Genetic Characterization of Hepatitis B Virus Genotypes among Patients with Chronic Hepatitis B Viral Infections in Sulaimaniyah Governorate, Kurdistan Region, Northern Iraq

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Background: The sequencing of the HBV genome gives rise to different genotypes. This study aimed to identify the current genotypes that circulate in the blood of patients with chronic HBV infections in Sulaimaniyah governorate, Kurdistan Region of Iraq.

Methods: Thirty-three adult chronic HBV patients were included in this HBV genotyping. Polymerase Chain Reaction (PCR)-based genotyping technique by using type-specified primers for genotypes determination was applied. Two amplicons (2/33) have been sequenced by the Sanger method in the Microgen sequencing facility in South Korea. Phylogenic trees were constructed using 36 HBV strains Pre-S1/Pre S2/S genes nucleotide sequences.

Results: All the patients had HBV genotype D. Additionally, two samples were further analyzed by sequencing and recorded in GenBank as HBV/Sul-1/2021 accession number MZ077051 and HBV/Sul-2/2021 accession number MZ077052. The HBV/Sul-2/2021 had two deletion mutations; the first mutation was at 61/G-87/T (27 a.a.), while the second mutation was at 104/Q-113/R (10 a.a.). Substitution mutations were recorded in both amplicons. The two amplicons were subgenotype D1/serotype ayw/subtype ayw2. Finally, we concluded that in Sulaimaniyah governorate, the HBV genotype D is the genotype that is circulating among patients with chronic HBV, of which subgenotype D1/serotype ayw/subtype ayw2 is recorded to present among them. HBV genotype D is subjected to deletion mutations. There are no mixed infections with different HBV genotypes. This study detected two new isolates, HBV/Sul-1/2021 and HBV/Sul-2/2021, different from previously recorded HBV isolate in 2016 in Sulaimaniyah governorate.

Keywords: HBV virus, genotyping, gene sequencing, chronic hepatitis, new viral isolates, Sulaimaniyah city.

Introduction

The chronicity of HBV infection and the absence of curable medications for this disease made it a nightmare in health care facilities. HBV infections are common worldwide; it is estimated that more than 275 million people were infected with HBV in 2015, with a prevalence rate of nearly 3.5% globally [1]. The prevalence of HBV infections in Mediterranean countries ranges between 2%-7%. In Iraq, the prevalence rate in 2005-2006 was 1.6% when HBsAg was the only marker for this measurement; this prevalence was underestimated as HBsAg was the only marker for measurement and anti-HBcAb was not tested yet [2].

The outcome of HBV infection is affected by several factors, including the viral load, HBV genotype, HBV mutations, host factors, and environmental factors [3]. The HBV genotypes emerge due to the absence of the HBV reverse transcriptase enzyme; this feature also contributes to the appearance of HBV sub-genotypes and HBV mutants [4]. The sequencing of the HBV genome gives rise to different genotypes. In addition, the presence of >8 other nucleotides give rise to a specific genotype. Eight different HBV genotypes have been identified; they have been named alphabetically from A-H. Recently, two additional genotypes have been documented as I and J. Sub-genotypes are determined for some genotypes, and >30 sub-genotypes have been defined [5].

Many researchers reported the relation between different genotypes and clinical course of infection, disease progression, response to treatment, and prognosis of the disease. Moreover, some studies mentioned other geographical distributions for different HBV genotypes [6]. The pathological impacts of different HBV genotypes are now partially recognized. Genotypes B and C are associated with higher intracellular and extracellular viral DNA than genotypes A and D. In addition, genotype C is associated with high replication capacity, which has increased genotype-related liver damage [7].

The HBV genotypes have different responses to anti-viral medications. It was observed in an experimental study that IFN/Peg-IFN was more effective in treating genotypes A and B than in treating genotypes C, D, or I [8]. In addition, the development of liver cirrhosis and hepatocellular carcinoma differs among different HBV genotypes. Many studies reported more severe disease

progression to liver cirrhosis and hepatocellular carcinoma in HBV genotype C than others [6]. Thus, this study was performed to identify the current genotypes that circulate in the blood of patients with chronic HBV infections in Sulaimaniyah governorate, Kurdistan Region of Iraq.

Materials and methods

Subjects

Thirty-three adult chronic HBV patients were included in this HBV genotyping in the current study. The patients consulted an outpatient clinic in Al-Shahid Hadi Consultation Clinic, Sulaimaniyah city, Iraq, from January 2020 to March 2021. All the patients have had HBsAg positive for more than six months.

Sampling

About 5.0 mL of coagulant blood from each patient was collected, and roughly 1.0 mL of serum was separated and stored at -20 °C. Hepatitis B viral DNA has been extracted with an addPrep Viral Nucleic Acid Extraction kit (Add Bio, Korea) using 200 µL serum as described by the manufacturer.

Experimental

A nested PCR-based genotyping technique was performed on the extracted viral DNA using specified primers for determining the HBV genotypes A to F [8]. The first-round amplification involved the identification of HBV itself (all HBV genotypes) in pre-S1 to S genes using the universal primer (Table 1) to produce one amplicon for the whole locus. The nested PCR primers were developed based on the conserved nature of the nuclear sequences in the pre-S1 through the S gene sequence of HBV. Different sizes of amplified DNA determined genetic characteristics in the HBV genotypes. Two nested PCRs were carried out in a respectful mixture for each sample. In the A, mix reaction allows specific detection of genotypes A (68 bp), B (281 bp), and C (122 bp), and in the B, mix reaction, genotypes D (119 bp), E (167 bp), and F (97 bp).

The PCR amplification reaction was done according to the manufacturer's instructions using Add Star Taq master mix PCR kit (Add Bio, Korea). Briefly, the simplex PCR reaction was completed up to a final volume of 20 µL by DEPC-H_2O (1.0 µL), 10 pmol universal P1 forward and S1-2

reverse primers and 5.0 µL of DNA sample. Initial denaturation of 5 min at 92 °C, 40 cycles of denaturation for 30 s at 94 °C, annealing at 50 °C for 30 s, extension for 30 s at 72 °C, and a final extension phase running at 72 °C for 5 min were the thermal cycler parameters.

The nested multiplex PCR amplification reaction was conducted in 0.2 mL tubes using Add Star Taq master mix PCR (Add Bio, Korea). Approximately 1.0 µL of the first-round PCR product was added to both A and B mix, and 1.0 µL of 10 pmol of each primer were also added (Table 1). The mixtures were compiled up to a final volume of 20 µL. The nested PCR reaction was carried out for 40 cycles (ESCO Thermocycler, Singapore) and with the following parameters, pre-heated to 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 40 s. The last extended to 72 °C for 5 min.

Finally, the PCR products were examined by loading 6.0 µL of PCR product on 2% agarose gel in 1× TBE buffer. The gel was stained with 10 µL safe gel stain dye (Add Bio, Korea). Electrophoresis was run at 120 volts for an hour on the electrophoresis system. The amplicons of PCR products (Figure 1) were analyzed according to the 100 bp DNA ladder migration pattern. The outer PCR products (first-round PCR product) of two amplicons (2/33) have been sequenced by the Sanger method in the Microgen sequencing facility in South Korea. Sequence from both ends using reverse and forward primers checked the identity for each nucleotide. Mega 10 program was used to perform multiple sequence alignment of amino acid HBsAg (pre S1, Pre S2, and large S) gene of Sulaimaniyah HBV isolates. It was compared to the peptide sequences of other HBsAg with closed percentage identities and reference HBV isolates that had been retrieved from NCBI.

Phylogenic trees were constructed using 36 HBV strains Pre-S1/Pre S2/S genes nucleotide sequences (Figure 2). The NCBI reference sequences for HBV genotypes and HBV D sub-genotypes were obtained. The Clustal W technique was used to perform multiple alignments of these sequences [9]. Neighbour-Joining phylogenetic analysis was performed using MEGA 10, and bootstrap values were calculated by 1000 replicates of the original data [10].

Ethical approval

Commented [za1]: , and

Commented [za2]: was

Commented [za3]: neighbor-joining

All procedures performed in this study were performed in accordance with the ethical standards of the national research committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards. On the other hand, written informed consent was taken from the patients to publish this data. The scientific and ethical committee approved the research of the Kurdistan Institute for Strategic Study and Scientific Research, Sulaimaniyah, the Republic of Iraq (No. MLD38-2020).

Statistical analysis

The Clustal W technique was used to perform multiple alignments of the obtained sequences. Neighbour-Joining phylogenetic analysis was also performed using MEGA 10, and 1000 replicates of the original data calculated bootstrap values.

Commented [za4]: neighbor-joining

Results

HBV detection and genotyping

A total of 33 chronic hepatitis B patients were examined by PCR assay using a universal primer to detect all HBV genotypes. All samples were positive and gave the expected amplicons (about 1063 bp). However, one sample had a smaller amplicon indicating a mutation in the genomic sequence (Figure 1) which was then verified by DNA sequencing. The positive samples were genotyped using type-specific primers (Table 1), and all the patients had HBV genotype D according to the migratory pattern of gel electrophoresis.

HBV Sequencing

The Sanger method sequenced the viral DNAs of the outer PCR amplicons of two positive specimens (2/33). They were submitted to GenBank as HBV/Sul-1/2021 accession number MZ077051 and HBV/Sul-2/2021 accession number MZ077052 (https://www.ncbi.nlm.nih.gov/nuccore/MZ077051). The HBV/Sul-2/2021 had two deletion mutations; the first mutation is at 61/G-87/T (27 a.a.), while the second mutation is at 104/Q-113/R (10 a.a.); the total amino acids deletion in HBV/Sul-2/2021 strain was 37 amino acids which represent a deletion mutation for 111 nucleotides. The HBV/Sul-2/2021 is unique in its nucleotides' sequence as it is not mentioned before (Table 2).

Phylogenic analysis

Phylogenetic analysis of the ten bp corresponding to the partial Pre S1/Pre S2/S genomic locus was done to identify HBV D sub-genotypes of the current isolates in comparison to the reference HBV sequences. According to the phylogenetic analysis, HBV sequences were classified into eight genotypes (A-H), and the reference HBV genotype D was classified into ten sub-genotypes (D1-D10) (Figure 2). Furthermore, the topology of the phylogenetic tree revealed that both HBV/Sul-1/2021 and HBV/Sul-2/2021 belonged to sub-genotype D1.

HBV serotype

HBV serotyping was determined by the deduced amino acids of the HBV S protein sequence. All Sulaimaniyah HBV serotypes were classified as serotype ayw2 based on Arg122 and Lys160, Pro127, Gly159, and Thr140 in the deduced amino acid sequences of the S gene (Table 2).

Genetic analysis of preS1/preS2 region

In the ongoing study, two isolates of Sulaimaniyah HBV had a similar 11 amino acid deletions in the beginning of pre S1 region as a categorization of genotype D. In HBV/Sul-2/2021, additional long-stretch deletions were observed in Pre S1 region from amino acid residue 61-87 as well as ten other mutations were identified in the 3-prim terminus of the pre-S1 from a.a. 104-113. Accordingly, 37 amino acids were deleted in the S promoter region (Table 2). HBV/Sul-1/2021 had no deletion mutation in Pre S1/Pre S2/S amino acids. However, there were various sense point mutations divergences between both isolates of the current study. HBV/Sul-1/2021 had M88L and T90A at the S promoter region and D114N at the carboxylic end of the PreS1 part. HBV/Sul-2/2021 had T26P and V55A V88L substitution at the S promoter region. There was no amino acid mutation in the hepatocellular binding site of the virus located at a.a. 21-46, and no frameshift mutation was detected.

Commented [za5]: mutation

Genetic analysis of S region

There was no premature stop codon mutation in the S gene in the current identified Sulaimaniyah HBV. However, a premature stop codon was found in previously identified Sulaimaniyah HBV residue 69 of the S gene. Only P113S substitution in the central hydrophilic region (MHR) was

found in a.a. 99-169 of S gene. No substation was detected in the 'a' determinant region (a.a. 124–147) (Table 2).

Discussion

The HBV infection is not uncommon in Iraq; the prevalence of this viral infection was estimated to be 1.1% in 2005-2006 [2]. However, there was an underestimation of the HBV prevalence in Iraq. Most studies detected only HBsAg as a marker of prevalence and did not measure the total anti-Hepatitis B core antibodies as an additional marker. The HBV infection can run in a chronic course and create a risky health situation, including organic, psychological, and social effects. To cope with this challenge, the Ministry of Health in Iraq introduced a free HBV vaccine for all newborns in Iraq. Unfortunately, there is little known data about the circulating HBV genotypes in Iraqi populations, including Iraq's Kurdistan Region.

There are more expectations about the correlations between different HBV genotypes with disease progression and/or response to treatment. In the current study, we analyzed the HBV genotypes distribution among 33 chronically HBV infected patients. The genetic analysis of HBV genotypes revealed the presence of only one genotype out of 6 genotypes investigated in the Sulaimaniyah governorate. All the 33 (100%) patients were positive for only genotype D. Our result is dissimilar to two earlier Iraqi studies; a previous HBV genotyping in Sulaimaniyah city on 4 HBV positive specimens was done in 2013 by Peshnyar et al., and it revealed mixed genotypes (A+B+C+ D) infections in all specimens [11], while Shakir et al. [12] detected five different genotypes (A-E) circulate among Iraqi HBV patients in Wasit Province, Iraq in 2016. Globally, genotype D was found in nearly 22.1% of the entirely HBV infected people, of which 61.9% were found in Asia, with 22% in Africa and 13.5% in Europe [13].

The HBV sequencing and its phylogenetic analysis showed that it belongs to genotype D; which was reported previously in Africa, Europe, Mediterranean countries, India, and Indonesia [6]. However, the genetic analysis of HBV in the current study showed that the two analyzed samples belong to sub-genotype D1/serotype ayw, subtype ayw2. These findings are similar to that reported in North Iran on 100 patients [14] and 24 specimens from Tehran, Iran [15]. However, two representatives from Tehran said genotype D2/ayw3; these findings may reflect the circulation of

genotype D1/ayw2 in these neighbouring regions (North Iraq and North Iran).

In the current study, one HBV out of the 33 specimens (HBV/Sul-2/2021) had relatively long two deletion mutations in the pre-S1-S locus. Its unique sequence raises the question of whether this strain is formed locally in Sulaimaniyah city due to the local mutation, or it is introduced from outside the city or even outside Iraq, as the HBV/Sul-2/2021 accession number MZ077052 was nearly similar to the Iran HBV isolates KC339792 [16]. HBV/Sul-1/2021 accession number MZ077051 was closely grouped with GU186046 isolate previously detected in Sulaimaniyah in 2013 [11]. Moreover, this long deletion mutation may partly describe the presence of different genotypes and subtypes of HBV; thus, the virus is frequently subjected to mutation. These deletion mutations might represent immune escape mutations [17] and may be associated with low expression of HBsAg [18]. Furthermore, data from a previous study demonstrated the prognostic significance of preS deletion in carboxylic terminus mutants in developing hepatocellular carcinoma [19].

Moreover, there was no amino acid mutation in the hepatocellular binding site of the virus located at a.a. 21-46, and no frameshift mutations were detected. Usually, these mutations are significantly associated with hepatocellular carcinoma in chronic HBV infections [20]. In addition, there was no premature stop codon mutation in the S gene in the current identified Sulaimaniyah HBV. However, a premature stop codon was found in previously identified Sulaimaniyah HBV residue 69 of the S gene that might increase the current pathogenicity of HBV now.

Genetic analysis of preS1/preS2 region

The HBV/Sul-2/2021 has two point mutations in the S promoter region (M88L and T90A) and one-point mutation (D114N) in the carboxylic end of the PreS1 region; these point mutations may be useful mutations for viral pathogenicity and may have negative effects on disease progression by enhancing liver injury and increasing viral load of HBV. In addition, HBV/Sul-2/2021 has two substitution mutations (T26P and V55A V88L) at the S promoter region; the effects of these mutations should be further analyzed to reveal their impact on protein expression and disease severity. Ogura et al. found G2765A substitution in the pre-S1 promoter of HBV genotype C, which caused reduced L protein expression and low viral load in CHB patients [21].

Conclusions

In Sulaimaniyah governorate, the HBV genotype D is the genotype that is circulating among patients with chronic HBV, of which sub-genotype D1 is recorded to present among them. HBV genotype D is subjected to deletion mutations, of which HBV/Sul-2/2021 accession number MZ077052 was detected as unique HBV genotype D with long deletion mutations. There are no mixed infections with different HBV genotypes. The second documented HBV is HBV/Sul-2/2021 accession number MZ077052, with few substitution mutations in the Pre-S1 region and S promoter. In Sulaimaniyah city, The HBV genotype belongs to ayw serotype/subtype ayw2. More should be known about substitution point mutations in S promoter and pre-S1 region in chronic HBV infection.

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Conflict of interest

The authors declare no conflict of interest in this study. No fund/grant was obtained to conduct this study.

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