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Diagnosis of HBV and HCV in Blood Samples of Different Regions at KSA and the Genetic Variability of the Polymerase (*poly*) Gene.

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ABSTRACT

The present study aims to diagnose both of hepatitis B virus (HBV) and hepatitis C virus (HCV) in some blood samples of different areas (Jeddah, Riyadh, Hafer El-Baten and Taif) at KSA and to focus on the genetic variability of the polymerase (*poly*) gene. Both of HBV and HCV were detected in the collected blood samples using the BIO-ELISA as well as real-time PCR kits specific to both of them. The nucleotide sequences of *poly* gene of HBV was determined using the DNA extracts of a positive-ELISA sample. Results concluded that three (representing 6.66%) and five (representing 11.11%) blood samples out of the 45 samples were infected with both of HBV and HCV, respectively. Both of HBV-DNA and HCV-RNA were not detected in 100% of blood samples of Hafr El-Batten. Detection of HCV in blood samples *via* ELISA showed that five blood samples (representing 11.11%) and 40 samples (representing 88.89%) out of the 45 were positive and negative, respectively. Data showed that a total number of three (6.67%) out of the 45 blood samples were positive *via* PCR detection of HBV-DNA. On the other hand, HBV-DNA was not detected in 93.33%. Results confirmed that the male was more available to be infected with hepatitis viruses than female. Correlation between age and HBV & HCV infection based on real-time PCR in blood samples was discussed. The partial nucleotide sequences of *poly* gene of HBV-isolate 8 (from Taif) was 676 nucleotides (Accession number LC101676 .1) and its analysis gave an open reading frame (ORF) encoding 224 amino acids. Genetic variability between the obtained ORF and those similar strains, recorded in GenBank was addressed.

Keywords: Hepatitis, HBV, HCV, ELISA, Real Time-PCR, polymerase (*poly*) gene.

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INTRODUCTION

Viral hepatitis is liver inflammation due to a viral infection. The most common causes of viral hepatitis are the six unrelated hepatotropic viruses HAV, HBV, HCV, HDV, HEV and HGV [1,2,3,4,5,6,7,8,9,10,11]. HBV infection (2-5%) is a major public health problem in the Middle East [12]. In Saudi Arabia, the prevalence of HBV infection among pregnant women at the antenatal clinic of a tertiary care center was determined and identified the target group for postpartum immunization. Sero-positivity among those aged 40 years and over was seven times as it was among people <30. People 30-39 were women aged ≥40 were five times more likely to be susceptible to infection with hepatitis as compared to young people aged <20. Moreover, those aged 20-40 are about 4.5 times more vulnerable to hepatitis as compared to their counterparts aged <20 [13]. Al-Knawy *et al*. [14] determined the HCV genotypes and the co-infection rate with HGV in 32 Arab patients infected with HCV. They concluded that HCV genotypes four and 1b were the major pathogenic strains, accounting for greater than 90% of HCV across ethnic groups in the southern region of Saudi Arabia. HBV belongs to the Hepadnaviridae family and is an enveloped virus with a double-strand DNA genome. They reported that HBV circular genome is nearly 3200 bp long, with four partially overlapping open reading frames (ORF); named surface (S), core (C), polymerase (P), and X. HBV is classified into eight genotypes (A-H) based on divergence of 8% or more of the complete HBV genomes [15,16]. The role of chronic HBV and HCV infection in adult patients as risk factors of celiac disease were assessed by Mostafa *et al*. [17] in Qassim University, between September 2012 and November 2014.

This study was designed to diagnose HBV and HCV in blood samples of different areas at KSA and focus on the genetic variability of the polymerase (*poly*) gene.

MATERIALS AND METHODS

Blood samples collection

A total of 45 serum samples of different age groups, 20-50 years, were collected (after obtaining the legal guardian consent) from different hospitals and blood bank of Jeddah (13), Riyadh (10), Hafr El-Baten (6) and Taif (10). Age groups were divided into three categories: group (I): from 20-29 years; group (II) from 30-40 years and group (III) from 41-48 years. In Jeddah, numbers of 5, 7 and 1 samples belonging to groups I, II and III, respectively, were recorded. In case of Riyadh locations, 4, 3 and 3 samples were belonging to groups I, II and III, respectively, were collected. Regarding the Hafr El-Baten region, 1, 3 and 2 samples belonging to groups I, II and III, respectively, were shown. Finally, 6, 7 and 3 samples belonging to groups I, II and III, respectively, were collected from Taif. Based on the gender, 9, 9, 4 and 12 blood samples of males, while, 4, 1, 2 and 4 blood samples of females were collected from Jeddah, Riyadh, Hafr El-Baten and Taif, respectively.

Serological and molecular diagnosis of HBV

The Monolisa™ HBsAg ULTRA kit for the detection of the surface antigen of the hepatitis B in human serum or plasma by the enzyme immunoassay technique was used. Regarding PCR diagnosis, Cobas *Taq*Screen test was used for examination of blood samples for HBV-DNA by Real Time-PCR Automated Detection.

Serological and molecular diagnosis of HCV

Monolisa™ HCV Ag-Ab ULTRA 1 as a screening kit for the detection of HCV infection in human serum/plasma by enzyme immunoassay from Bio-Rad was used. HCV RNA was amplified and detected using automated, Real Time-PCR on the COBAS® TaqMan® Analyzer.

Partial sequencing of polymerase (poly) gene

QIAamp MinElute Virus Spin Kit (Catalog # 57704) was used for extraction and purification of viral DNA from serum. Two primers (F: 5'-TTC CTG CTG GTG GCT CCA G-3' and R: 5'-CCA ATA CAT ATC CCA TGA ACT-3' were used to amplify the *poly* gene from HBV-DNA. PCR reaction was conducted in a volume of 25 µl (12.5 µl Go*Taq* Green Master Mix, 2X, 1.25 µl Sense Primer, 20 µM, 1.25 µl Antisense Primer, 20 µM, 2.50 µl HBV-DNA Template, and 7.50 µl Nuclease-Free water. The PCR program was 95^{o}C/5 min (one cycle); 35 cycle each

consists of 95°C, 55°C and 72°C, one min for each; and extension cycle of 72°C for 10 min. PCR product was gel purified and sequenced using automated DNA sequencing system (ABI 3100) and BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the Manufacturer's instructions. DNA sequence was then analyzed using BLASTN 2.2.23+ software (http://www.ncbi.nlm.nih.gov /blast/) against all sequences in the database for genotyping of each sample. The sequence that showed the lowest e-value and maximum identity was taken as the genotype of the sample analyzed.

RESULTS AND DISCUSSION

In this study, we are focusing on both of HBV and HCV as they remain important public health problem in Saudi Arabia as well as all over the world. This aim was supported by some investigators [18,19,20,21,22,23]. Due to the importance of detection, both of serological and molecular markers were recommended to diagnose HBV for appropriate management of disease [22], the present study diagnosed the presence of HBV and HCV in 45 blood samples collected from different regions (Jeddah (13), Riyadh (10), Hafer El-Baten (6) and Taif (16)), and represent four major locations of KSA *via* ELISA and Real Time-PCR. The nucleotide sequences of *poly* gene of HBV genome were also studied as shown in Table 1. Swellam *et al*. [24] evaluated the efficacy of the ELISA-3 for detection of anti-HCV in comparison with RT-nested PCR to detect HCV RNA for the diagnosis of HCV.

ELISA detection of hepatitis viruses in collected blood samples

The experimental results showed that HBV was ELISA-diagnosed by detection the presence of HBsAg in the collected blood samples as recommended by some researchers [22,25,26,27]. In case of Jeddah blood samples (Table 1) only one sample (No. 8) representing 7.69% appeared high ELISA reading (9.000) (three folds more than that of positive control (2.6170)) in comparison with both of Cut Off (0.0715) and negative control (0.0215). Regarding Riyadh blood samples, results shown in Table 1 showed that all the collected blood samples were negative when tested for the detection of the presence of HBV *via* ELISA. The ELISA reading was ranged from 0.0110 to 0.0260 compared to Cut Off (0.0715); negative control (0.0215) and positive control (2.6170). In case of samples from Hafr El-Batten, no positive ELISA values were obtained. At the level of Taif blood samples, results of detecting the HBV showed that HBV was detected in one (No. 9) out of two blood samples of King Fasial Hospital with ELISA reading 9.0000 in comparison with Cut Off (0.0715) and negative control (0.0215). Sample No. (1) collected from Blood Bank, representing (14.28%), showed positive ELISA reading (0.1210). It was obvious that serological detection of HBV in blood samples collected from four different regions of KSA *via* ELISA showed that three blood samples (representing 6.67%) and 42 samples (representing 93.33%) out of the 45 were positive and negative, respectively, when subjected to detection of HBV as shown in Table 2.

The experimental results showed that no positive ELISA readings were recorded in case of samples collected from Children Birth Hospital and King Fahd Hospital. While, two samples (No. 03 and No. 06) out of the five samples of King Abdul Aziz Hospital recorded positive ELISA results as they appeared readings of 3.0690 and 3.0460, respectively, in comparison with both of Cut Off (0.3145) and negative control (0.0315), when subjected to HCV detection. This was in harmony with that reported by [28,29,30,31], who concluded that serological diagnosis of patients infected with the HCV can be performed using indirect tests, which detect antibodies against HCV. On the contrary of HBV in Riyadh blood samples, one sample (No. 9) out of the 10 blood samples was positive as shown in Table 1. Its ELISA reading was 3.0460 in comparison with Cut Off (0.3145), negative control (0.0315), and positive control (1.5723). All blood samples of Hafr El-Batten appeared negative ELISA readings ranged from 0.0150 to 0.0240 in comparison with Cut Off (0.3145), negative control (0.0315), and positive control (1.5723) when subjected to HCV detection. In Taif samples, two out of 16 blood samples (No. 04 and No. 11) were infected with HCV when samples were subjected to ELISA test, and they showed ELISA readings 3.0690 and 0.3370, respectively, compared to Cut Off (0.3145), negative control (0.0315), and positive control (1.5723).

Based on the finding results one can conclude that ELISA detection of HCV in blood samples collected from four different regions of KSA *via* ELISA showed that five blood samples (representing 11.11%) and 40 samples (representing 88.89%) out of the 45 were positive and negative, respectively, when subjected to detection of HCV as shown in Table 2.

Concerning the gender and its correlation to HBV and HCV infection, results as illustrated in Fig. 1A showed that of the three HBV positive blood samples under investigation, two were male and one was female. In case of HCV positive blood samples one was female and four were male. In the present study, data as shown in Fig. 1B appeared that the positive HBV blood samples that were determined by the ELISA were distributed into the three age groups as follows: one of both of 20-29 & 41-48 years and two of 30-40 years. While, one of both of 30-40 & 41-48 years and two of 20-29 years were HCV positive samples.

PCR detection of hepatitis viruses in collected blood samples

In this study, the presence of HBV in blood samples was confirmed by Real-Time PCR test, this because serological diagnosis has its limitations: acute hepatitis most often precedes seroconversion; the results do not date the disease nor do they specify infectivity, evolution or recovery; sero-negativity does not exclude a positive diagnosis with any certainty, especially in such populations as haemodialysis patients [41].

HBV DNA of the sera of 45 blood samples was used as templates for PCR diagnosis of HBV. Results shown in Table 1 revealed that HBV DNA was detected in only one blood sample (No. 08) with 358,674 IU/ml PCR reading out of the 13 samples of Jeddah. The HBV-DNA was not detected in the blood samples collected from Riyadh (10 samples) and Hafr El-Batten (6 samples). In case of the 16 blood samples collected from Taif region, PCR results confirmed the presence of HBV DNA in only two samples, (No. 01 from Blood Bank and No. 09 from King Fasial Hospital) representing 12.5%. The PCR readings were 399 and 4420 IU/ml, respectively. We can conclude that a total number of three (6.67%) out of the 45 blood samples were positive *via* PCR detection of HBV DNA. On the other direction HBV DNA was not detected in 93.33% as shown in Table 2.

Direct tests, which detect, quantify, or characterize components of the viral particle, such as HCV-RNA testing and testing for detection of the HCV core antigen were reported by several investigators [29,32,33,34]. Therefore, in this study, Real-Time PCR was became the real reference test for hepatitis C diagnosis as it allows the evaluation of the number of viral genomes as recommended by [20,24,35,36,37,38,39]. The experimental results showed that HCV-RNA was detected *via* Real-Time PCR in the blood samples of Jeddah, two (samples No. 03 and No. 06 from King AbdulAziz Hospital). These samples recorded PCR readings of 2,775,621 and 1,766,898, IU/ml, respectively. Results shown in Table 1 reveal that HCV-RNA was detected in tenth of blood samples collected from Riyadh. Where sample No. 09 collected from Prince Mohamed Hospital recorded PCR reading of 41,454 IU/ml. On the other hand, all six blood samples collected from Hafr El-Batten were negative PCR, as no HCV-RNA was detected *via* PCR test. At the level of Taif region, sample No. 04 collected from Blood Bank appeared PCR reading of 729.354 IU/ml, while the other 15 blood samples were negative. One can conclude that, the HCV was successfully diagnosed in only four blood samples collected from three regions (Jeddah, Riyadh and Taif).

It was noted that eight samples out of the 45 blood samples were infected with either HBV or HCV. The three samples which were gave positive ELISA values for HBV were also gave positive PCR readings. On the other hand, four out of the five HCV-ELISA positive samples were confirmed *via* PCR. Results shown in Fig. 2 confirmed that the male was more avaliable to be infected with hepatitis viruses than female. This was clear from the high number of viral infected samples as five and two male and female patients, respectively, proved to be infected with HBV or HCV. Results showed that HBV was not detected in the samples of the patients of 40-48 years. Both of HBV and HCV were equally detected in samples of 20-29 year and 30-39 years, respectively. Only one sample of the age group 40-48 years was infected with HCV.

Genetic variability of *poly* gene of HBV

It is well known that HBV is a incomplete double-stranded DNA virus which replicates in a unique manner that involves reverse transcription [40], its genotypes are geographically distributed [41] and HBV-polymerase region overlaps pre-S/S genes with high epitope density and plays an essential role in viral replication [42]. In the present study, the HBV-DNA of the positive ELISA sample No. 08 from Jeddah was extracted and used as template for PCR isolation of polymerase (*poly*) gene of HBV. Mutations in the catalytic domain of the *poly* gene can affect the amino-acid sequence of the envelope protein (HBsAg) and vice versa [43]. In this study, the partial sequence of *poly* gene of HBV-isolate 08 was 676 nucleotides (Accession number LC101676). Results showed that a high percent identity of 96% was recorded between the nucleotide

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sequence of partial sequence of *poly* gene of HBV-isolate 08 and eight overseas strains of HBV in GenBank (KC792690.1, KC792695.1, KC792962.1, KC792704.1, FJ715407.1, FJ715383.1, FJ715414.1 and FJ715412.1). Phylogenetic tree revealed that the nucleotide sequence of partial sequence of *poly* gene of the HBV-isolate 8 was felled in a cluster contains HBV isolate EP0057 *poly* and HBV isolate EP0043 *poly* gene as shown in Fig. 3.

When the partial sequence of *poly* gene of HBV-isolate 8 (Query) was compared with the eight GenBank, a number of 27 differences were found between the Query (isolate under investigation) and the eight overseas strains of HBV. Open reading frame (ORF) number 1 in reading frame 3 on the reverse strand extends from base 3 to base 674 was obtained, and translated into 224 amino acids. Results shown in Fig. 4 showed presence of genetic variability of 18 amino acids were recorded between the ORF and the eight overseas strains of HBV in GenBank. HBV variants with mutations in the surface and polymerase genes of HBV have now been described following vaccination [44]. The HBV polymerase has a mutation rate of approximately, $<3X10^{-4}$ base substitutions/site/year, which is a rate similar to the retroviral *gag* gene [45, 46,47]. The HBV genome encompasses four overlapping reading frames [48] with the polymerase gene overlapping the envelope gene.

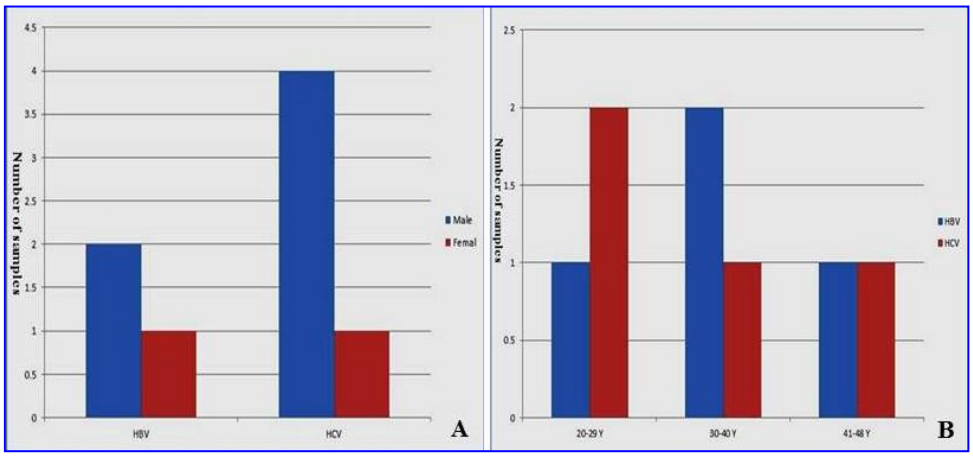

Figure 1: A): HBV and HCV positive blood samples of male and female detected *via* ELISA. B) Correlation between age and HBV & HCV infection based on ELISA in blood samples collected from different regions of KSA.

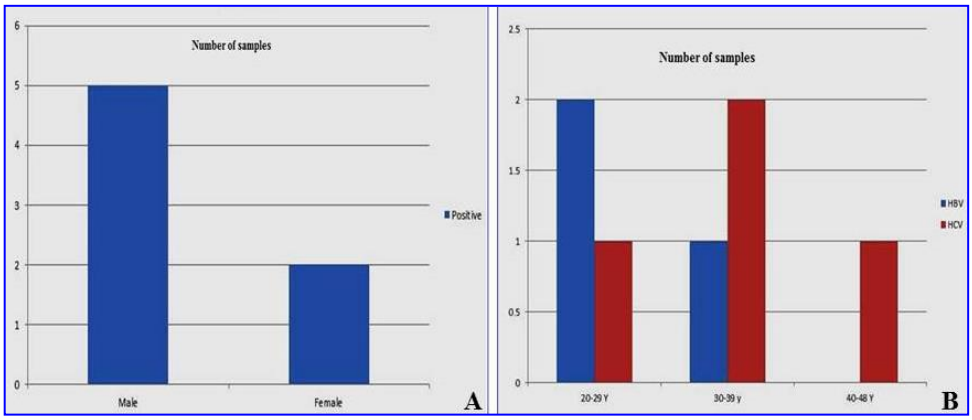

Figure 2: A): HBV and HCV positive blood samples of male and female detected *via* PCR. B): Correlation between age and HBV & HCV infection based on PCR in blood samples collected from different regions of KSA.

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Table 1: Source of blood samples collected from Jeddah, Riyadh, Hafr El-Batten and Taif for detection of the presence of both HBV and HCV.

Serial #	HBV diagnosis				HCV diagnosis			
	ELISA		PCR		ELISA		PCR	
	Reading	Result	Reading	Result	Reading	Result	Reading	Result
01Jed	0.0140	-	DND	-	0.0240	-	RND	-
02Jed	0.0160	-	DND	-	0.0360	-	RND	-
03Jed	0.0130	-	DND	-	**3.0690**	**+**	2,775,621	+
04Jed	0.0130	-	DND	-	0.0210	-	RND	-
05Jed	0.0140	-	DND	-	0.0190	-	RND	-
06Jed	0.0140	-	DND	-	**3.0460**	**+**	1,766,898	+
07Jed	0.0130	-	DND	-	0.0550	-	RND	-
08Jed	**9.0000**	**+**	**358,674**	**+**	0.0360	-	RND	-
09Jed	0.0140	-	DND	-	0.0260	-	RND	-
10Jed	0.0140	-	DND	-	0.0170	-	RND	-
11Jed	0.0140	-	DND	-	0.0320	-	RND	-
12Jed	0.0140	-	DND	-	0.0390	-	RND	-
13Jed	0.0140	-	DND	-	0.0180	-	RND	-
01Riyd	0.0140	-	DND	-	0.0240	-	RND	-
02Riyd	0.0150	-	DND	-	0.0280	-	RND	-
03Riyd	0.0150	-	DND	-	0.0220	-	RND	-
04Riyd	0.0260	-	DND	-	0.0220	-	RND	-
05Riyd	0.0140	-	DND	-	0.0390	-	RND	-
06Riyd	0.0160	-	DND	-	0.0250	-	RND	-
07Riyd	0.0130	-	DND	-	0.0260	-	RND	-
08Riyd	0.0130	-	DND	-	0.0220	-	RND	-
09Riyd	0.0110	-	DND	-	**3.0460**	**+**	41,454	+
10Riyd	0.0130	-	DND	-	0.0120	-	RND	-
01HB	0.0130	-	DND	-	0.0170	-	RND	-
02HB	0.0140	-	DND	-	0.0150	-	RND	-
03HB	0.0190	-	DND	-	0.0166	-	RND	-
04HB	0.0160	-	DND	-	0.0220	-	RND	-
05HB	0.0140	-	DND	-	0.0240	-	RND	-
06HB	0.0140	-	DND	-	0.0210	-	RND	-
01TF	**0.1210**	**+**	**399**	**+**	0.0220	-	RND	-
02TF	0.0150	-	DND	-	0.0170	-	RND	-
03TF	0.0150	-	DND	-	0.0150	-	RND	-
04TF	0.0130	-	DND	-	**3.0690**	**+**	729.354	+
05TF	0.0190	-	DND	-	0.0180	-	RND	-
06TF	0.0140	-	DND	-	0.0550	-	RND	-
07TF	0.0140	-	DND	-	0.0390	-	RND	-
08TF	0.0130	-	DND	-	0.0280	-	RND	-
09TF	**9.0000**	**+**	**4420**	**+**	0.0210	-	RND	-
10TF	0.0170	-	DND	-	0.0220	-	RND	-
11TF	0.0140	-	DND	-	**0.3370**	**+**	RND	-
12TF	0.0140	-	DND	-	0.0190	-	RND	-
13TF	0.0180	-	DND	-	0.0240	-	RND	-
14TF	0.0190	-	DND	-	0.0220	-	RND	-
15TF	0.0160	-	DND	-	0.0260	-	RND	-
16TF	0.0190	-	DND	-	0.0330	-	RND	-

-: Negative. +: Positive. DND: DNA not detected.

Table 2: The percentage of samples that showed positive ELISA and PCR results for HBV and HCV.

Source of samples	Total samples		HBV		HCV	
			ELISA	PCR	ELISA	PCR
Jeddah	No.	13	1	1	2	2
	%		7.7	07.70	15.4	15.39
Riyadh	No.	10	0	0	1	1
	%		0.00	00.00	10	10.00
Hafr El-Batten	No.	6	0	0	0	0
	%		0.00	00.00	0.00	00.00
Taif	No.	16	2	2	2	1
	%		12.5	12.50	12.5	6.25
Total	No.	45	3	3	5	4
	%		6.67	6.67	11.11	8.89

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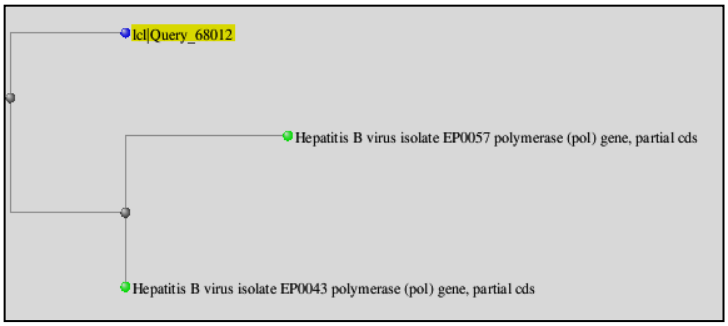

Figure 3: Phylogenetic trees between the nucleotide sequence of partial sequence of polymerase (*poly*) gene of HBV-isolate No. 01 and No. 08 overseas strains of HBV in GenBank (KC792690.1, KC792695.1, KC792962.1, KC792704.1, FJ715407.1, FJ715383.1, FJ715414.1 and FJ715412.1).

```
Query      1     WNAGILYKRETTRSASSCGSPYSWEQELQHGRLVFQTSIRNLDESFCSQSSGIISRSPVG  60
KC792690   338   .K.............F.....................T.HG...........L......  517
KC792695   410   .K.............F.....................T.HG...........L......  589
KC792962   421   .K.............F.....................T.HG...........L......  600
KC792704   396   .K.............F.....................T.HG...........L......  575
FJ715407   1155  .K.............F.....................HG...........L......  1334
FJ715383   1155  .K.............F.....................T.HG...........L......  1334
FJ715414   1155  .K.............F.....................T.HG...........L......  1334
FJ715412   1155  .K.............F.....................T.HG...........L......  1334

Query      61    PCVRSQLKQFRLGLQLQQGSPTRGKSGRSGSFWARVHPTTRRSCGVEPLGSGRIDNSASR  120
KC792690   518   .........S.....P....LA.........IR..........F....S...H......S  697
KC792695   590   .........S.....P....LA.........IR..........F....S...H......S  769
KC792962   601   .........S.....P....LA.........IR..........F....S...H......S  780
KC792704   576   .........S.....P....LA.........IR..........F....S...H......S  755
FJ715407   1335  .........S.....P....LA.........IR..........F....S...H.N....S  1514
FJ715383   1335  .........S.....P....LA.........IR..........F....S...H.N....S  1514
FJ715414   1335  .........S.....P....LA.........IR..........F....S...H......S  1514
FJ715412   1335  .........S.....P....LA.........IR..........F....S...H......S  1514

Query      121   TSSCLRQSAVRKTAYSHLSTSKRQSSSGHAVELHNIPPSYARSQSEGPIFSCWWLQFRNS  180
KC792690   698   .....H.................................S.....................  877
KC792695   770   .....H.................................S.....................  949
KC792962   781   .....H.................................S.....................  960
KC792704   756   .....H.................................S.....................  935
FJ715407   1515  .....H.................................S.....................  1694
FJ715383   1515  .....H.................................S.....................  1694
FJ715414   1515  .....H.................................S.....................  1694
FJ715412   1515  .....H.................................S.....................  1694

Query      181   KPCSDYCLTHIVNLLEDWGPCTEHGEHNIRIPRTPARVTGGVFL  224
KC792690   878   ..............................................  1009
KC792695   950   ..............................................  1081
KC792962   961   ..............................................  1092
KC792704   936   ..............................................  1067
FJ715407   1695  ..............................................  1826
FJ715383   1695  ..............................................  1826
FJ715414   1695  ..............................................  1826
FJ715412   1695  ..............................................  1826
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Figure 4: Genetic variability between the ORF number 1 in reading frame 3 on the reverse strand extends from base 3 to base 674 of HBV-isolate No. 08 (Query) and 8 overseas strains of HBV in GenBank (KC792690.1, KC792695.1, KC792962.1, KC792704.1, FJ715407.1, FJ715383.1, FJ715414.1 and FJ715412.1).

CONCLUSION AND RECOMMENDATIONS

Both of ELISA and Real Time-PCR were successfully used for detecting the presence of HBV and HCV in 45 blood samples collected from different regions: Jeddah, Riyadh, Hafer El-Baten and Taif. Variation between the nucleotide sequences of *poly* gene (Accession number LC101676 .1) of HBV and those similar in GenBank was determined at the level of nucleotides and open reading frames.

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