pre Core gene; X, HBV X gene; *HBsAg*, HBV surface antigen; IFN, interferon; *Pre S1/Pre S2/S*, *HBsAg* genes; PCR, polymerase chain reaction; DDBJ, DNA Data Bank of Japan; EMBL, European Molecular Biology Laboratory.

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Jeddah, Saudi Arabia and those previously isolated worldwide. Additional studies with large numbers of subjects in other areas might lead to the discovery of the specific HBV strain genotypes or even additional new sub-genotypes that are circulating in Saudi Arabia.

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1. Introduction

Many nations have a heavy financial load from the high percentage of both individuals with chronic hepatitis B virus (HBV) infection and the approximately 400 million HBV carriers worldwide. HBV infection leads to the development of severe liver diseases including cirrhosis and hepatocellular carcinoma (HCC) (Norder et al., 1992, 1994). Human HBV is the prototype member of the family *Hepadnaviridae* that contains a circular, partially double-stranded DNA genome of about 3200 bp. HBV DNA contains four overlapping open reading frames encoding the pre S1/pre S2/S, pre C/C, pol, and X viral proteins (Fig. 1) (Magnius and Norder, 1995).

Compared to most DNA viruses, HBV has a high rate of nucleotide substitution although this is lower than the mutation rate of RNA viruses (Okamoto et al., 1987). Previously, HBV genomes were classified into four genotypes, but recently have been categorized into eight genotypes designated as A-H. This categorization was based on inter-genotype divergence of at least 8% of the complete nucleotide sequence or on more than 4% divergence in the *HBsAg* gene (Arauz-Ruiz et al., 1997; Okamoto et al., 1987). These genotypes possess different geographical distributions as illustrated in Fig. 2 (Arauz-Ruiz et al., 1997; Chu et al., 2003; Miyakawa and Mizokami, 2003). Recently, a complex recombinant of genotypes A, C, and G has been referred to as representing a new genotype (I), which was described and sequenced in northwestern China, Vietnam, and Brazil (Santos et al., 2010).

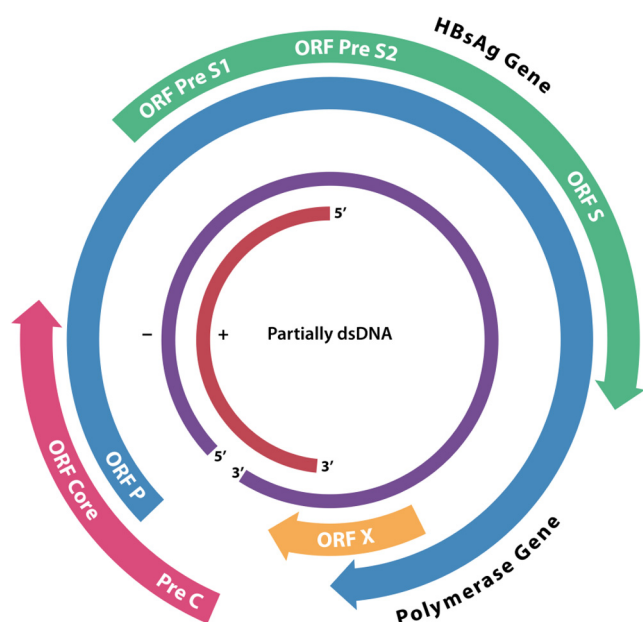


Figure 1 Genome organization of HBV. The colored arrows indicate overlapping genes: (ORF P) polymerase gene; (ORF C/pre C) core/pre core gene; (ORF X) X gene and (ORF Pre S1/ORF Pre S2/ORF S) *HBsAg* gene (Jalali and Alavian, 2006).

In addition, sub-genotypes have been described in certain HBV genotypes; these are A1-A6 in genotype A (HBV/A), and B1-B8, C1-C16, and D1-D8 in HBV/B, C, and D, respectively (Abdou Chekaraou et al., 2010; Mulyanto et al., 2009).

HBV genotypes show a direct correlation with the severity of liver disease. HBV/C, HBV/D, and HBV/B are associated with severe liver cirrhosis (Banerjee et al., 2006; Huy et al., 2006; Kramvis et al., 2008; Sakamoto et al., 2006) and appear to have a higher incidence of HCC (Chan and Sung, 2006; Liu et al., 2007; Liu et al., 2011; Sumi et al., 2003; Yuen et al., 2003, 2009). Additionally, patients with either HBV/C or HBV/D have a lower response rate to treatment with interferon (IFN) γ when compared with those carrying other HBV genotypes (Zöllner et al., 2001). The HBV genotype might also influence the emergence of lamivudine resistance mutations, which appear to be more strongly associated with HBV/A than HBV/D (Wen, 2004).

HBV surface antigen (HBsAg) can induce protection against HBV infection as it is related directly to B-cell epitopes and is considered as the major target of neutralizing antibodies; therefore, it is used as an HBV vaccine (Kramvis et al., 2005). The complete *HBsAg* gene consists of three regions: *large S*, *Pre S2*, and *Pre S1*, which share their C-terminal 226 amino acid residues (Carman, 1997; Szmuness et al., 1981). Mutant *HBsAg* nucleotides might cause amino acid substitutions (EL Hadad et al., 2013), which could affect the binding of specific anti-HB antibodies and the detection by conventional diagnostic assays (Torresi, 2002). In addition, a relationship has been observed between low antigenicity of HBV (leading to HBV reinfection) and the increased incidence of HCC in Egyptian patients with chronic HBV (Tian et al., 2007). Furthermore, correlations between mutations in the *HBsAg* gene, particularly in the *Pre S* regions, and the development of HCC have been verified in patients with chronic HBV (Yang et al., 2010).

The prevalence of HBV infection is generally high in Asian and African countries (Lee, 1997). Saudi Arabia reported HBV as the most predominant type (53%) of hepatitis infection followed by HCV (30%) and HAV (17%) (Alshabanat et al., 2013). Furthermore, the Jeddah province of Saudi Arabia hosts large numbers of HBV infected individuals originating from countries with a high-HBV burden because Jeddah is a main entry point as well as being the largest commercial port in the country. For these reasons and because little is known regarding the *HBsAg* genotypes/sub-genotype sequences circulating in Saudi Arabia, and in particular in the Jeddah region (Al-Faleh et al., 1992; 1999), the present study was conducted to provide sequence data of the most common *HBsAg* genotypes/subgenotypes circulating in Jeddah for subsequent phylogenetic analysis.

2. Materials and methods

2.1. Patient samples

Serum samples were collected from 17 Saudi patients with chronic HBV (5 women and 12 men, mean age 32.7 years)

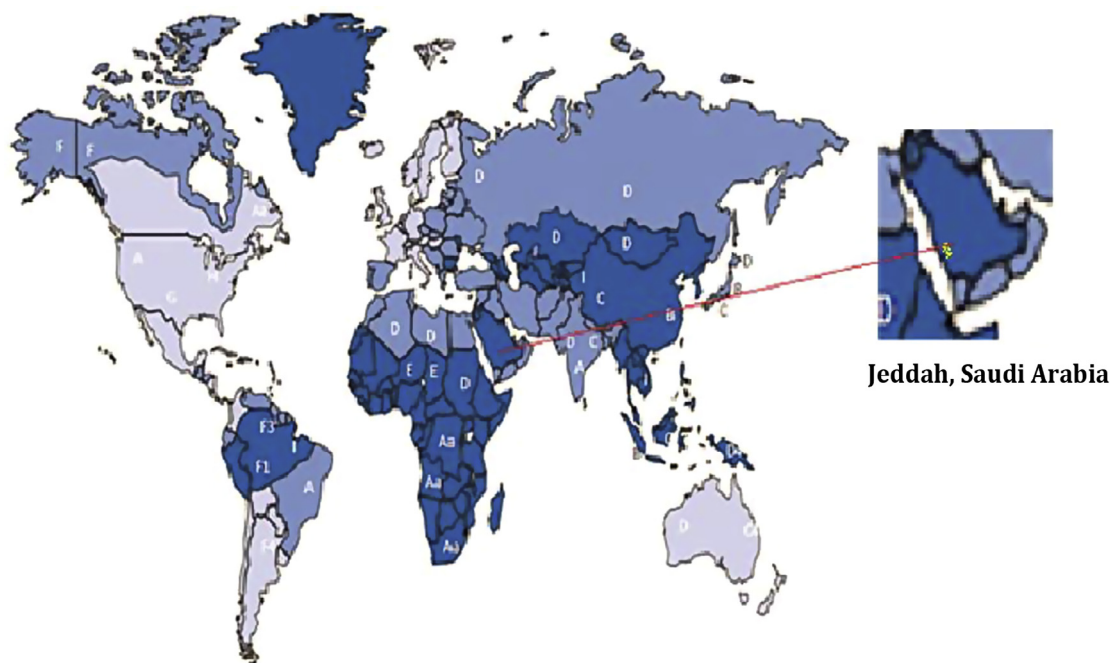


Figure 2 Worldwide distribution of *HBsAg* genotypes (A-I) and some subgenotypes. Dark blue represents regions where the genotypes are highly endemic (>8%), whereas blue and light blue represent regions with intermediate and low endemicity (2.7% and <2%), respectively. Saudi Arabia was identified as an *HBsAg* high endemic region (Hou et al., 2005).

and were randomly selected as they became available from different hospitals in Jeddah; review of the medical records confirmed that all samples were positive for HBsAg with no co-infection with either HIV or HCV. The samples were divided into aliquots and stored at -70°C until used.

The study protocol was reviewed and approved by the Deanship of Scientific Research Ethics Committee of King Abdulaziz University and the King Abdulaziz Hospital Ethics Committee. Written consent was obtained from all patients after full explanation of the purpose of the study.

2.2. HBV-DNA extraction, amplification, and *HBsAg* genotype/sub-genotype determination

HBV DNA was obtained from 17 patient sera samples using the Mini Elute viral Extraction Kit (QIAGEN, Inc., Valencia, CA, USA) according to the manufacturer's instructions. The resultant HBV-DNA samples were stored at -70°C until use (McElhinney et al., 2011). The presence of HBV DNA was determined by nested PCR using a Hot start Taq plus PCR

Master Mix Kit (QIAGEN) using the primers reported in Table 1. The first round PCR amplification reaction was performed according to the manufacturer's instructions using $10\ \mu\text{l}$ HBV-DNA and 50 pmol each of primers SBFO10 and SBRO20. The cycling conditions were performed with an initial 5 min of preheating at 95°C , followed by 35 cycles of denaturing for 30 s at 95°C , annealing for 30 s 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 using the SBF130 and SBRI40 primers was performed on $10\ \mu\text{l}$ of the samples negative for PCR products in the first round amplification. The second round of amplification was performed with an initial 5 min preheating step at 95°C , followed by 35 cycles of denaturing for 30 s at 95°C , annealing for 30 s at 55°C , and 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 from healthy individuals who were not positive for any HBV markers were included (EL Hadad et al., 2013; Mulyanto et al., 2011).

Table 1 Primers for full length *HBsAg* amplification and their nucleotide position.

Primer code	Primer sequence	Primer length	Nucleotide position
SBFO10	5' GGGTCACCATATTCCTGG 3'	19	2859–2878
SBRO20	5' CCCACCTTAGAGTCCAAGG 3'	19	873–892
SBFI30	5' GAACAAGAGCTACCGCATGGG 3'	21	2877–2898
SBRI40	5' CAAGAGACAAAAGAAAATTGG 3'	21	810–789

Nucleotide positions are based on the HBV reference sequence (contained in 3221 bp) that was retrieved from DDBJ/EMBL/GenBank.

2.3. Interpretation of *HBsAg* nucleotide sequences and phylogenetic analysis

Purified PCR products were sequenced in both directions using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems (ABI), Foster City, CA, USA). The ABI Prism 310 genetic analyzer was used for electrophoresis and data collection according to the manufacturer's protocol. All isolate sequences were assembled using SeqMan II software (DNASTar Inc., Madison, WI, USA) and multiple alignments with the reference sequences of *HBsAg* genotypes and sub-genotypes (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 (1000 replicates) using the neighbor joining method (Saitou and Nei, 1987; Tamura and Nei, 1993).

2.4. Amino acid composition analysis

The protein coding regions of the *HBsAg* (*pre S1*, *Pre S2*, and *large S*) gene were translated into amino acid sequences using the standard and universal genetic codes, respectively, and were compared to the peptide sequences of the surface antigens of other *HBsAg* strains that had been retrieved from the DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and GenBank databases (Tallo et al., 2008).

2.5. Nucleotide sequence submission

The base sequence data reported in this study has been submitted to DDBJ/EMBL/GenBank and assigned accession numbers KP191641–KP191650 for the 1.2 Kb partial sequences.

3. Results

3.1. Amplification of the *HBsAg* gene using nested PCR

Nested PCR confirmed the presence of HBV-DNA in 15 (88.3%) out of 17 *HBsAg* positive samples whereas in the remaining 2 (11.7%), the absence of HBV DNA was verified even after the second round of PCR amplification. All positive

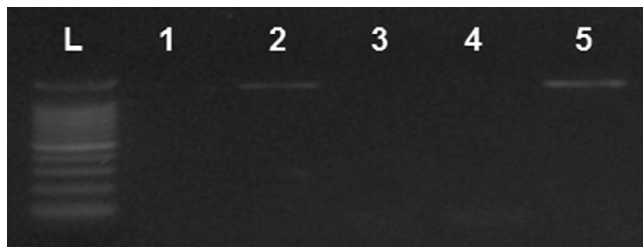


Figure 3 PCR amplification of the *HBsAg* gene using nested PCR. “L” represents the 100 bp DNA ladder ranging from 100 to 1200 bp. Lanes 1, 2, and 5 contain DNA fragments of the *HBsAg* gene, whereas lanes 3 and 4 show negative results.

PCR products were at the expected size of approximately 1.2 Kb that included almost the entire *Pre S1/Pre S2* and *S* genomic region (Fig. 3).

3.2. Identification of *HBV* genotypes and sub-genotypes based on the complete nucleotide sequence of the *HBsAg* gene

Phylogenetic analysis of the 1.2 Kb genomic regions from each sample, corresponding to the complete *HBsAg* gene and containing approximately the entire *Pre S1/Pre S2/S* genomic locus was performed in order to analyze the nucleotide heterogeneity of the present isolates with respect to the reference HBV genotypes (A-I). The completed tree demonstrated nine distinct clusters comparable to the known HBV genotypes (A-I); 11 (73%; S028, S118S, S18014, S318S, S140014, S20014, S140914, S02E12, S2312E, S07E, and S10612E) out of the 15 new isolates seemed to be more closely related to HBV/D by nucleotide distance identity, means ± 0.034 , whereas 4 (27%) isolates (S4S214, S5S014, S34014, and S04S) were identified as HBV/C with a nucleotide distance means ± 0.036 that verified this relationship (Fig. 4).

Whole *HBsAg* reference sequences of 117 HBV/D sub-genotype strains including the present 11 isolates were used to generate another phylogenetic tree. The reference sequences were retrieved from the DDBJ/EMBL/GenBank databases along with their accession number and country of origin for identification. All 117 isolates were grouped into clusters that represented the nine sub genotypes of HBV/D (D1–D8 and D10). An exclusive subset of eight HBV/D isolates (S18014, S140014, S20014, S140914, S02E12, S2312E, S07E, and S10612E) (72%) were observed to be closest to reported HBV/D1 strains with identities of 97.5–99%, indicating that these eight HBV/D isolates belonged to sub-genotype D1. The three remaining isolates (S028, S118S, and S318S) formed a particular cluster that belonged to subtype DA, despite segregating into a larger cluster related D5 with identities of 94–96% (Fig. 5).

3.3. Determination of the amino acids specific for *HBV/D1*

In the present study, all eleven HBV/D isolates exhibited a common 11 amino acid deletion in the *pre S1* region consistent with the categorization into genotype D. The amino acid sequence of S18014, S140014, S20014, S140914, S02E12, S2312E, S07E, and S10612E isolates were compared against those of HBV/D1 reference sequences retrieved from the DDBJ/EMBL/GenBank databases representing strains from different countries such as Egypt, Turkey, Germany, Uzbekistan, India, USA, Iran, and China. As indicated in Table 2, the lack of any amino acid insertion or deletion was confirmed in all eight HBV/D1 isolates, despite the identification of substitutions established across the entire *Pre S1/Pre S2/S* region, and particularly within the *Pre S1* region. Therein, one novel amino acid substitution was demonstrated in S01612E, S07S, and S140014 isolates. Two amino acid substitutions were found to be specific to S02E12 and S07S isolates. Four and five amino acid substitutions were characterized in S2312E and S140914 isolates, respectively, while S18014 and S20014 showed six amino acid substitutions. In the *Pre S2* region, one amino acid residue substitution was verified in both

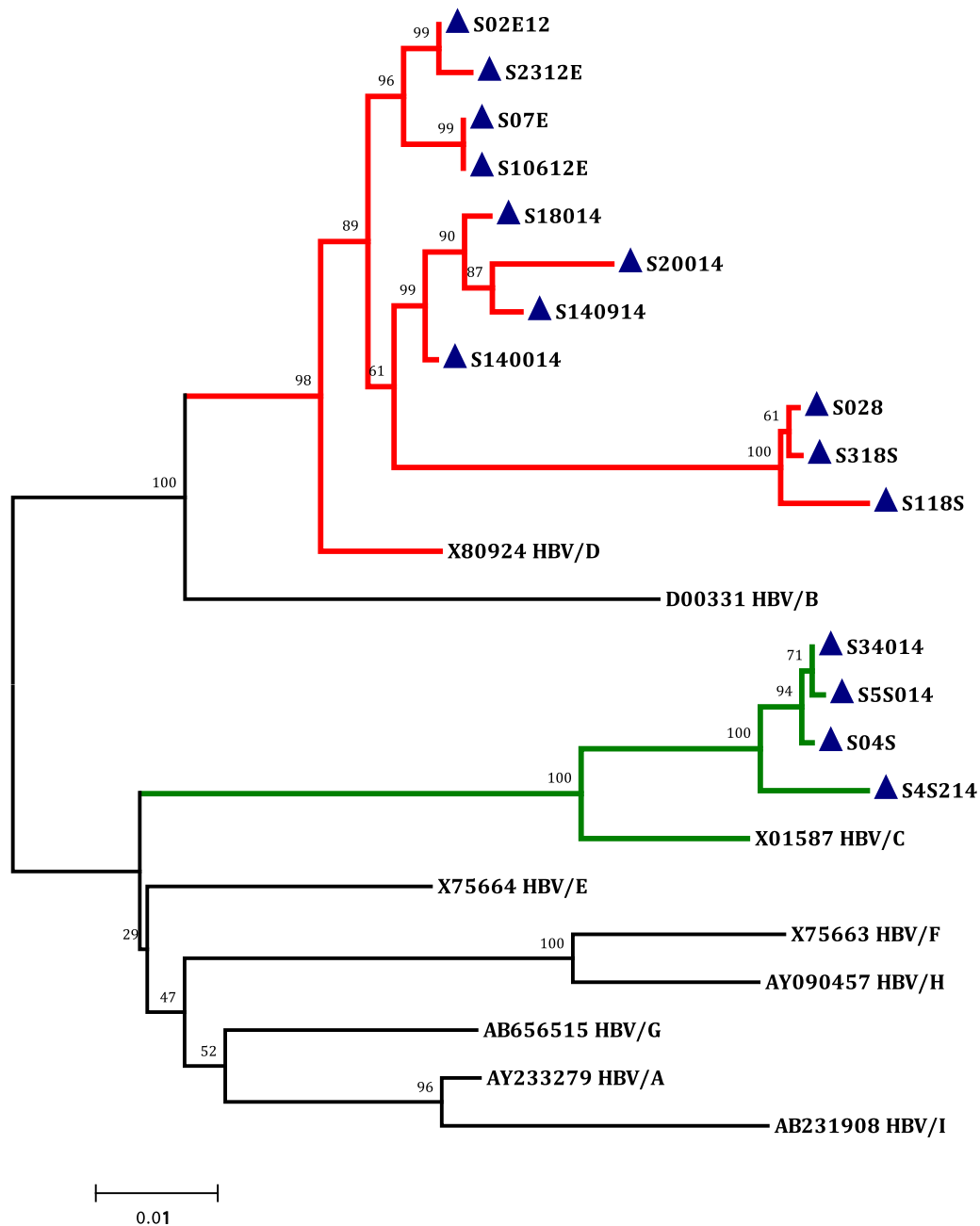


Figure 4 Phylogenetic tree constructed using the neighbor-joining method, based on nine 1.3-kb, full length *HBsAg* reference sequences retrieved from the DDBJ/EMBL/GenBank Database representing all HBV genotypes (A-I). All the reference isolates are indicated with their accession nos. In addition, the 15 Saudi isolates whose nucleotide sequences were determined in the present study (indicated with a blue closed triangle) are shown; 11 isolates clustered with HBV/D and are represented by the red branches, while 4 isolates were grouped with HBV/C and are represented by the green branches. Bootstrap values indicate the major nodes as a percentage of the data obtained from 1000 replications.

S07S and S140914, and the S20014 isolate showed two amino acid residue changes in the S region.

3.4. Amino acid changes of HBV/DA isolates in comparison with other isolates of HBV/D5

Comparison of the amino acid sequences among the complete *HBsAg* genes of two reference isolates (DQ315779 India and AB033558 Indonesia) belonging to D5 with the present HBV/

DA isolates verified 10 DA-specific amino acid substitutions in all HBV/DA strains. In the Pre S1 region, these were Arg28Ala, Pro81Ala, and Gln89Lys; in the Pre S2 region, Pro162Leu; and in the S region, Phe171Leu, Met288Thr, Thr290Pro, Gly348Glu, Leu349Val, and Met361Arg were found. In addition, two unique amino acid residues; Pro149-His in the Pre/S2 gene and Pro377Leu in the S gene, were observed in the S318S and S118S strains, respectively (Table 3).

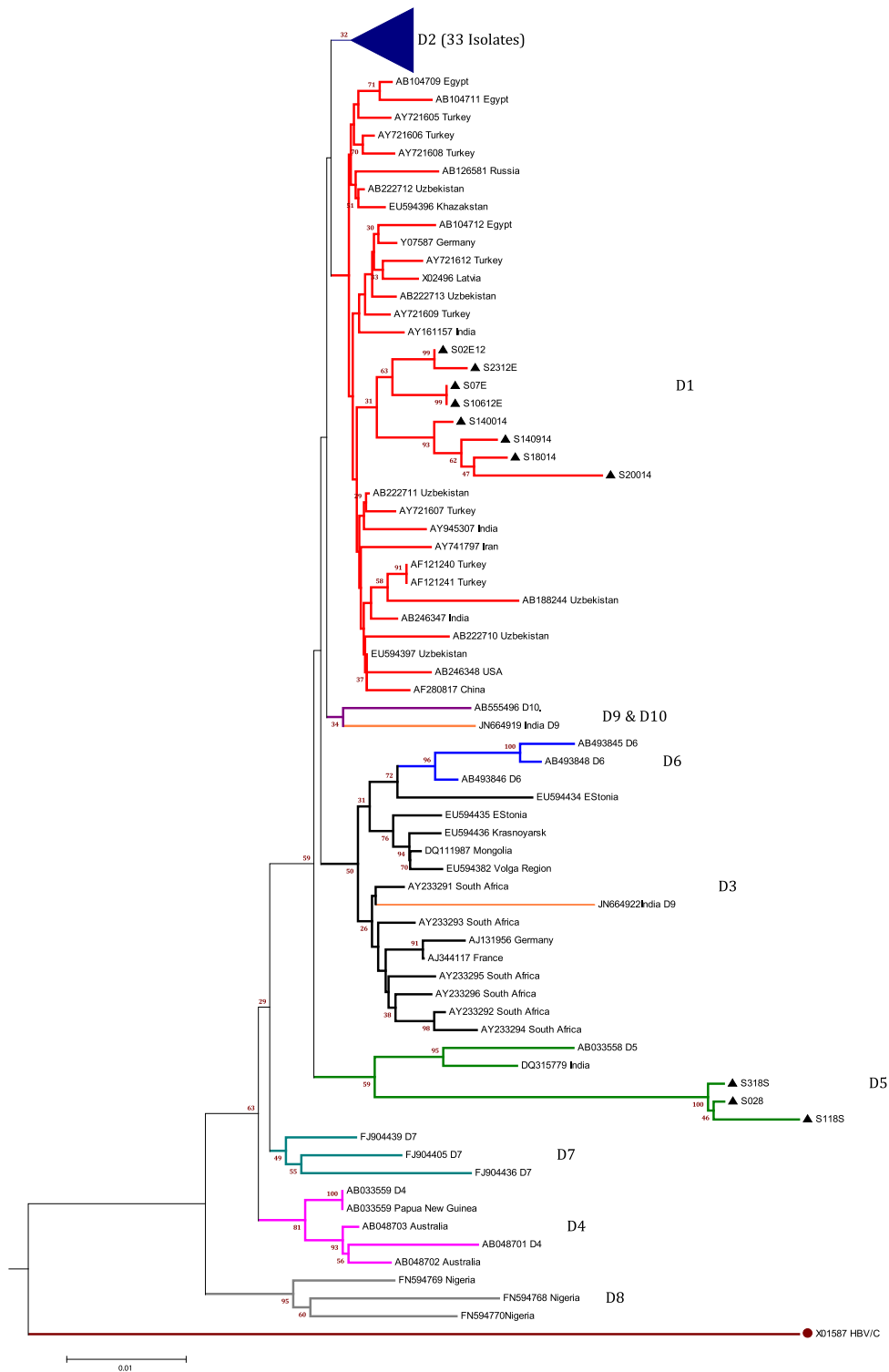


Figure 5 Phylogenetic tree constructed by the neighbor-joining method, based on the entire nucleotide sequences of the 106 reported *HBsAg* genes of sub genotypes D1-D10. The gene sequences are represented by different colored branches as indicated by the accession no., followed by the country of isolation. The 11 Saudi HBV/D isolates whose entire *pre/S1*, *pre/S2*, and *S* gene sequences were determined in the present study are indicated with black closed triangles. The HBV/C isolate (X01587) was used as an out group and was indicated with a brown closed circle. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1000 replicates.

Table 2 Conserved amino acid residues in HBV/D1 isolates.

Gene/Amino Acid. Position	Reference Isolate							Saudi Isolate							
	Egypt (n = 3)	Turkey (n = 8)	India (n = 2)	USA (n = 1)	China (n = 1)	Latvia (n = 1)	Germany (n = 1)	S02E12	S2312E	S07E	S10612E	S140014	S18014	S140914	S20014
<i>PreS1/3</i>	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Arg	Gln
<i>PreS1/4</i>	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Gln	Glu	Asn
<i>PreS1/5</i>	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Ser	Ser	Leu
<i>PreS1/6</i>	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Phe	Phe	Ser
<i>PreS1/9</i>	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asp	Asn	Asn	Asn	Asn	Asn	Asn
<i>PreS1/16</i>	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	His	Asp	Asp
<i>PreS1/22</i>	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Pro	Pro	Pro	Pro	Ala	Ala	Ala	Ala
<i>PreS1/40</i>	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Asn	Asn	Thr	His
<i>PreS1/45</i>	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	His	Asn	Asn	Asn	Asn	Asn	Asn
<i>PreS1/49</i>	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Val
<i>PreS1/51</i>	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Thr
<i>PreS1/54</i>	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Pro
<i>PreS1/62</i>	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Arg
<i>PreS1/63</i>	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Ile	Ile	Gly
<i>PreS1/77</i>	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Val
<i>PreS1/88</i>	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Leu	Leu	Arg	Arg	Arg	Arg	Arg	Arg
<i>PreS2/130</i>	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Ile	Phe
<i>PreS2/163</i>	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Thr	Asn	Asn	Asn	Asn	Asn
<i>S/244</i>	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Phe	Leu
<i>S/252</i>	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Phe

Conserved amino acid residues in the HBsAg region (Pre S1, Pre S2, S) were derived from the different HBV/D reference sequences retrieved from DDBJ/EMBL/GenBank and from the eight isolates described in the present study (S18014, S140014, S20014, S140914, S02E12, S2312E, S07E, and S10612E). The amino acid sequence was derived from the nucleotide sequence.

n = No. of sub-genotypes isolates.

Subgenotype-specific substitutions are shown in red.

Table 3 Conserved amino acid residues in HBV/D5 isolates.

Gene/amino acid position	Reference isolate		Saudi isolate		
	DQ315779 India	AB033558 Indonesia	S028S	S118S	S318S
<i>PreS1/28</i>	Arg	Arg	<i>Ala</i>	<i>Ala</i>	<i>Ala</i>
<i>PreS1/81</i>	Pro	Pro	<i>Ala</i>	<i>Ala</i>	<i>Ala</i>
<i>PreS1/89</i>	Gln	Gln	<i>Lys</i>	<i>Lys</i>	<i>Lys</i>
<i>PreS2/149</i>	Pro	Pro	<i>Pro</i>	<i>Pro</i>	<i>His</i>
<i>PreS2/162</i>	Pro	Pro	<i>Leu</i>	<i>Leu</i>	<i>Leu</i>
<i>S/171</i>	Phe	Phe	<i>Leu</i>	<i>Leu</i>	<i>Leu</i>
<i>S/288</i>	Met	Met	<i>Thr</i>	<i>Thr</i>	<i>Thr</i>
<i>S/290</i>	Thr	Thr	<i>Pro</i>	<i>Pro</i>	<i>Pro</i>
<i>S/348</i>	Gly	Gly	<i>Glu</i>	<i>Glu</i>	<i>Glu</i>
<i>S/349</i>	Leu	Leu	<i>Val</i>	<i>Val</i>	<i>Val</i>
<i>S/361</i>	Met	Met	<i>Arg</i>	<i>Arg</i>	<i>Arg</i>
<i>S/377</i>	Pro	Pro	<i>Pro</i>	<i>Leu</i>	<i>Pro</i>

Conserved amino acid residues in *HBsAg* (Pre S1, Pre S2, S) were derived from 2 reference sequences of HBV/D5 indicated by their DDBJ/EMBL/GenBank Accession numbers and 3 isolates of the present study (S028, S118S, and S318S). The amino acid sequence was derived from the nucleotide sequence.

Sub-genotype-specific substitutions are italicized.

Sub-genotype-unique amino acids are shown in red.

4. Discussion

HBV has drawn attention for the peculiar geographic distribution of its nine distinctive immunologic genotypes (Kao et al., 2003; Liu et al., 2002). Saudi Arabia, and especially Jeddah, receives millions of pilgrims from various countries annually because of the Hajj and Umrah seasons. However, despite the import of the infection, molecular epidemiological data of the specific HBV genotype and sub-genotype strains circulating in Jeddah are limited (Al-Faleh et al., 1992, 1999). Phylogenetic analysis of 15 current isolates demonstrated that HBV/D is the most dominant HBV genotype (73%) among Saudi patients with chronic HBV, followed by HBV/C (27%). This result is consistent with many previous observations that postulated that the HBV genotype D appeared to be predominant in the Mediterranean basin, including the Middle Eastern countries (EL Hadad et al., 2013; Norder et al., 2004; Saady et al., 2003), whereas HBV/C was dominant in Southeast Asian countries (Lusida et al., 2008).

Recently, ten sub-genotypes of HBV/D (D1 to D10) have been reported, each showing distinct geographical clustering (Tran et al., 2014). In the present work, eight isolates belonging to HBV/D (S18014, S140014, S20014, S140914, S02E12, S2312E, S07E, and S10612E) segregated with sub-genotype D1 reference isolates, and the inter distances equaling 0.006 verified this effect. This result is in agreement with recent studies that reported the prevalence of HBV/D1 in regions of the Middle East through North Africa (EL Hadad et al., 2013; Tran et al., 2014). Additionally, a possible new HBV sub-genotype (DA) was suggested by 3 isolates, S028, S318S, and S118S, which showed identity similarities of 94–96%.

Many conflicting reports have arisen in the past few years regarding the identification of novel HBV/D-sub-genotypes. Recently, new HBV/B3 sub-genotypes have been sorted out using partial sequence of the *S* gene (Ghosh et al., 2013). In

addition, HBV/D9 has been classified from isolates showing recombination between genotypes D and C in the pre core/core regions, although the *pre-S/S* open reading frame did not possess any unique motifs that could distinguish HBV/D9 isolates from the other eight sub-genotypes of D. Furthermore, the frequent practice of sequencing only the *pre-S/S* region for defining genotype/sub-genotype can lead to their improper classification within sub-genotype D3 or D2 (Ghosh et al., 2013; Tran et al., 2014). The former phenomenon was confirmed by our present phylogenetic tree, wherein current *Pre S/S* sequence analysis of the JN664922 India isolate placed it as belonging to HBV/D3 instead of as an HBV/D9 subtype, as characterized by previous analysis. In the present study, three current isolates (S028, S318S, and S118S) were clustered with reference strains representing HBV/D5. However, this observation did not affect the proposed establishment of a novel HBV/DA sub-genotype, as the sub-genotype HBV/D6 strains clustered within the HBV/D3 branch. In addition, the phylogenetic analysis provided corroboration of both HBV/D9 and HBV/D10 (Yousif and Kramvis, 2013). Thus, whole genome sequence analysis is required to substantiate whether the present isolates actually belong to HBV/D5 or represent a novel sub-genotype, despite that HBV classification was initially built on the premise of inter-genotype divergence of at least 8% of the complete nucleotide sequence or more than 4% in the *HBsAg* gene, which stringencies these isolates meet (Magnius and Norder, 1995).

The demonstrated importance of illustrating the difference in the *HBsAg* nucleotide sequences of the present isolates urges further study on the differences in the sequences of amino acids. No alterations in amino acid sequence result from “non-sense” nucleotide mutations (Rodriguez-Frias et al., 1999; Thuy le et al., 2005), whereas “sense” mutations might lead to alterations in the amino acid sequence either by substitution, insertion, or deletion (Weinberger et al., 2000). Therefore, sense mutations might lead to the creation of mutants with the specific ability to escape antibody detection and neutralization. These mutations thus might also lead to re-infection because HBV replicates through an RNA intermediate synthesized by the reverse transcriptase of viral genomes (Kfoury Baz et al., 2001; Kohno et al., 1996; Kreutz, 2002; Miyake et al., 1996; Ohishi et al., 2004).

As shown in Table 2, the present HBV/D1 isolates (S18014, S140014, S20014, S140914, S02E12, S2312E, S07E, and S10612E) showed the presence of 1–8 unique amino acid residues out of the 390 that represent the entire Pre S/S region. Amino acid sequence alignments were also performed between the three HBV/DA isolates (S028, S318S, and S118S) and two reported reference sequences related to HBV/D5 (DQ315779 India and AB033558 Indonesia) to ensure that the proposed sub-genotype harbored nucleotide and amino acid motifs that were specific to the novel sub-genotype. The current HBV/DA isolates harbored 3, 1, and 6 amino acid residues, respectively in the *Pre S1/Pre S2/S* locus that were unique to the proposed sub-genotype. In addition, two unique amino acid residues, Pro149His in the *Pre/S2* gene and Pro377Leu in the *S* gene, were observed in the S318S and S118S strains, respectively (Table 3). The relatively large number of sense mutations identified in HBV suggests that this virus might be better at avoiding elimination than HAV and HCV; in addition, HBV might be more stable than the latter, which are RNA viruses. Therefore, our result might explain one of the reasons why in Saudi

Arabia; the incidence of viral hepatitis is decreasing for both HAV and HCV, but not for HBV, which showed a minimal increase. Overall, between hepatitis A, B, and C, HBV was the most predominant type, accounting for 53% of identified cases, followed by HCV (30%) and HAV (17%) (Alshabanat et al., 2013).

5. Conclusion

This study highlighted the sequence analysis of *HBsAg* genes isolated from Saudi patients with chronic HBV. The sequence results obtained from these isolates verified that HBV/D and HBV/C are the most common genotypes in this population. This result confirmed previously published findings demonstrating that HBV/D was the dominant type in the Middle East (EL Hadad et al., 2013; Liu et al., 2002). In addition, the current study revealed the presence of multiple sub-genotypes of HBV within the D genotype, with the dominant distribution of the seemingly indigenous sub-genotype D1. Therefore, the availability of complete *HBsAg* sequences might serve as a reference for future epidemiological studies of HBV viruses. Additionally, this study identified a new sub-genotype, tentatively designated DA in Jeddah, KSA, which meets the proposed rules for HBV/D classification (Norder et al., 2004). For further confirmation whether the three current isolates belong to HBV/D5 or represent a novel sub-genotype, HBV/DA, whole HBV genome sequences would be required. Similarly, additional studies with large numbers of cases in previously examined and unexamined areas will likely lead to the discovery of the HBV strains and even possibly additional new sub-genotype strains circulating in Saudi Arabia.

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