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Epidemiological Genetic Study for Novel World Records of Hepatitis B Virus Strains Detected by DNA Sequences in the South of Iraq/Al-Basrah Province

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Abstract
Hepatitis B virus (HBV) infection is one of the global and major health problems which possess a serious threat to humans and normally associated with the presence of hepatitis B surface antigen (HBsAg) (i.e. HBsAg small (S) protein particle). The small hepatitis B virus envelope protein S has the intrinsic ability to direct the morphogenesis of spherical 20-nm subviral lipoprotein particles. The DNA sequence analysis of hepatitis B virus surface protein S gene samples was amplified by PCR for 36 blood samples collected from Al-Basrah Province patients. Seven of 36 (19.4%) strains of HBV were identical (100%) to those previously isolated from Italy (1), Iran (3) and India (3), while the other 29 (80.6) strains which closely related with those strains from Sudan (24), India (2), Iran (1), Turkey (1) and Australia (1) were recorded in GenBank as novel strains in the world. In the present study, several point mutations in the sequence of s gene upon over-expression of full-length surface proteins, which may possibly contribute to HBV genome replication. These results suggest that indicated s gene rather than the surface protein was involved in regulation of HBV genome replication and could be developed into a safe and promising adjuvant of HB vaccine. The study investigation indicates that all new strains were closely related (99% identity) with the pervious mentioned global strains.

Keywords Novel · HBV · Surface S protein · DNA sequences · New records · Basrah · Iraq

For approval

1 Introduction

Hepatitis B virus (HBV) is related to the *Hepadnavirus* family and considered a prototype member of a non-cytopathic enveloped viruses containing a small enveloped DNA virus with a virion diameter of 42 nm [1, 2]. The genetic materials of HBV consist from a relaxed circular molecule of double-stranded DNA which encoded to four types of overlapping open reading frames (ORFs); they are named C, S, P and X coding for core protein, surface proteins (pre-S1, pre-S2 and S), DNA polymerase and X protein, respectively [3]. The small hepatitis B virus surface protein S gene has the intrinsic ability to direct the morphogenesis of spherical 20-nm lipoprotein particles. Such particles expressed in yeast or mammalian cells represent the antigenic component of current hepatitis B vaccines. The knowledge about the steps leading from the genetic engineered product of surface protein is very limited, as in this study, the information on the structure of the mutant type of surface protein (s gene) epitope that induces the formation to product antibodies after vaccination. As many researchers have described and studied the role of surface proteins in HBV envelopment and secretion, there are several causes of death as a result of HBV consequence, starting with hepatocyte infection which lead to acute and chronic liver diseases such as liver cirrhosis of the liver causing liver failure and liver cancer, in particular. According to the report of the World Health Organization (WHO), globally, there are more than 450 million people who have chronic HBV infections, and around one million people die every year due to the acute or chronic HBV infection [4]. Currently, there are many studies which have been reported on the molecular mechanisms for relation between HBV infection and pathogenesis of hepatic diseases, but the mechanisms are still not fully

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understood and the aetiologic agent of human hepatitis B, with man as its only natural host [5]. If exposed to HBV in early infancy or if the individual is immunocompromised, there is a high risk of the infection tending toward chronicity. [6]. In controlling HBV infection, cytotoxic T lymphocyte (CTL) response against different epitopes of HBV proteins is considered key factors [7]. The HBV genome consists of partial double-stranded DNA, encoding viral envelope proteins, core antigen (HBcAg), viral DNA polymerase and the X protein. The HBV viral envelope consists predominantly of the small or major envelope proteins (S), referred to as the HBV surface antigen, HBsAg; middle envelope proteins (preS2/S); and large envelope proteins (preS1/preS2/S) [8]. The majority of hepatitis B surface antigen (HBsAg)-positive individuals, especially those with circulating HBeAg, have easily detectable levels of hepatitis B virus (HBV) DNA in the serum [9]. HBsAg alone can assemble into 22-nm subviral spherical particles that are non-infectious and abundant in the blood stream of HBV chronically infected patients [10]. HBV vaccines consisted initially of HBsAg (22-nm spherical particles) purified from the plasma of HBV chronic carriers and at present contain purified recombinant HBsAg expressed in yeast [11]. Anti-HBs antibody induced by vaccination has neutralization activity and provides immunity against HBV infection [12]. The hepatitis B virus (HBV) is the main human pathogen responsible for severe hepatic diseases such as cirrhosis and hepatocellular carcinoma [13]. Even though infection can be prevented by immunization with an efficient vaccine, still about 2 billion people have been infected worldwide, resulting in 450 million chronic carriers who are prone to liver diseases [14]. Consequently, the reliable methods of HBV infection detecting are required especially in the high risk of infection rate. Generally, HBV surface antigen (HBsAg), HBV e antigen (HBeAg), HBV core antibody (HBcAb) and HBV DNA in serum have been used as standard markers of HBV infection and HBV replication [15]. In some cases, HBV DNA test is performed in HBsAg-negative patients with continuing chronic hepatic inflammation. The host's immune system strongly suppresses viral replication and gene expression of HBV, possibly aided by mutations in the surface antigen gene and precore region of the viral genome [16, 17]. The S-ORF encodes three surface proteins, L, M and S protein (HBsAg), through alternative translation initiation (ATI) from three in framed start codons [18]. In the last years, the identification of surface protein mutations has occurred due to the expanded use of molecular investigation and molecular clinical screening for HBV diagnosis [19]. However, certain amino acid residues bearing critical function for virus viability are highly conserved, and mutations rarely occur among the various HBV genotypes, making clinical screening insufficient to address the importance of these highly conserved residues [20]. This work is paving the way for further molecular study of all the mutations at the s protein region which could lead to synthesis and fabricate the epitope sequence of HBV proteins in vitro and design the novel s gene vaccine.

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2 Materials and Methods

2.1 Specimens

All blood specimens were collected from thirty-six patients with HBV infection which diagnosed by serological test were recruited from the Al-Basrah Province laboratory, 5ml blood samples were used for molecular testing. Serum sample was separated and purified by centrifugation at 3000×*g* for 10 min and stored at −70°C prior to DNA extraction, PCR amplification and sequencing analysis.

2.2 DNA Extraction

In this study, the standard method of DNA extraction from serum was used according to the procedure of proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. All viral DNA extraction was done in a flow safety cabinet using appropriate procedures to avoid contamination [21]. The DNA extraction was incubated at 70°C for 3 h with 100 pg/ml of proteinase K, 0.5% sodium dodecyl sulphate (SDS), 5 mM EDTA and 10 mM Tris (pH 8). After phenol/chloroform extraction, the DNA was precipitated with chilled absolute ethanol. Finally, The DNA was dissolved in 100 μl of 10 mM TE, pH 8.0, and stored at −70°C.

2.3 HBV DNA Quantitation

The concentration of extracted DNA was determined by measuring the absorbance at 260 and 280 nm wavelength using UV spectrophotometer Nanodrop (Optizn-Japan), and then PCR amplification of s gene with 50 pMol/μl of extracted and purified DNA was carried out in the next step.

2.4 PCR Amplification

The DNA amplification by PCR was carried out in BIONEER-MyGenie™ 96/384 Gradient Thermal Block (Daejeon, Korea),using 30 cycles of the following protocol: denaturation at 95°C for 2 min, primer aneling at 52°C for 2 min and elongation at 72°C for 5 min [22]. All runs included positive and negative controls. Ten microlitres of the PCR reaction mixture was run on 2% agarose gel and stained with ethidium bromide. The expected product is 387 bp. A selected genomic region of the polymerase gene as well as the hepatitis B surface antigen (s gene) was amplified and sequenced with primers sense (5′-CCTGCTGGTGGCTCCAGTCCCGGAACAGTA-′3) and antisense (5′-TTGGTAACAGCGGTATAAAGG-′3) [23]. The PCR products

were sequenced (ABI3730XL-BIONEER Gene analyzer, Daejeon, Korea) using dye terminator chemistry and analysed on an automated sequencer.

3 Results

In this study, the sequence of highly conserved site of s gene in the surface protein revealed several point mutations which regulate HBV genome replication level without compromising the overlapping s gene product. As mentioned previously, S gene (hepatitis B surface antigen) of 36 hepatitis virus serotype D isolates from patients' blood was amplified by PCR technique and sequenced via Genetic analyzer automated sequencer ((ABI3730XL-BIONEER Gene analyzer, Daejeon, Korea). The first group including five strains forming 45.5% which are IRQBASv7:HG810924.1(24) (Fig. 1), IRQBASv8:HG810925.1(1) (Fig. 2), IRQBASv9:HG810926.1(1) (Fig. 3), IRQBASv10:HG810927.1(1) (Fig. 4) and IRQBASv11:HG810928.1(1) (Fig 5) in the present study was similar in 99% to their reference strains; therefore, they are recorded in the GenBank of NCBI as a novel and new isolate in the world. Interestingly, the group under the strain named IRQBASv7:HG810924.1(24) (1,4,6,7,10,13,14,23, 24,25,26,27,28,31,32, 34,36, 38, 40,41,42,45,47 and 50) forming 66.6% of the total strains, since, there was a Gen or Point mutation type Transition (C instead T) at the position 249bp comparing with the reference strain SDAC_010 (Fig. 1). The group of strain No. 9 is IRQBASv8 (HG810925.1) which has Gen or point mutation type transversion (A instead C) at the position 309 bp and C instead A at 368 bp compared with the reference strain IR-37 (Fig. 2). The group of strain No. 18 and 35 is IRQBASv9 (HG810926.1) due to Gen or point mutation type transition (C instead T) at the position 248 bp compared with the reference strain THB_IC (Fig. 3). The group of strain No. 15 is IRQBASv10 (HG810927.1) which presented three Gen or point mutations: one type of transversion (G instead T) at the position 161 bp and two types of transition (A instead G) at the position 239 bp and (T instead C) at the position 253 bp compared with the reference strain TK6 (Fig. 4). Finally, the group of strain No. 12 is IRQBASv11 (HG810928.1) containing two Gen or point mutation type transversion (A instead C) at the position 235 bp and (C instead A) at the position 368 bp and one type of transition (C instead T) at the position 447 bp compared with the reference strain GS430 (Fig. 5).

Depending on Rooted Neighbour Joining phylogenetic tree constructed from concatenated sequences of 268 bp for each isolate, then produced from a MAFFT alignment and visualized using forester version1027. This N-J tree shows the

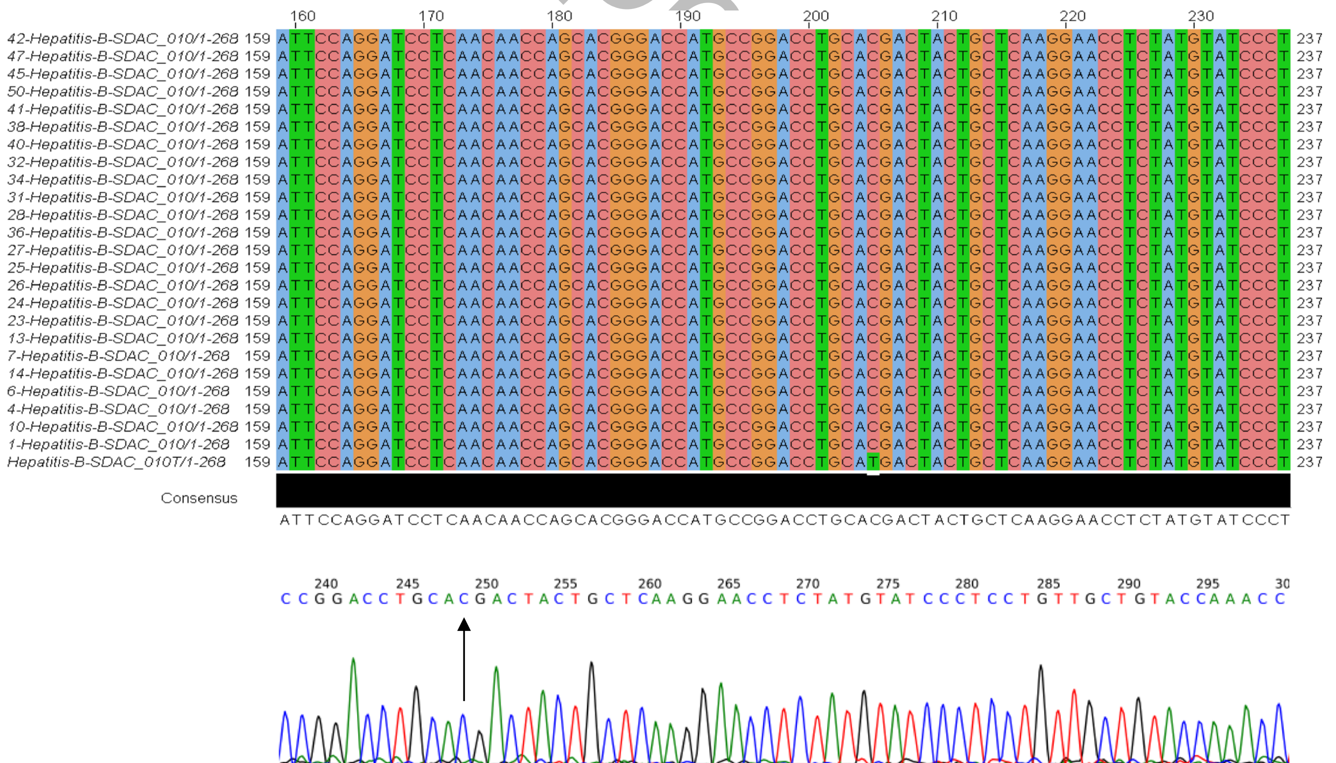

Fig. 1 Comparison of the 24 HBV isolates (including peaks) with the reference strain HB SDAC_010 containing gene or point mutation transition type (C instead T) at 249 bp

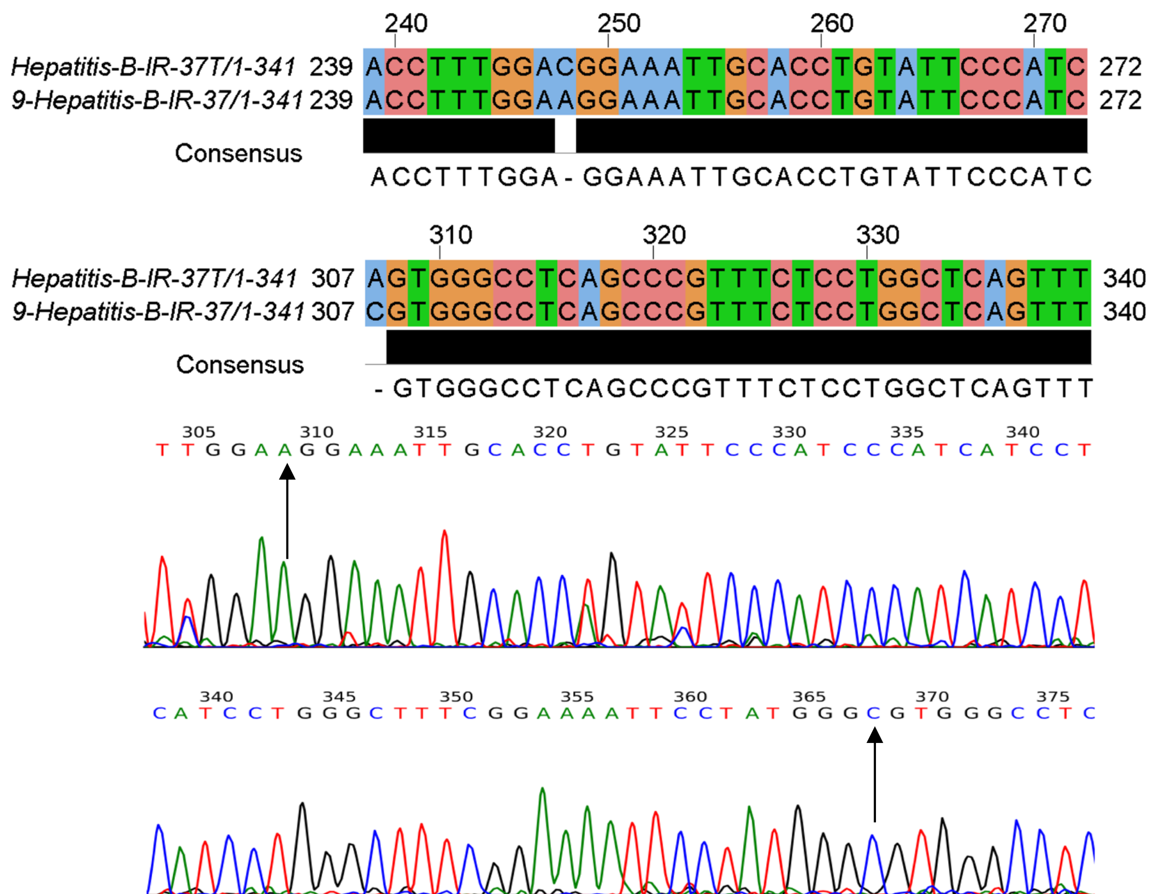

Fig. 2 Comparison of nucleotide sequences for the isolate of hepatitis B (with peaks) from present study and the reference strain hepatitis B IR-37. Gene or point mutation type transversion (A instead C) at the position 309 bp and C instead A at 368 bp

distribution and phylogenetic relationships of 36 isolates in this study and their 11 reference strains (T). All horizontal branch lengths were drawn to scale. Bootstrap values after 1000 repetitions are indicated. Out of 11 different strains groups, six (54.5%): 3, 37, 30, (16 and 43), 5 and 44 were identical in100% sequence similarity to their reference strains

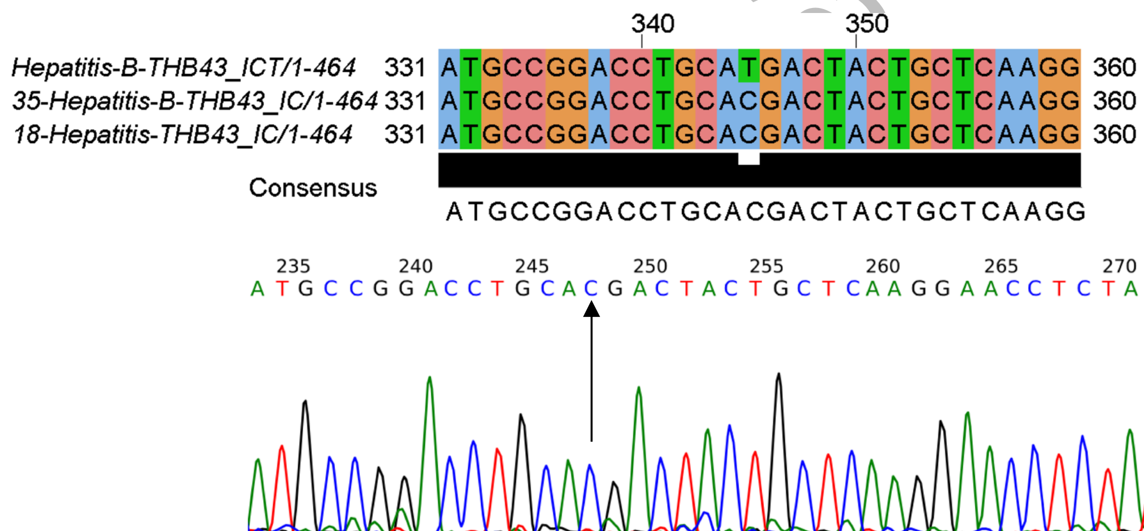

Fig. 3 Comparison of nucleotide sequences for the 2 isolates of hepatitis B (with peaks) from present study and the reference strain hepatitis B THB43_IC. A gene or point mutation type transition (C instead T) at the position 248 bp

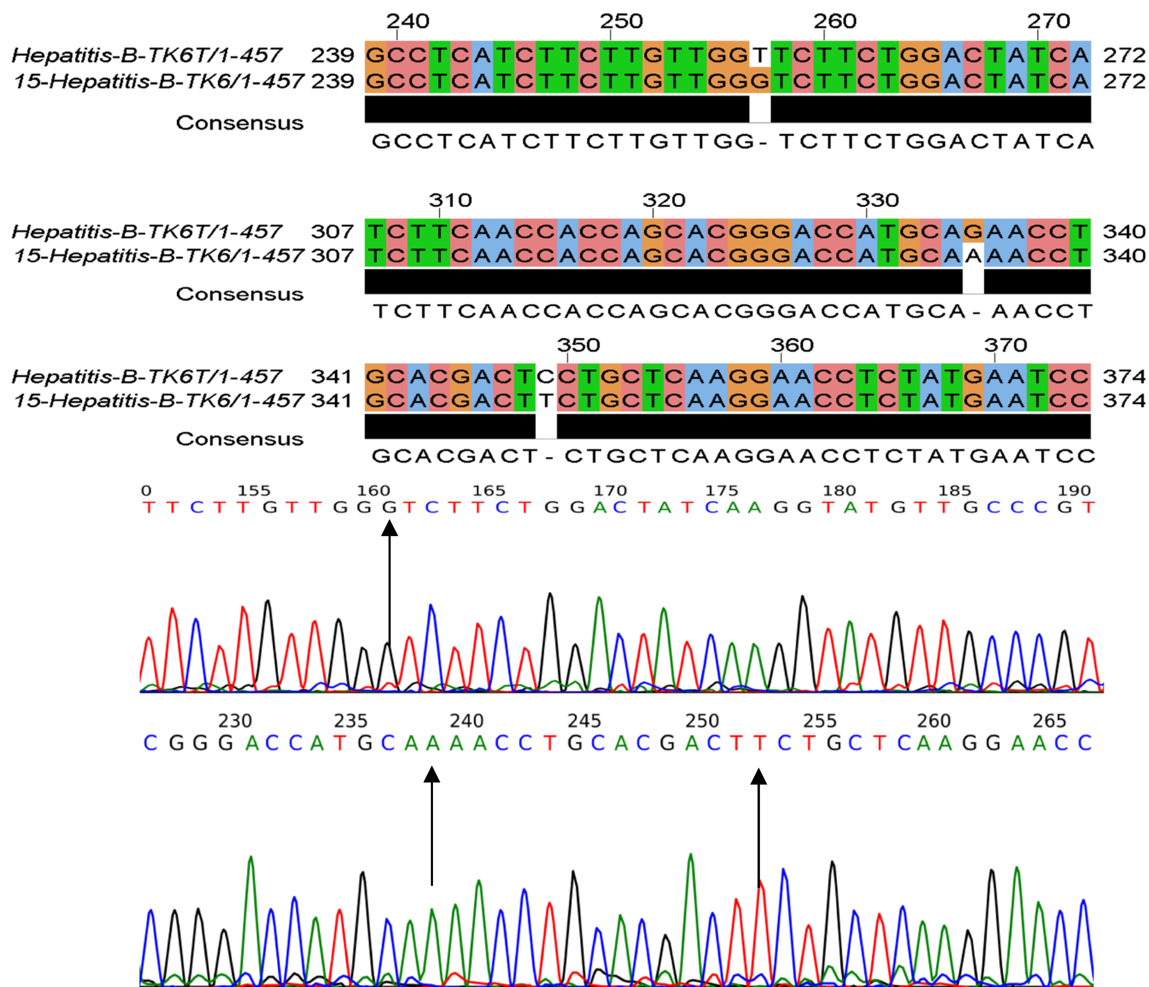

Fig. 4 Comparison of nucleotide sequences for the isolate hepatitis B (with peaks) from present study and the reference strain hepatitis B TK6. One gene or point mutation type transversion (G instead T) at the position 161 bp and two transitions (A instead G) at the position 239 bp and (T instead C) at the position 253 bp

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PTV031_D, F, Mashad-462, H, 919042 and IR-14 respectively as shown in (Fig. 6).

The unrooted phylogenetic tree (Fig. 7) shows some information about the distribution and the source of hepatitis virus serotype D in Basrah city. Since, isolates No. (16 and 43) and 37 (Clade 1) were genetically related to each other and identical to the reference strains H and F (respectively) from India, whereas with wide phylogenetic distance from other clades, confirming that this country is the source of them. However, isolates No. 5, 44 and 30 showed wide zone of genetical relationships among them within clade 2 as the Iran is the source of them, since they are identical to the reference strains 919042, IR-14 and Mashad-462 (respectively) from that country. Nevertheless, isolate No. 3 was from Italy as a result of its 100% similarity with the Italian strain PTV031_D.

On the other hand, although IRQBASv7 and IRQBASv11 (clade 3) were genetically closely related with the reference strains SDAC_010 and THB43_IC from Sudan and India (respectively), but they appeared closely with each other; alternatively, they genetically so far distance from other new isolates. Similarly, isolate IRQBASv8 was closely related with strain IR-37 from Iran. However, all these three new isolates severing only one nucleotide mutation (for each) reveal that these countries could be their origin.

Besides, each of IRQBASv9 and IRQBASv10 isolates (clade 4) has 3 different nucleotide mutations compared with the reference strains GS430 and TK6 from Australia and Turkey (respectively) revealing that these new isolates could be the Iraq which is the predominance origin of them. However, with the exception of relationship between IRQBASv7 and IRQBASv11, all new isolates in the present study showed genetically wide distance among each other.

According to the geographical map of the world (Fig. 8), there was no limited direction or route of transmission between the Iraqi HBV isolates (neither identical nor closely

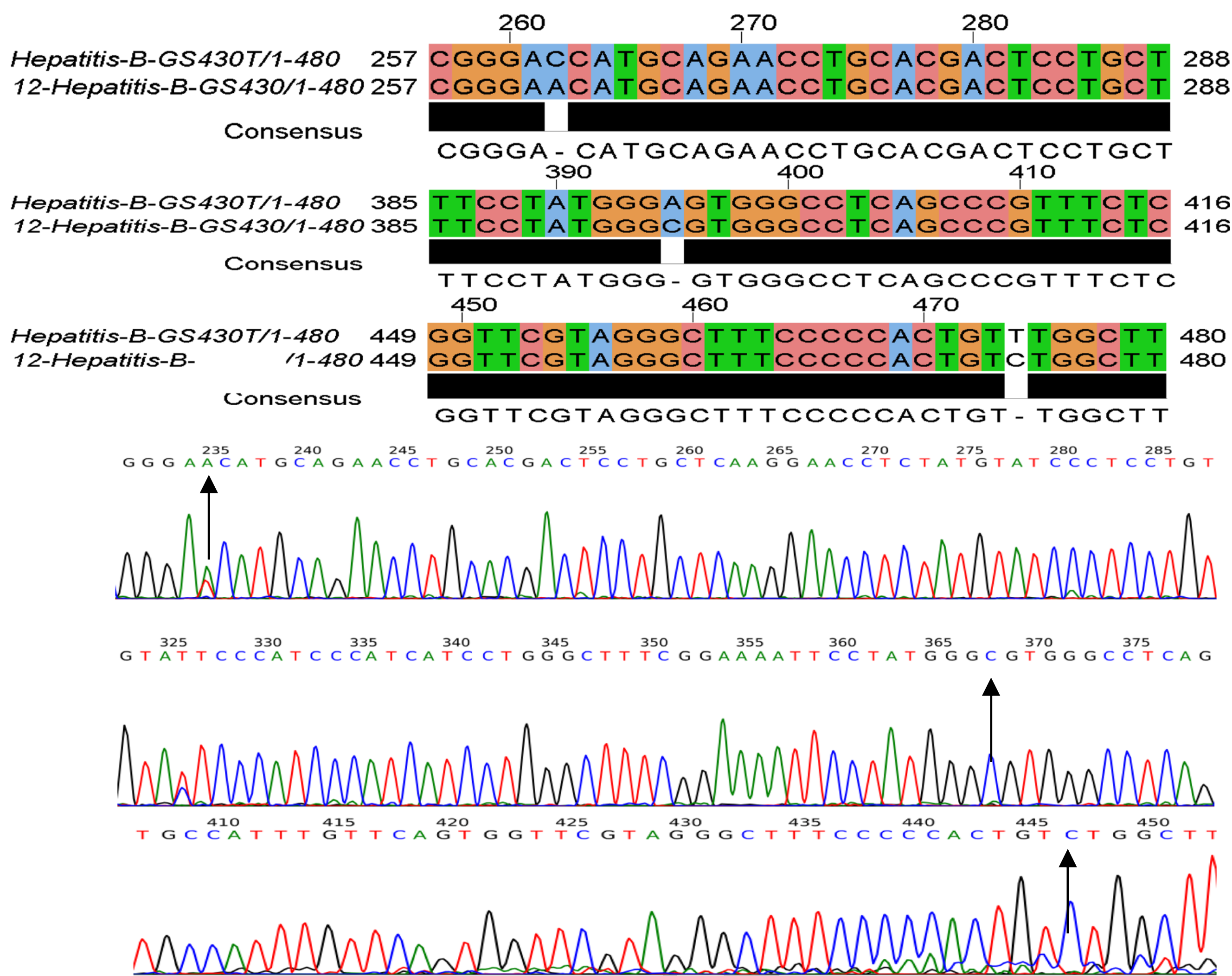

Fig. 5 Comparison of nucleotide sequences for the isolate hepatitis B (with peaks) from present study and the reference strain hepatitis B GS430. Two gene or point mutation type transversion (A instead C) at the position 235 bp and (C instead A) at the position 368 bp and one transition (C instead T) at the position 447 bp

related "new strains") and other countries, even those with so far distance.

4 Discussion

There are many researchers who have described and studied the role of surface proteins in HBV envelopment and secretion [2, 24]. The regulation of HBV genome replication is a complicated process controlled by various genes and several factors [25]. Although the surface proteins mainly exert their functions in assisting virus envelopment and secretion, there are also data refer to it is role in the regulation of HBV genome replication [26]. The current study demonstrated several novel point mutations in the S gene that could help to attenuate HBV replication level by decreasing the surface protein and shared by all the three types of surface proteins. Although the molecular genetic study was focused on the sequence of s gene which is responsible for the surface proteins that were involved in the regulation of HBV genome replication, the respective roles of each surface protein remained unstudied. Combined with the results from previous reports and other sequence of many countries' HBV strain that all three types of surface proteins are important for HBV DNA replication [27], it is possible that they play a regulatory role during HBV replication and the suppression of HBV replication by the effects of the mutations on S protein. This work is paving the way for further molecular study of all the mutations at the s protein region which could lead to synthesis and fabricate the epitope sequence of HBV proteins in vitro and design the novel vaccine. The defect in s gene can also hamper the secretion of HBsAg [28]. Moreover, many mutations occur in s gene of HBsAg which are reported to enhance the regulation [29]. The results of current study found that each mutation was

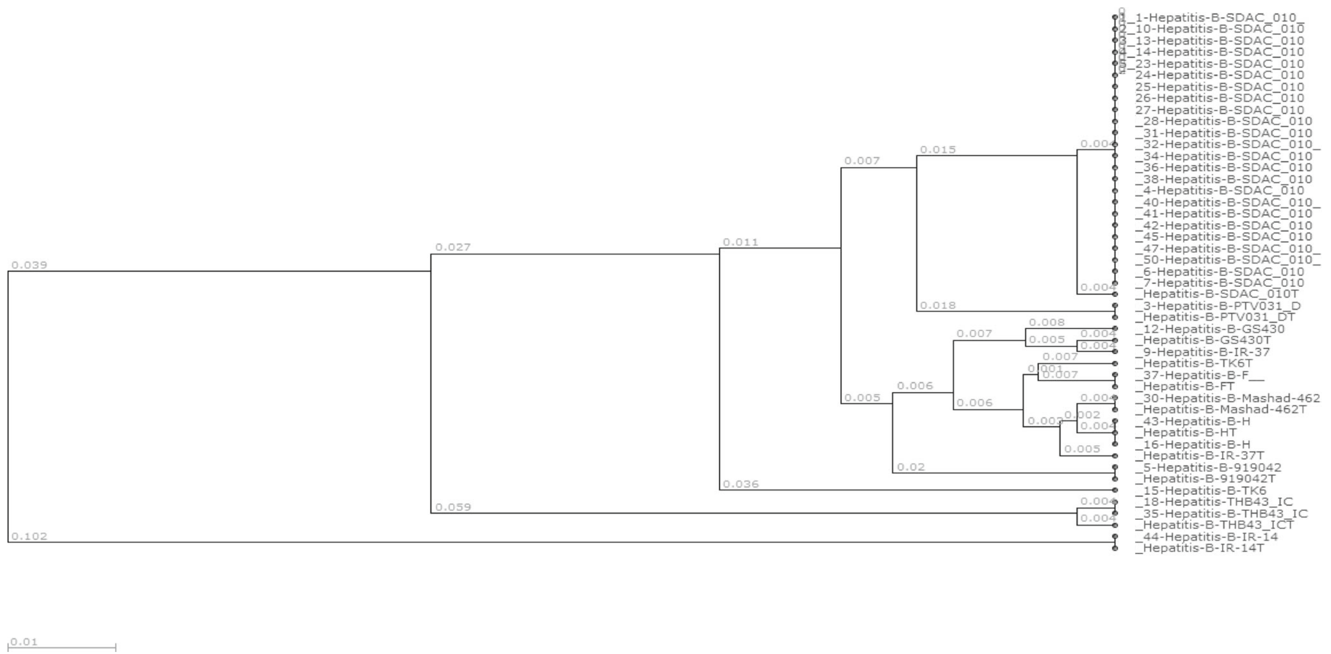

Fig. 6 Rooted neighbour joining phylogenetic tree constructed from concatenated sequences of 268 bp for each isolate and then produced from a MAFFT alignment and visualized using forester version1027. This N-J tree shows the distribution and phylogenetic relationships of 36 isolates in this study and their 11 reference strains (T). All horizontal branch lengths were drawn to scale. Bootstrap values after 1000 repetitions are indicated

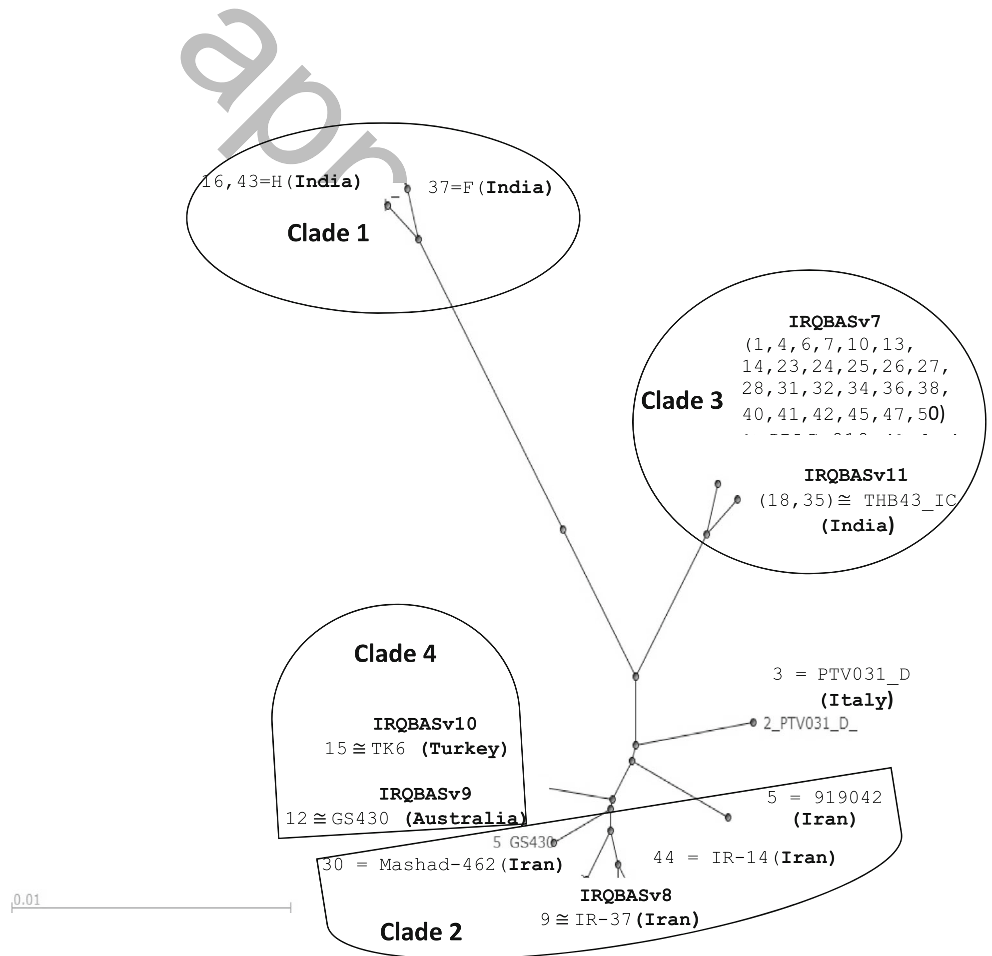

Fig. 7 Unrooted phylogenetic tree constructed from the concatenated (451bp) nucleotide sequences of S gene from 11 different groups of HBV serotype D isolates (from present study) and their 11 reference strains. Isolates No. 16 and 43, 37, 3, 5, 44 and 30 were identical with reference strains H (India), F (India), PTV031_D (Italy), 919042 (Iran), IR-14 (Iran) and Mashad-462 (Iran), respectively, while isolates IRQBASv7, IRQBASv8, IRQBASv9, IRQBASv10 and IRQBASv11 (new) were closely related to isolates SDAC_010 (Sudan), IR-37 (Iran), GS430 (Australia), TK6 (Turkey) and THB43_IC (India), respectively

Fig. 8 World map showing the geographical distribution of HBV serotype D isolates (from Basrah city) which are either identical (black spot) or closely related "new isolates" (white spot) to the reference strains from other countries

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essential for the secretion of HBsAg. Whether the point mutation in HBsAg plays a regulatory role in HBV replication needs to be further focussed and studied.

Abbreviations HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; PCR, polymerase chain reaction; WHO, World Health Organization; CTL, cytotoxic T lymphocyte; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBcAb, hepatitis B core antibody; ORFs, overlapping open reading frames; ATI, alternative translation initiation; Gen, gene; bp, base pair; SDS, sodium dodecyl sulphate; EDTA, ethylenediamine tetra acetic acid

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Author Contribution H.F. M. carried out majority of experimental work, specially DNA extraction and PCR amplification, and A.H.I developed the concept and involved in sample preparation and the serological investigation also discussion and writing. M.J.A was involved in all aspects of work, but mostly in sequence data analysis and results writing. All authors have read and agreed to the published version of the manuscript.

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Declarations

Due to the samples collected from human patients, the committee of Ethical Standards of University of Basrah depended for all samples, and all procedures and all participants were agreed to be part of this work and filled written consent form for the current study.

Conflict of Interest None.

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