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Molecular epidemiology of hepatitis B virus in Iran

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ABSTRACT

Hepatitis B virus (HBV) infection is a major cause of liver disease worldwide. Eight genotypes and 24 subgenotypes of HBV have been identified. The aim of this study was to determine the distribution of HBV genotypes, subgenotypes and subtypes, and to understand HBV genetic variability in the HBV genome circulating in Iranian provinces. Two hundred and forty-nine sera from HBV-infected patients living in 25 provinces of Iran were collected (2004–2007). A part of the HBV *S/pol* and whole *BCP/C* genes were amplified, sequenced and then subjected to phylogenetic, recombination and genetic variability analysis. Results revealed genotype D of HBV in all samples and subgenotypes D1 (98.52%), D2 (0.74%) and D3 (0.74%) among Iranian patients living in different provinces of Iran. Subtypes *ayw2* (94.4%), *ayw1* (2.8%), *ayw3* (2%) and *ayw4* (0.4%) were deduced, on the basis of HBV small surface antigen (HBsAg) amino acid sequences. The mean percentage intra-genotypic distance of *S* plus core regions was 2.8%; the mean percentage inter-genotypic distance of this region between Iranian strains and genotype D isolates was 3.1%; and this rate for other genotypes was 5.2–11.4%. Various rates of point mutations have been found within different HBV genes, e.g. HBsAg (17.2%), precore-G1896A (59.5%) and Basal core promoter (BCP) double mutations (49.2%), whereas no recombination was found. In conclusion, these results indicate that the only genotype circulating in the provinces of Iran is genotype D. There exist high genetic variabilities in the *S/pol* and *BCP/C* regions among the Iranian HBV isolates.

Keywords Genetic variability, genotype, hepatitis B virus, Iran, phylogenetic analysis

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem, with more than 350 million people being chronically infected worldwide. HBV is the major aetiological agent of acute and chronic liver disease, including fulminant hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [1]. Traditionally, HBV has been classified into nine serological types [2]. Currently, on the basis of a nucleotide diversity of >8% in the entire genome, eight genotypes of HBV (A–H) have been identified [3–5]. HBV isolates have also been further classified into 24 subgenotypes on the basis of a >4% (but <8%) difference in the complete nucleotide sequences [6]. These genotypes show distinct variation in their geographical distribution in the world.

Genotyping and serotyping assays are useful tools in understanding the epidemiology of HBV infection. Indeed, there is increasing evidence showing a correlation between the clinical outcomes of HBV infection and genetic diversity [7,8]. With knowledge of the HBV genomic diversities, clinical practice and outcomes will definitely improve.

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Iran is a Middle Eastern country located in south-west Asia. A recent report showed that over 35% of Iran's population has been exposed to HBV and approximately 2% are chronically infected [9]. The genotypes and genome sequences of HBV prevalent in Iran have not been sufficiently evaluated in all provinces, and only limited information is available from Tehran, the capital of Iran [10,11]. In the present study, HBV genotype distribution and its genetic variability were determined among Iranian HBV-infected individuals from the provinces of Iran.

MATERIALS AND METHODS

Patients

The aim of the study was to determine HBV genotype/subgenotype/subtype distribution and also to understand HBV genetic variability in the whole of Iran. Samples were collected from throughout the country. Iran consists of 30 provinces, which can be divided into five different geographical regions (Fig. 1). In this regard, a cooperative network was set up to connect provincial health centres, which were asked to collect HBV-positive sera from patients living in their own province between January 2004 and July 2007. Samples were regularly sent to the Research Centre for Gastroenterology and Liver Diseases in Tehran. Unfortunately, samples from five provinces could not be obtained, despite frequent requests. In the end, 280 blood samples were collected from patients with HBV infection that covered the major regions of Iran (25/30 provinces, 85% of the country). The numbers, percentages and geographical distribution of these patients are shown in Fig. 1. Informed consent was obtained from all the patients, and the study protocol was reviewed and approved by the Ethics Committee at the Research Centre for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

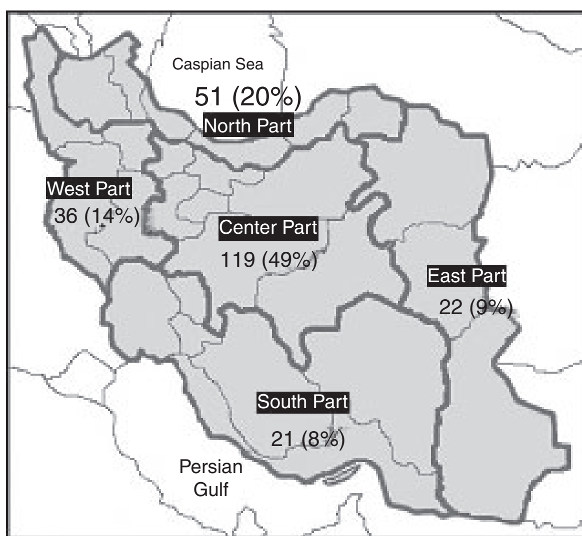

Fig. 1. Division of Iranian provinces into five parts on the basis of geographical location. Numbers and percentages of patients in each part are shown.

Serological and biochemical markers

Serological markers of HBV infection, which included HBsAg, anti-HBV core (anti-HBc), HBV pre-core Antigen (HBeAg), and anti-HBe, were determined with commercially available ELISA kits (DIA PRO Diagnostic Bioprobes, Srl., Italy). The sera were also tested using liver function tests such as alanine aminotransferase, aspartate aminotransferase, direct bilirubin total bilirubin and alkaline phosphatase.

Amplification and sequencing

HBV DNA was extracted with an QIAamp UltraSens Virus kit (Qiagen, Hilden, Germany). A 402-bp fragment covering a region of the *S/Pol* genes was amplified (with marked heterogeneity among genotypes), flanked by a conserved region to allow equal amplification of all genotypes. HBV *BCP/preC/C* gene sequences were chosen for amplification (784 bp), in order to determine the basal core promoter and any precore mutations [12]. The partial HBV *S* genes of 249 and 136 HBV *BCP/preC/C* gene sequences were amplified successfully. Amplicons were cleaned up using a QIA Quick Gel Extraction kit (Qiagen). The BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was employed for sequencing. The ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems) was utilized for electrophoresis and data collection. All sequences were analyzed bi-directionally.

Phylogenetic analysis

The combined *S* plus *BCP/preC/C* sequences (1186 bp) of 136 isolates were compared to their corresponding regions of 71 reference sequences retrieved from the GenBank database and representing all major HBV genotypes and, in particular, different subgenotypes of genotype D (D1–D5). The sequences were aligned using CLUSTALX software [13]. The genetic distances were estimated by a Kimura two-parameter algorithm, and a phylogenetic tree was constructed by the neighbour-joining method [14]. The reliability of the phylogenetic tree was confirmed by a bootstrap resampling test [15]. The analyses and calculated nucleotide differences within and between the isolate sequences were carried out with the MEGA program, version 3.1 [16]. Recombination was assessed using SIMPLOT software [17]. Woolly monkey HBV (GenBank accession number AF046996) was utilized as an outgroup.

Statistical analysis

Statistical differences were evaluated by chi-square test and Student's *t*-test. Differences were considered significant when a p value was ≤ 0.05.

RESULTS

Demographic and clinical data

In this study, 280 sera were collected from HBV-infected individuals from different ethnic groups

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in 25 provinces of Iran. After successful PCR amplification (the partial *S* gene of 249 patients and the *BCP/preC/C* gene of 136 patients), a study population totalling 249 patients with a mean age of 40.1 ± 14.5 years (range: 11–80 years), including 69.8% males and 30.2% females, was enrolled. The clinical profile of these patients is presented in Table 1.

Phylogenetic analysis and genetic distance

The 1085-bp fragment used for phylogenetic tree analysis was generated from two different blocks of the HBV *BCP/preC/C* gene with 731-bp sequences plus a partial HBV *S* gene with 355-bp gene sequences taken from 136 HBV Iranian isolates. The phylogenetic tree analysis revealed that all Iranian isolates were classified as genotype D and the majority belonged to subgenotype D1 (97%). One isolate was grouped as subgenotype D2 and another belonged to subgenotype D3 (Fig. 2). Genetic distance was estimated, based on a 1085-bp region within and between the HBV isolates. The mean percentage of nucleotide distance (intra-genotype) for Iranian isolates was 2.8%, whereas the nucleotide distance (inter-genotype) with other D genotypes was 3.1% (Table 2). The mean percentage of intra-genotype nucleotide distance between D1 Iranian isolates and other D1 subgenotypes from the GenBank database was 2.4%.

On the basis of the phylogenetic tree results, the Iranian isolates were, interestingly, grouped into two distinct branches within subgenotype D1 (Fig. 2, shown by arrows). Comparison of these two groups revealed a correlation in three residues within open reading frame C, exactly in the nucleotide positions 2080, 2107 and 2167, respectively. Nucleotide T at position 2080 co-appeared with C2107 and T2167 (called D1a), whereas, when A/C/G was located at position 2080, the coincidence of T2107 and C2167 was observed (called D1b) (Fig. 3). These patterns were found among 123 isolates (90.4%). The mean percentage of nucleotide distance between these two groups was 2.9%, close to the overall inter-genotype distance of all Iranian isolates. Interestingly, these two groups were also linked with another nucleotide position in ORF S of 104 from 123 isolates (84.5%). The nucleotide C499 (in the HBsAg) was detected in 94.7% of group D1a isolates, whereas this rate for group D1b was only 31.2%. Interestingly, the BCP double mutation (A1762T/G1764A) was more common within group D1a, and the other BCP double mutation (G1764T/T1766G) was clearly more common in group D1b (Table 2). Analyses of demographic and clinical properties of patients among groups D1a and D1b did not show a significant difference. Moreover, no specific geographical association was found between these two groups.

HBV subtyping

The HBsAg gene from 249 Iranian HBV isolates was amplified, which covered the major immunogenic region 'a' determinant domain. Subtypes were deduced from the sequence of the viral genome region encoding the HBsAg [18]. The majority of Iranian isolates were *ayw2* (94.4%), based on Arg122, Lys160 and Pro127; seven isolates were *ayw1* (2.8%), based on Arg122, Lys160 and Pro127 plus Phe134 and/or Ala159; five isolates were *ayw3* (2%), based on Arg122, Lys160 and Thr127; finally, one isolate was *ayw4* (0.4%), based on Arg122, Lys160 and Leu127. Interestingly, the subtype of one isolate was unknown because of atypical substitution at subtype-specifying residues (Ser127 because of a CCT → TCT mutation).

Table 1. Demographic, clinical, serological and biochemical information on the 249 patients infected by hepatitis B virus

Clinical status	Number (%)	Sex		Age (years)	$HBsAg^+$	$Anti\text{-}HBc^+$	$HBeAg^+$	$Anti\text{-}HBe^+$	ALT (IU/L)	AST (IU/L)	T-Bil (mg/dL)	D-Bil (mg/dL)	ALP (U/L)
		M	F										
Asymptomatic carriers	31 (12.4)	18	13	36.7 ± 11.8	30	31	0	31	26.2 ± 9.6	24.1 ± 6.4	0.81 ± 0.35	0.18 ± 0.12	148.7 ± 46.9
Chronic hepatitis	196 (78.8)	136	60	39.1 ± 13.6	194	196	43	153	54.8 ± 41.6	47.9 ± 22.6	1.4 ± 0.9	0.6 ± 0.5	204 ± 68.5
Acute hepatitis	5 (2)	5	0	42.6 ± 14.1	5	5	4	1	328.6 ± 177.2	244.4 ± 71.4	10.1 ± 12.4	4.1 ± 3.1	338.2 ± 31.5
Liver cirrhosis	14 (5.6)	12	2	54.4 ± 12.5	13	14	3[a]	10[a]	86.6 ± 109.4	107.9 ± 69.8	7.4 ± 7.3	2.5 ± 3.5	436.5 ± 272.8
HCC	3 (1.2)	3	0	70.6 ± 9.01	3	3	0	3	71 ± 24.6	174.3 ± 142.2	5.4 ± 7	1.6 ± 2.2	458.3 ± 274.5
Total	249 (100)	174 (69.8%)	75 (30.2%)	40.1 ± 14.5	245	249	50	198	57.7 ± 75.4	53.7 ± 49.1	1.8 ± 3.1	0.7 ± 1.2	216.4 ± 130.1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; D-Bil, direct bilirubin; T-Bil, total bilirubin; ALP, alkaline phosphatase; HCC, hepatocellular carcinoma.
[a]One patient's test results were negative for both HBeAg and anti-HBe antibody.

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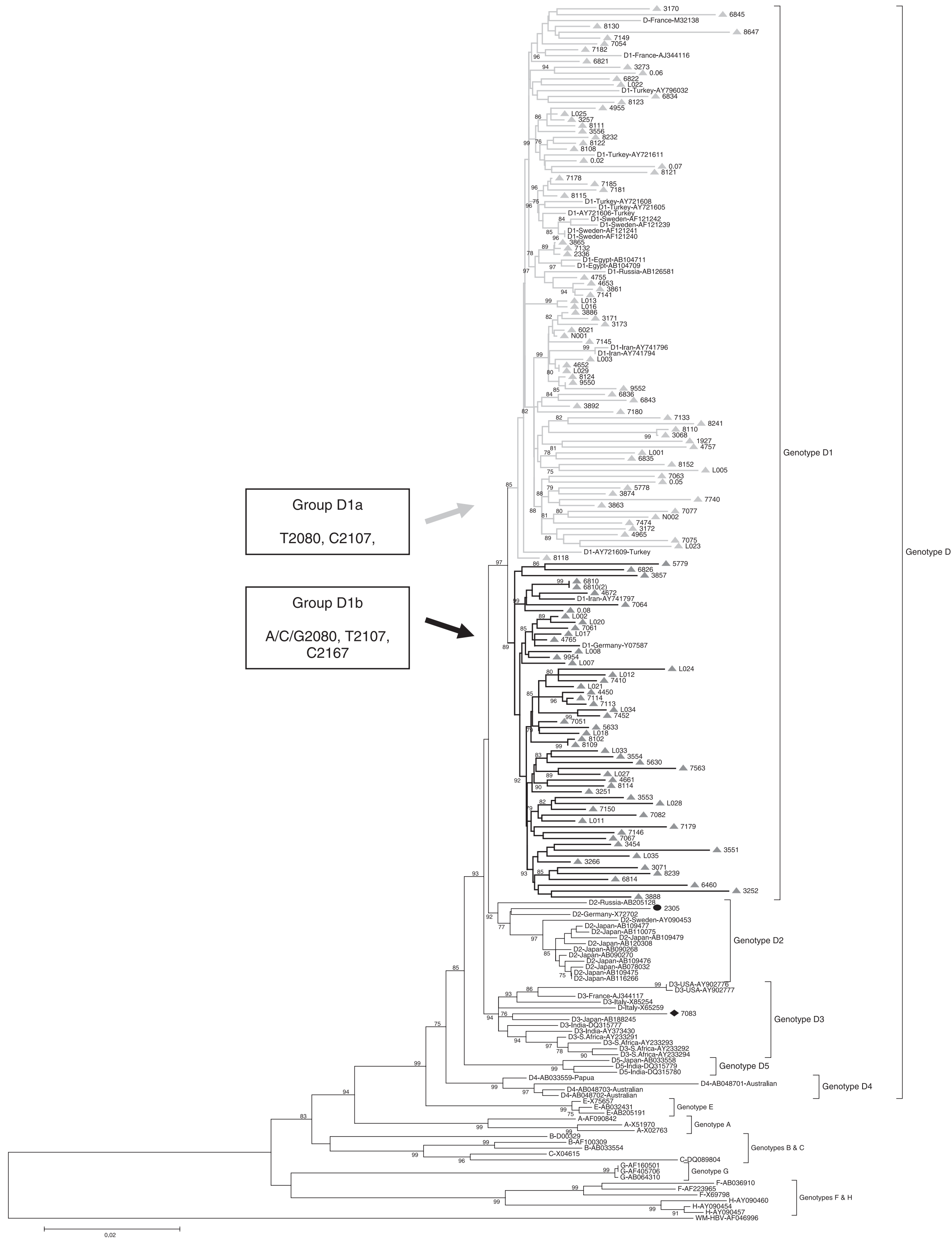

Fig. 2. A phylogenetic tree constructed using the neighbour-joining method, based on two different blocks of the HBV. BCP/preC/C with 731-bp sequences plus partial HBV HBsAg with 355-bp gene sequences taken from 136 HBV Iranian isolates were compared with the corresponding region of 71 reference sequences retrieved from the GenBank database representing all major HBV genotypes and subgenotypes of genotype D.

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Table 2. Demographic, serological, biochemical and mutation information on the D1a and D1b patient groups

Group properties (number of patients)	D1a 75 of 136 (55.1%)	D1b 48 of 136 (35.2%)	p-value
A1762T/G1764A double mutation in BCP	16 (21.3%)	3 (6.2%)	0.02
G1764T/T1766G double mutation in BCP	13 (17.3%)	21 (43.7%)	0.01
C nucleotide in position 499 of HBsAg	71 (94.7%)	15 (31.2%)	<0.001
T/G/A nucleotide in position 499 of HBsAg	4 (5.3%)	33 (68.8%)	<0.001

Genetic variability in the *S/pol* and *BCP/C* genes

Various point mutations in the sequence of HBsAg have been found among Iranian HBV isolates (Fig. 4). The most frequent mutations in Iranian isolates were Ser143Leu (5.2%), Ile10Leu (4.4%), Gln101Arg (1.6%), Pro120Ser (1.6%), Thr118Ala (0.8%), Ser136Tyr (0.8%) and Thr118Lys (0.8%). Interestingly, one isolate had an insertion (15 nucleotides) between nucleotides 222 and 223 which resulted in an LLPGS amino acid insertion. The TGG → TGA stop mutation was detected in two isolates, and led to a premature stop codon at position Trp172 in the *S* gene.

In the studied population, 54 patients (21.6%) had received lamivudine (100 mg/day) for more than 1 year. Thirty-one treated patients (57%) had HBV isolates with drug resistance mutations in the HBV pol region. Twenty-eight of 31 isolates (90.32%) had lamivudine resistance mutations, and three patients had isolates with both lamivudine resistance and adefovir dipivoxile resistance

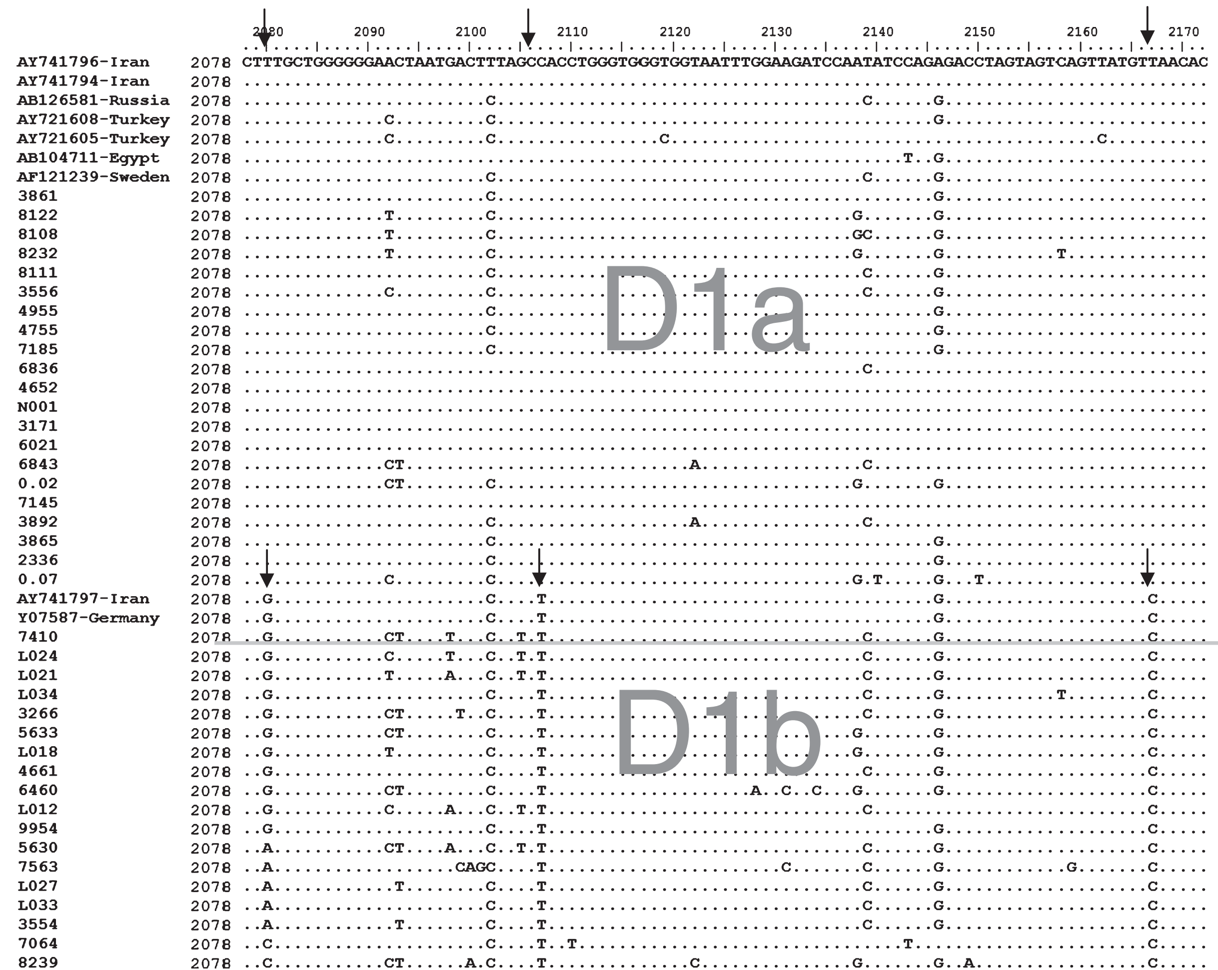

Fig. 3. The nucleotide sequence alignment of the *C* gene (nucleotides 2078–2174) deduced from the nine GenBank subgenotype D1 strains and the 39 Iranian isolates from this study. Three positions (2080, 2107 and 2167) are linked together in 91.2% of Iranian isolates. If T is located at position 2080, there will be C2107 and T2167 (group D1a). If A/C/G is at position 2080, there will be T at position 2107 and C at position 2167 (group D1b).

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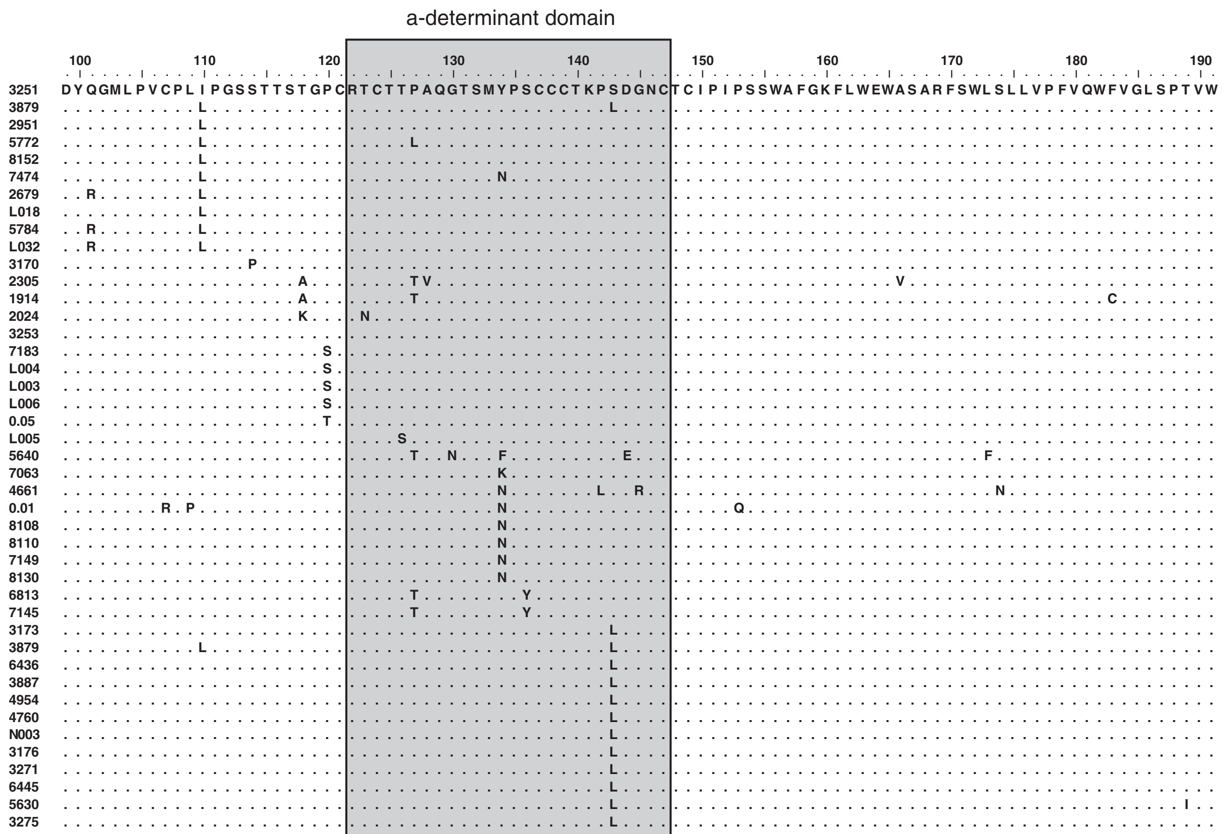

Fig. 4. Alignment of amino acids 99–191 of the partial S amino acid sequence of 43 Iranian isolates. The first (3251) is a wild-type isolate and is depicted in detail.

mutations (9.67%). Analysis of the RT region of the *pol* gene revealed M204I in 19 patients (61.29%), L180M + M204I in six patients (19.35%), L180M + M204V in two patients (6.45%) and V173L + L180M + M204V in one patient (3.22%). The L180M + M204V + A181V mutations and V173L + L180M + M204I + A181T mutations (lamivudine and adefovir resistance) were observed in one and two patients, respectively.

The *BCP/preC/C* gene from the Iranian HBV isolates was amplified to identify a double mutation at the BCP region and other mutations at the preC/C region. The double mutation A1762T/G1764A was detected in 22 isolates (16.1%), the mutation G1764A in four (2.9%), the double mutation G1764T/T1766G in 40 (29.4%), and the mutation T1766G in two (1.4%). There were 81 isolates (59.5%) with the G1896A stop precore mutation. The G1899A mutation was also found in 29 isolates (21.3%). Most of the Iranian isolates had Thr1858, Gly1862 and Gly1888 in the pregenomic encapsidation signal sequences; these are common in HBV genotype D. Interestingly, there were the following mutations: T1858C in one isolate, G1862A in two isolates, and G1862T in five isolates. The direct repeat 1 sequence at position 1824–1834 was conserved in all the Iranian isolates. An eight-nucleotide deletion (position 1762–1770) in two isolates, a nine-nucleotide deletion (position 1741–1749) in one isolate and a single base pair deletion in position 1846 in one isolate have been found and confirmed by resequencing.

Comparison of liver function test (LFT) results and HBeAg status between BCP/and precore mutants and wild-type isolates in chronic patients was done. There were no significant differences in mean values of LFT results between these groups. Among the HBeAg-positive patients, 25.8% showed wild-type isolates, whereas 15.3% of patients with BCP mutant isolates were still detected as being HBeAg-positive as well. All patients with precore mutants and BCP/precore mutants were HBeAg-negative. When mean age

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and LFT results were compared between the HBeAg-positive/anti-HBe-negative group and the HBeAg-negative/anti-HBe-positive group, there were no significant differences in the mean values of LFT results, but the mean age was significantly less ($p < 0.001$) in the HBeAg-positive/anti-HBe-negative patients than in the HBeAg-negative/anti-HBe-positive patients.

DISCUSSION

HBV genotyping has been extensively used throughout the world; but the results are incomplete in some parts and, in particular, the Middle East region. There is little information on HBV genotypes in Iran, and this is the first study to cover the majority of Iranian provinces. On the basis of a phylogenetic analysis of the *S* and *C* regions of 136 Iranian patients, genotype D was found to be the predominant genotype of HBV and was circulating in all provinces of the country. The majority of Iranian strains were subgenotype D1, subtype *ayw2*, whereas small numbers of D2/*ayw3* and D3/*ayw2* isolates were detected as well. Previous molecular epidemiological studies among Iranian chronic HBV patients, based on the *S* and *C* regions from chronic patients [11] and on the five complete genome sequences [12], also demonstrated that genotype D was the only HBV genotype in the Iranian population. Additionally, these data were in accordance with a previous study based on 109 HBV-infected patients, all living in Tehran [19]. HBV genotype D is found worldwide, although it is relatively rare in northern Europe and in the Americas [6]. There are reports of the presence of genotype D in Iran's neighbouring countries, including Turkey [20], Afghanistan [12], Pakistan [21] and Saudi Arabia [22].

Studies on subgenotypes in the Middle East region are limited to Turkey and Afghanistan; subgenotypes D1 and D2 were found in Turkey [20] and subgenotype D1 in Afghanistan [12]. Subgenotypes D1, D2 and D3 have been described as widespread throughout the world, subgenotype D3 was found in Asia (East India), South Africa, and Europe, subgenotype D4 was found in Australia [6], and recently, a new subgenotype, D5, was found in eastern India and Japan [23]. Although subgenotype D1 was predominant in this study, subgenotypes D2 and D3 were also found.

Genetic variability analyses of Iranian isolates have shown a number of mutations, insertions and deletions within the sequences of the *S* and the *C* regions. Amino acid substitutions within the 'a' determinant domain can lead to conformational changes. Some of these changes may lead to important medical and public health issues, such as vaccine escape, failure of hepatitis B immune globulin to protect liver transplant patients, babies being born to carrier mothers, and failures in detection of HBV carriers with some diagnostic tests [24,25]. In this study, HBV isolates with P120T, P120S, T123N and T126S mutations in the HBV *HBs* gene were identified, which may cause problems in diagnostic assays or treatment failures in hepatitis B immune globulin therapy, according to previous surveys [24–30]. The most important mutation in the 'a' determinant of HBsAg is G145R [24]. This mutation is stable over time and can be transmitted horizontally, despite the presence of high levels of anti-HBs [24,25]. Only one isolate with the G145R substitution was detected in this survey. Another mutation outside the 'a' determinant is F183C, which was found in one isolate. Recently, Oon *et al.* [28] have shown that F183C mutation may reduce affinity for monoclonal antibody. The present results showed that the frequency of HBsAg mutants in Iranian HBV-infected patients is 17.2% (43/249 isolates). As a viral HBV population infecting a host is usually distributed as a quasi-species, variants are expected to coexist with wild-type strains in most carriers. Direct sequencing of the PCR products cannot reveal a variant present in the sample as a minor population, and the true proportion of patients carrying HBsAg variants may be higher [31]. Recent studies indicated that the prevalence of HBsAg escape mutants among random chronic carriers could be as high as 6–12% [32].

As lamivudine is the only antiviral agent prescribed for HBV infection treatment in Iran, lamivudine resistance mutations may be observed among Iranian patients. Analysis of the HBV RT region of the HBV *pol* gene revealed various point mutations for lamivudine drug resistance in Iranian patients who received lamivudine. Thirty-one patients (57%) from among 54 treated individuals showed a variety of lamivudine drug resistance mutations. This rate is relatively high compared with the 15–20% drug resistance per year after lamivudine antiviral therapy [7].

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Other important mutations in the HBV genome can occur in the HBV *C* region. There were two major groups of mutations, including precore mutation and BCP double mutations, that may affect HBeAg expression. In the present survey, G1896A was found in 59.5% of the patients (81/136). G1896A can create a stop codon in the precore region that prevents production of HBeAg. Other important mutations are BCP mutations that can occur at positions 1762 and 1764 (A1762T/G1764A). These double mutations also decrease HBeAg production [33]. In this study, the A1762T/G1764A double mutations were detected in 15.4% (21/136); moreover, the G1764T/T1766G double mutations were identified in 29.4% (40/136). There were no significant difference in mean values of LFT results between chronic patients with BCP/precore mutant isolates and chronic patients with wild-type isolates. Recent studies indicated that the A1762T/G1764A mutation correlates with development of HCC [34,35], which was in agreement with the patients who had HCC in this study. The G1764T/T1766G double mutation has been proposed to form a putative new binding site for the transcription factor hepatocyte nuclear factor 3, and is thought to be a rare mutation [36]. In contrast, this rare mutation was found in 31% of patients in an independent study [37], which was close to the present findings (29.4%).

In conclusion, HBV genotype D and subgenotype D1/*ayw2* were predominant in Iranian patients, and subgenotypes D2/*ayw3* and D3/*ayw2* were also found in the country. These results indicate that there is high genetic variability in the HBV HBs, pol and core regions among Iranian HBV isolates.

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TRANSPARENCY DECLARATION

The authors declare that they have no conflicts of interest.

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