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

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ABSTRACT
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_1/pre S_2/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 (Magnius 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; Chekaraou *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-α 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/sub genotype 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 (GGGTCACCATATTCTTGG) 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 SBFI30 (GAACAAGAGCTACCGCATGGG) and SBRI40 (CAAGAGACAAAAGAAAATTGG) 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 from subjects with no HBV markers were included (Mulyanto *et al*., 2011; El Hadad *et al*., 2013).

Determination of HBsAg nucleotide sequence and the 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).

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 under accession number KP191641-KP191650 for the 1.2 kb partial sequences.

RESULTS

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).

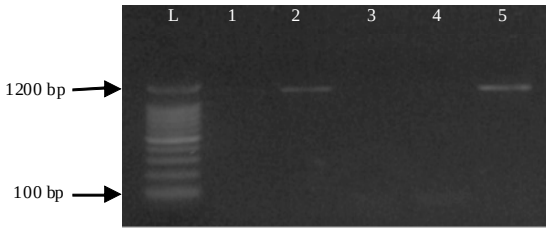

Fig. 1: PCR amplification of HBsAg gene using nested PCR. L: 100 step DNA ladder that ranged from 100-1200 bp. Lane 1, 2 and 5: DNA fragments of the HBsAg gene. Lane 3 and 4: Negative results

Investigation of HBV genotypes/subgenotype based on the full nucleotide sequence of HBsAg gene: 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) 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, 140914, 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

HBV genotypes and present isolates	HBV/(A)	HBV/(B)	HBV/(E)	HBV/(F)	HBV/(G)	HBV/(H)	HBV/(I)	HBV/(C)	HBV/(D)	S04S	S4S214	S023E12	S07E	S028	S118S	S318S	S2312E	S10612E	S18014	S20014	S140014	S140914	S34014
S028	0.133	0.139	0.109	0.178	0.127	0.172	0.163	0.139	**0.067**	0.064	*												
S118S	0.145	0.151	0.121	0.191	0.139	0.184	0.175	0.157	**0.084**	0.075	0.016	*											
S318S	0.133	0.139	0.109	0.178	0.127	0.172	0.163	0.139	**0.067**	0.064	0.000	0.016	*										
S07E	0.104	0.104	0.081	0.160	0.095	0.157	0.133	0.112	**0.029**	0.010	0.064	0.075	0.064	*									
S10612E	0.104	0.104	0.081	0.160	0.095	0.157	0.133	0.112	**0.029**	0.010	0.064	0.075	0.064	0.000	*								
S140014	0.092	0.098	0.070	0.148	0.084	0.145	0.121	0.104	**0.024**	0.016	0.059	0.070	0.059	0.016	0.016	*							
S140914	0.104	0.104	0.078	0.160	0.095	0.157	0.133	0.109	**0.029**	0.021	0.064	0.075	0.064	0.021	0.021	0.016	*						
S18014	0.104	0.109	0.081	0.160	0.095	0.157	0.133	0.115	**0.034**	0.026	0.070	0.081	0.070	0.026	0.026	0.010	0.013	*					
S20014	0.107	0.121	0.084	0.163	0.098	0.160	0.127	0.115	**0.048**	0.040	0.084	0.095	0.084	0.040	0.040	0.032	0.024	0.029	*				
S2312E	0.112	0.112	0.089	0.169	0.104	0.166	0.142	0.121	**0.037**	0.008	0.073	0.084	0.073	0.018	0.018	0.024	0.029	0.034	0.048	*			
S04S	0.107	0.151	0.089	0.154	0.101	0.154	0.107	0.032#	0.121	0.127	0.154	0.172	0.154	0.127	0.127	0.118	0.127	0.127	0.121	0.136	*		
S4S214	0.121	0.163	0.104	0.178	0.115	0.175	0.121	0.056#	0.136	0.139	0.157	0.175	0.157	0.139	0.139	0.133	0.139	0.139	0.133	0.148	0.029	*	
S34014	0.107	0.151	0.089	0.154	0.101	0.154	0.107	0.032#	0.121	0.127	0.154	0.172	0.154	0.127	0.127	0.118	0.127	0.127	0.121	0.136	0.005	0.024	*
S5S014	0.109	0.154	0.092	0.157	0.104	0.157	0.109	0.034#	0.124	0.130	0.157	0.175	0.157	0.130	0.130	0.121	0.130	0.130	0.124	0.139	0.008	0.026	0.003

Bold figures represents the inter nucleotide 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), *Aslericks represents the inter nucleotide distance equations (0)

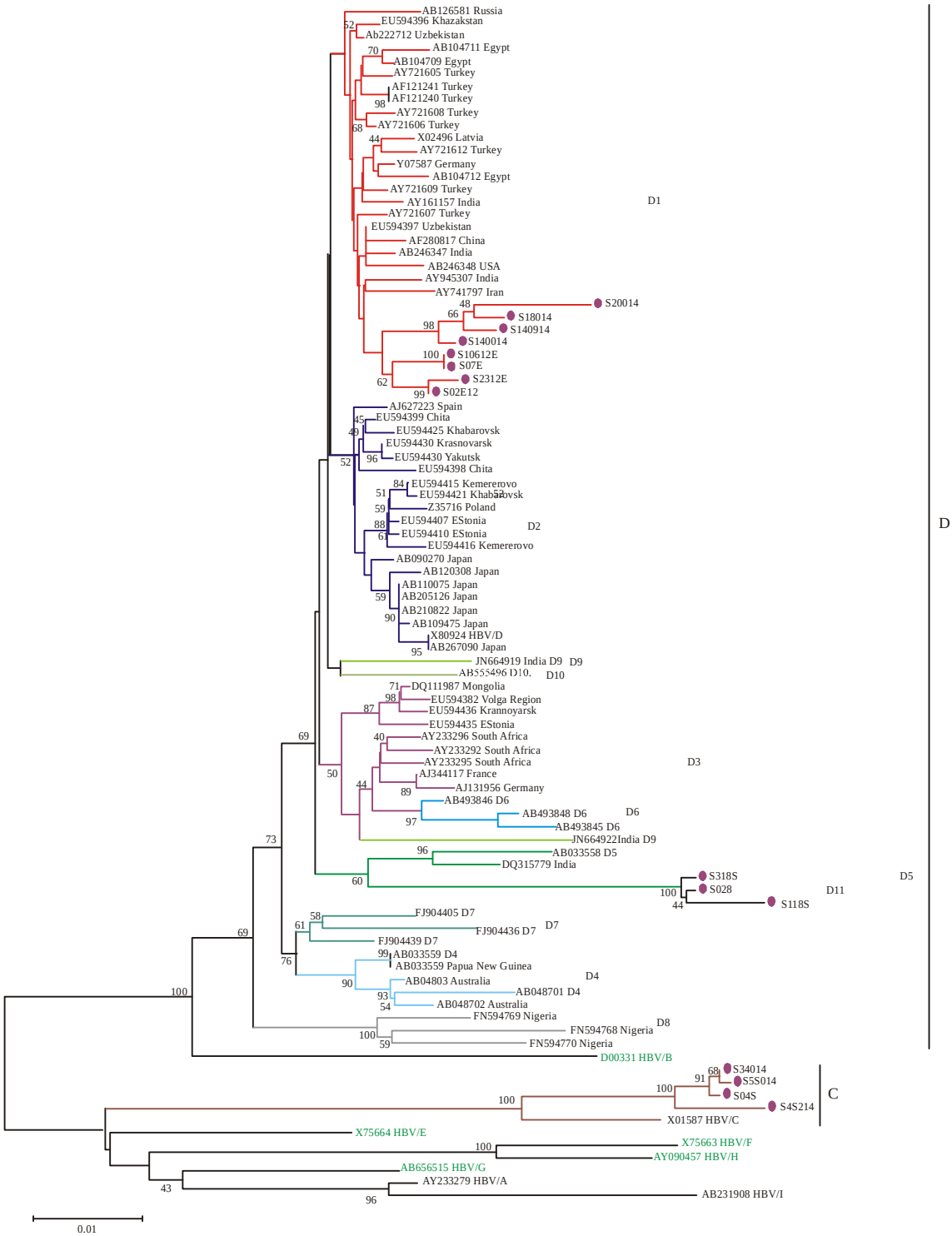

Fig. 2: Phylogenetic tree constructed by the neighbor-joining method (NJ), based on the 1.2 kb full length of HBsAg reference sequences retrieved from GeneBank database and represented all HBV genotypes (A-I) and HBV/D subgenotypes. HBV/D subgenotypes (D1-D10) represented by different colored branches 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)

		HBV subgenotypes												
												New D11		
HBsAg regions	Amino acid position	D1 n = 23	D2 n = 43	D3 n = 7	D4 n = 6	D5 n = 2	D6 n = 4	D7 n = 3	D8 n = 3	D9 n = 2	D10 n = 1	S028S	S3118S	S118S
PreS1	7	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	*	*	*
	75	Gln	Gln/Glu/His	Gln	Gln	Gln	Gln	Gln	Gln	Gln/Glu	Gln	Pro**	Pro**	Pro**
PreS2	112	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Thr	Ile**	Ile**	Ile**
	161	Ala/*	Ala/Val	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Gly**	Gly**	Gly**
S	196	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Glu**	Glu**	Glu**
	197	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ala**	Ala**	Ala**
	238	Met	Met	Met	Met	Met	Met	Met	Met	Met	Met	Ser**	Ser**	Ser**
	259	Val	Val	Val	Val	Val	Val	Val	Val	Val	Val	Cys**	Cys**	Cys**
	373	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser**	Ser**	Ile[#]
	374	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro**	Pro**	Ala[#]
	381	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile**	Ile**	Thr[#]

n: No. of isolates in the subgenotypes, *Amino acid deletion, Amino acid sequence was derived from the nucleotide sequence. **Subgenotypes-specific substitution, [#]Unique specific amino acids

substitution among the three D11 strains (S028, S118S and S318S) in the following regions: 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 residue in the S gene (373Ile, 374Ala and 381Thr) were specified S118S isolate. Also, there were in-phase deletions in the preS1 region in the three novels isolates, thus resulting in the loss of one amino acid Thr7 within the stretch of 108 amino acid that represent the preS1 region product (Table 2).

DISCUSSION

Although Saudi Arabia, especially Jeddah receives millions of people from different countries of the five continents annually in the Hajj and Umraha seasons, molecular epidemiological data to monitor Saudi HBV genotype/subgenotype strains are limited. Phylogenetic analysis of 15present isolates were demonstrating that HBV/D (73%) is most predominant HBV genotype, followed by HBV/C (27%) genotype C among HBV Saudi chronic Patients. This result is consistent with many previous observations postulated that genotype D appears to be predominating in the Mediterranean basin and the Middle East countries (Saudy *et al*., 2003; Norder *et al*., 2004; Zekri *et al*., 2007; El Hadad *et al*., 2013), whereas HBV/C is predominant in Southeast Asia countries (Lusida *et al*., 2008).

Recently, ten subgenotypes of HBV/D (D1 to D10) have been reported, each subgenotype showed distinct geographical clustering (Tran *et al*., 2014). Because little is known about the prevalence, distribution and sequences of HBV subgenotypes in Saudi Arabia, particularly Jeddah, no previous data was available (Al-Faleh *et al*., 1992, 1999). In the present study, eight isolates belonging to HBV/D (S18014, S140014, S20014, S140914, S02E12, S2312E, S07E and S10612E) are segregated with subgenotypes D1 reference isolates and inter distance equal 0.006 that was verified this result. Those in agreement with, recent studies reported the prevalence of HBV/D1 in regions of the Middle East through North Africa (Tran *et al*., 2014; El Hadad *et al*., 2013).

Moreover, it have identified a possible new HBVsubgenotype in 3 isolates S028, S318S and S118S (D11) with similarity identity 94-96%. As shown in Table 1, comparing S028, S318S and S118S against other HBV/D subgenotype verified inter genomic divergence between 4% and 6.7% based on the entire gene sequence of preS1/preS2/S regions. The designation of new HBV subgenotypes in particular D subgenotypes reported many conflicts in the last few years. Previously, analysis of the whole HBsAg gene sequence of some HBV strain alone might be insufficient to classify HBV/D into sub genotypes (Norder *et al*., 2004). Recently, new HBV subgenotype has been classified and designated as HBV/B3 using the partial sequence of the S gene (Lusida *et al*., 2008). Also the novel HBV/D9 have been classified from isolates showed recombination between genotype D and C in the pre core/core region, although the pre-S/S ORF didn't possess any unique motif that can distinguish HBV/D9 isolates from the other eight subgenotypes of D. Moreover, the sequencing of only the pre-S/S region that is frequently practiced for defining genotype/subgenotype will lead to their improper classification as subgenotype D3 or D2 (Ghosh *et al*., 2013). The previous suggestion confirmed by present phylogenetic tree, where pre S/S sequence analysis of HBV/D9 previously revealed that one isolate was belonged to do HBV/D2 while the other was clustered in specific branch. In the present study, the new three isolates were clustered with reference strains represented HBV/D5, despite this observation did not affect the identification of new HBV/D11, where subgenotype HBV/D6 strains clustered within the HBV/D3 branch as well as corroboration of both HBV/D7 with HBV/4 and HBV/D9 with HBV/D10 in three different clads respectively. These observations have been interpreted as not a separate subgenotype of genotype D but rather a clade of subgenotype HBV/D3 (Yousif and Kramvis, 2013). Therefore, the entire genome sequence analysis is needed to confirm whether the present isolates were belonged to HBV/D5 or they represent a novel subgenotype thought, we believe that the identification of HBV subgenotypes should be dependent only on the entire S gene sequencing.

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; Seddigh-Tonekaboni *et al*., 2001; Wakil *et al*., 2002). As shown in Table 2, the present amino acids HBV/D11isolates 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 D11in 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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