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Short communication

Epidemiological history and genomic characterization of non-D1 HBV strains identified in Iran

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ABSTRACT

Background: Hepatitis B virus (HBV) has been classified into eight genotypes and forty subgenotypes. Genotype D of HBV is the most worldwide distributed genotype and HBV subgenotype D1 has been isolated from Iranian patients.
Objective: To characterize for the first time complete genomes of recently emerged non-D1 strains in Iran.
Study design: HBV complete genomes isolated from 9 Iranian HBV carriers were sequenced. Different diversities of the ORFs were mapped and evolutionary history relationships were investigated.
Results: Phylogenetic analysis identified four D2 subgenotypes and five D3 subgenotypes of HBV in the studied patients. Of note, D2 strains clustered with strains from Lebanon and Syria. The time of the most recent common ancestor (TMRCA) of the first cluster of D2 was dated at 1953 (BCI = 1926, 1976) while the second cluster was dated at 1947 (BCI = 1911, 1978). All five Iranian D3 strains formed a monophyletic cluster with Indian strain and dated back to 1967 (BCI = 1946, 1987). Surprisingly, two D3 strains had an adw2 subtype. Interestingly, more than 80% of the present strains showed precore mutations, while two isolates carried basal core promoter variation.
Conclusion: Iranian D2 and D3 isolates were introduced on at least two and one occasion in Iran and diverged from west and south Asian HBV strains, respectively. Considering the impact of the different (sub) genotypes on clinical outcome, exploring the distinct mutational patterns of Iranian D1 and non-D1 strains is of clinical importance.

1. Background

Hepatitis B virus (HBV) infection is a worldwide public health problem. Phylogenetic analysis of the full-length genome has classified HBV into 8 genotypes (A-H) and 40 subgenotypes [1,2]. HBV (sub) genotypes display distinct geographical distributions [3] and impact clinical and antiviral therapy outcome [4]. Thus, understanding the molecular epidemiology of HBV is critical to improve control and treatment of the infection [5]. HBV genotype D is the most widely distributed genotype and its nine subgenotypes present diverse ethno-geographic patterns. Briefly, subgenotype D1 is prevalent in South-Eastern Europe, North Africa, the Middle

Abbreviations: HBV, hepatitis B virus; ORF, open reading frame; anti-HBc, antibody to hepatitis B core antigen; HBeAg, hepatitis B e antigen; anti-HBe, antibody to hepatitis B e antigen; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PCR, polymerase chain reaction; ML, maximum likelihood; MCC, maximum clade credibility; TMRCA, time to most recent common ancesto; PP, posterior probabilitym; MCMC, Markov chain Monte Carlo; BCI, Bayesian credible interval; MHR, major hydrophilic region.

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Table 1
Demographical and serological data of the studied population.

No (%)	HBsAg positive	Anti-HBs positive	Anti-HBc positive	HBeAg positive	Anti-HBe positive	Anti-delta Ag positive	Age
9 (100%)	9 (100%)	0 (0%)	9 (100%)	1 (11.1%)	6 (66.7%)	2 (22.2%)	32 ± 13[a]

[a] Mean age ± SD.

East, West and Central Asia. Subgenotype D2 is frequently identified in North-Eastern Europe; D3 has been isolated from Serbia and India; D4–D7 are found in Oceania, Southeast India, Indonesia and Tunisia. Subgenotype D8 and D9 which are "recombino-subgenotypes" have been reported from Niger and India [1]. In Iran, D1 is the main HBV subgenotype circulating in the entire country. Interestingly, our recent nationwide studies identified 2.86% (9 out of 314 isolates) non-D1 strains [6–8]. In this study, we report and analyse diversity of these nine isolates and attempt to reveal the epidemiological history of these HBV strains in Iran.

2. Objectives

Here, we characterized the evolutionary history of non-D1 HBV strains based on complete genomes isolated from Iranian asymptomatic carriers using a Bayesian phylogenetic framework.

3. Study design

Serum samples of Iranian HBV carriers were collected. Liver enzymes level and HBV serological markers were evaluated (Table 1). Viral DNA was extracted and HBV full-length genome sequence was determined [9,10]. The sequences (submitted to NCBI GenBank under accession numbers KM577663-71) were compared to 1063HBV subgenotype D1–D9 strains retrieved from GenBank. Alignments were created using ClustalW [11]. Possible recombinants were investigated using SimPlot v.3.5.1[12]. A preliminary tree was constructed using the Neighbour-Joining method and Kimura-2-parameter model [13].

To determine the epidemiological history of these isolates, strains with uncertain locations and collection dates were excluded. Maximum likelihood (ML) trees were constructed with the GTR + Γ model and 1000 bootstraps replicate using MEGA v6.0 [14]. Time-scaled trees were reconstructed using BEAST v1.8.1 [15]. Preliminary analysis using Path-O-Gen (http://tree.bio.ed.ac.uk/software/pathogen/) indicated that temporal signal of each subgenotype dataset was limited (D2: $R^2 = 0.233$ and D3: $R^2 = 0.16$). To maximize the temporal signal, each subgenotype was allowed to have an independent tree, while sharing the same underlying substitution model [16], which allows for different rates at the 1st + 2nd and 3rd codon positions. The uncorrelated lognormal molecular clock model was applied to take into account rate variation along the tree branches [17]. A flexible non-parametric demographic tree prior was used [18]. Two Markov chain Monte Carlo (MCMC) chains were run for 100 million steps, sampling parameters and trees every 10,000th step and MCMC convergence was verified using Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/). TreeAnnotator was used to construct a maximum clade cred-

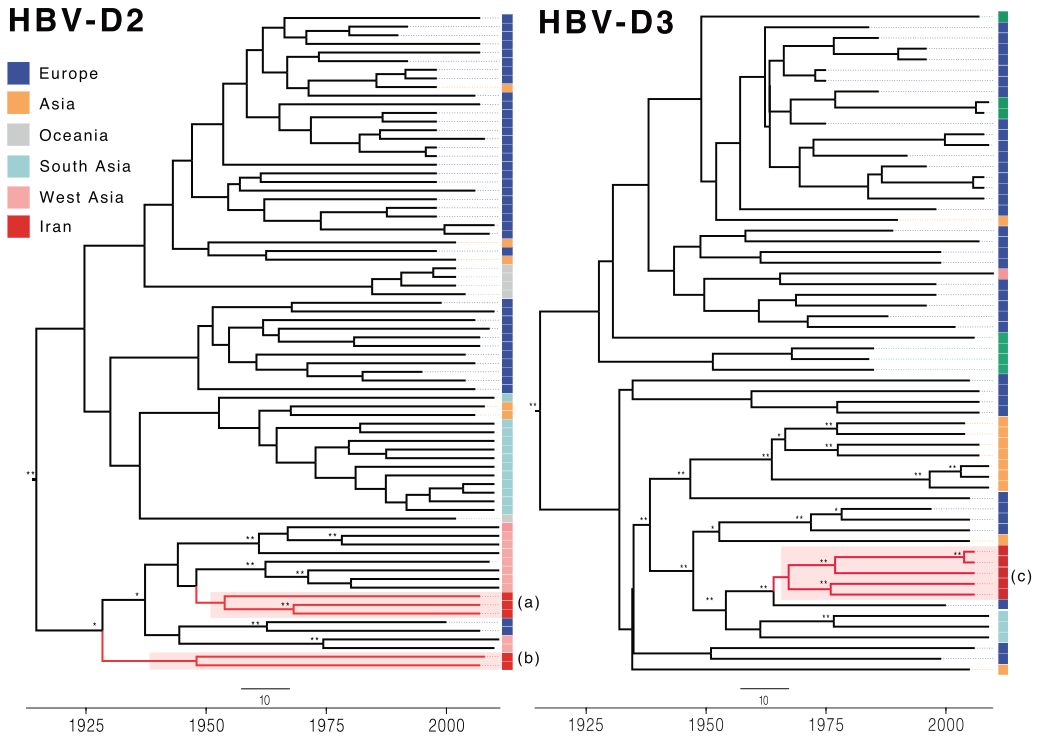

Fig. 1. Maximum clade credibility (MCC) tree of subgenotypes D2 and D3. The MCC tree of HBV D2 and D3 included the following regions and countries (number of sequences described as D2-D3): America: Argentina ($n = 0$–2), Canada ($n = 0$–3), Colombia ($n = 0$–1), Haiti ($n = 0$–1). Europe: Belarus ($n = 3$–3), Belgium ($n = 7$–16), Germany ($n = 2$–1), Latvia ($n = 5$–0), Poland ($n = 6$–0), Russia ($n = 14$–1), Serbia ($n = 1$–3), Sweden ($n = 0$–12), United Kingdom ($n = 1$–0). South Asia: India ($n = 12$–3). West Asia: Lebanon ($n = 7$–0), Syria ($n = 3$–1). Iran ($n = 5$–5 [One D2 strain (GU456635) was retrieved from the previous study by Garmiri *et al.*]). Southeast Asia: China ($n = 0$–1), Indonesia ($n = 0$–7), Japan ($n = 3$–1), Kazakhstan ($n = 1$–0), Malaysia ($n = 1$–0), Mongolia ($n = 0$–1) and Oceania: Caledonia ($n = 5$–0). Abbreviations: a–c: Iranian clusters. * posterior probability ≥ 0.70, and ** posterior probability ≥ 0.90 in the Iranian clusters."

ibility (MCC) tree, which was visualized in FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

4. Results

4.1. Patients information

The enrolled patients consisted of nine males, with a mean age of 32.6 years (range = 19–59 years). The mean of serum ALT and AST were 15.5 ± 4.8 IU/L, 15.7 ± 6.5 IU/L, respectively. Serological information of the studied population presented in Table 1.

4.2. Evolutionary analyses

Phylogenetic analysis showed that Iranian sequences clustered with the reference strains of subgenotypes D2 and D3, respectively. The nodes were supported by >70% bootstraps in the ML tree (data not shown). Also, these strains presented >4% nucleotide divergence. Further analysis of the Iranian D2 and D3 strains revealed similar topology in ML and MCC trees (data not shown). Fig. 1 shows the time-calibrated trees obtained using only D2 and D3 HBV full-length genome datasets (n = 76 and 62, respectively). Iranian D2 strains clustered with strains from Lebanon and Syria [Posterior Probability (PP) = 0.71] and formed two clusters, indicating at least two introductions. The most recent common ancestor (TMRCA) of cluster "a" was dated at 1953 [95% Bayesian Credible Interval (BCI) = 1926, 1976] while cluster "b" was dated at 1947 (BCI = 1911, 1978). All Iranian D3 strains formed a cluster with PP = 0.51 (Fig. 1). The TMRCA of cluster "c" was estimated at 1967 (BCI = 1946, 1987). The closest strain to cluster "c" was a Belgian strain (PP = 0.99) in which the Iranian strains shared a common ancestor in 1964 (BCI = 1941, 1984). This strain was sampled in Belgium from an Indian patient who had immigrated to this country five decades ago. The Belgian-Iranian D3 strains clustered strongly (PP = 0.98) with sequences from India (TMRCA = 1954, BCI = 1927, 1977).

4.3. Genome mapping

HBV-subtypes were determined based on particular motifs on the surface antigen [19]. All D2 strains carried ayw3, two out of five D3 strains showed adw2 and the rest presented ayw2 subtypes. The strains neither had recombination nor showed antiviral resistance mutations in the polymerase gene. One strain showed two substitutions in the MHR region, sG112R and sS113N, which may have clinical impact [8]. Also, three D3 and all four D2 strains showed the G1896A precore mutation, in which one D3, and three D2 isolates also presented G1899A. Furthermore, the single and double forms of basal core promoter (BCP) mutation [G1764A and (A1762T + G1764A/T)] were detected in two D2 isolates. In addition, clinically well-known core gene mutations, G1661A, G1732A and T1753C were observed in two D2 strains; A1760G, A1775G, A1837G in three D2 strains; T1849A, T1961A/G, C1962G and T1963C in two D3 and three D2 strains. X ORF mutations xI127T, xK130M, and xV131I were identified in three D2 strains.

5. Discussion

It has been demonstrated that different HBV subgenotypes carry different mutational patterns, which can have impact on diagnosis, therapy and prophylactic measures [4,20,21]. D1 is the most prevalent subgenotype circulating in West Asia where vertical transmission is a common route of HBV spread [1]. Although D2 and D3 have also been reported in Asia, there is a lack of data on these subgenotypes in Iran. Due to the clinical importance, characterizing emerging non-D1 subgenotypes in Iran is now becoming increasingly important.

Different HBV subtypes show epidemiological association with (sub) genotypes [22]. In line with previous reports, our study confirmed the association of ayw3 and ayw2 with HBV subgenotype D2 and D3. Surprisingly, in this survey two out of five D3 isolates carried adw2 subtypes, which have not been found associated with subgenotype D3 yet. Although no considerable mutation at the MHR of the HBsAg was found around 80% of strains (all D2 and some D3) presented precore mutations that abolish production of HBeAg. In contrast to the high substitution rate in the precore promoter, only two D2 strains (none of the D3 strains) showed a BCP mutation. This pattern in the core region of Iranian D2 and D3 strains differs with previous findings of Iranian strains with subgenotype D1 [23,24]. Furthermore, the association of BCP mutation with advance liver diseases [25] highlights the importance to study the HBV genome diversities [4].

In this study, transmission risk factors among the studied population were unclear. The mutation sT125M, which was related to the D3 epidemic of intravenous drug users in Europe in 1970s [26], was not detected in Iranian D3 strains. This suggests that divergence of Iranian D3 was through other routes of transmission in Asia. Interestingly, our results suggest multiple introductions of D2 around the 1950s in Iran, whereas a single introduction of D3 occurred around 1967. These findings are consistent with the estimates of Zehender et al. [27]. However, our results have slightly differences that could be attributed to the full-length genome analysis. Although present and previous studies highlighted the value of the using full-length genome of HBV in phylogenetic/phylogeography analysis [28–32] further research should be carried out to clarify the TMRCA of these subgenotypes. Non-European ancestors for Iranian D2 and D3 strains (West and South Asian ancestors) are another intriguing facet of our study.

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Competing interests

None declared.

Ethical approval

Not required.

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