



## Original Research Article

# Serological and molecular detection of hepatitis B viral antibodies and its surface antigen in blood samples of different age stages at Taif Governorate, KSA

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## ABSTRACT

### Keywords

Detection;  
Hepatitis B  
virus (HBV);  
ELISA;  
HBsAb;  
HBcAb;  
HBsAg;  
PCR.

Hepatitis B virus (HBV) infection is a major public health problem in the Middle East. This study was designed to evaluate the immune response to vaccination with the recombinant Hepatitis B virus surface antigen (HBsAg) after day(s), month(s) and year(s) of vaccine administration of different age groups of the general population of Taif Governorate, KSA either before or after the vaccine became compulsory in 1991 (1410H). For the valid tested blood samples (1056), enzyme-linked immune sorbent assay (ELISA) was used to determine the level of HBsAb, HBcAb and HBsAg in different age groups of the general population who had been previously vaccinated against HBV (since year 1410H) or non-vaccinated. Results showed that the 1056 samples were divided into 6 groups, as follows: Group **A** means that the persons are healthy due to lacking of immunity against the HBV. Group **B**, it has several explanations, *.i.e.*, i) may be recovery from acute HBV-infection and did not reach antibodies to a level that can be detected, ii) may be distantly immune and test not sensitive enough to detect very low level of anti-HBs in serum, iii) may be undetectable level of HBsAg present in the serum and person is actually a carrier and iv) may be susceptible with a false positive anti-HBc. Group **C** could be explained as acutely infected (IgM anti-HBc +ve) or chronically infected (IgM anti-HBc -ve). In case of group **D**, the persons are immune due to hepatitis B vaccination. On the other hand, persons of group **E** are immune due to natural infection. While, persons of group **F** are chronically infected. It was shown that HBsAg positive cases were also positive for viral DNA when detected by Real-Time PCR. The nucleotide sequence of HBsAg gene was partially determined using the DNA template of a HBV positive blood sample. Results showed the nucleotide sequences of the forward direction (892 nts) and compared with 5 universal viral isolates and suggested to be named Hepatitis B virus isolate AAM2012 S protein gene.

## Introduction

Hepatitis B virus (HBV) is the most important member of the taxonomic family *Hepadnaviridae* that causes

hepatitis B, liver cancer, and liver cirrhosis. Despite the progress made over the past 3 decades through vaccination,

HBV remains to be a major challenge and a constant threat in the field of public health; current estimates suggest that there are more than 350 million hepatitis B carriers worldwide (Zhu *et al.* 2014). HBV infection is a major public health problem in the Middle East. The majority of the countries in the region have intermediate (2 to <5%) or high (>5%) endemicity of HBV infection (Al Faleh *et al.* 1992).

Studies in Saudi Arabia showed prevalence of Hepatitis B surface antigen (HBsAg) that ranges from 7.4 to 17% denoting high endemicity (Beasley and Hwang 1993). The HBV is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It ranks as an important pathogen throughout the world (Fung and Lok 2004).

Hepatitis B immunization in the postpartum period is feasible and effective (Ljunggren *et al.* 2002). The availability of a safe and effective hepatitis vaccine encourages us to accelerate viral elimination, and additional intervention such as Hepatitis B immunization in postpartum women can thereon be undertaken. Thus, Hepatitis B immunization can be recommended, giving the first dose immediately on the first postpartum day before the mother gets discharged from the hospital; and the second dose to coincide with her child's first vaccination dose at the age of 2 months, and a third dose to be given to the mother when her child gets vaccinated at the age 6 months. However, further studies to assess the feasibility as well as effectiveness of such a program are necessary (Ocama *et al.* 2005).

Information on the epidemiology of viral hepatitis B, C and A in the Kingdom of Saudi Arabia (KSA) has accumulated over the last two decades. The authors

reviewed the changing epidemiology of these infections and suggest possible strategies for eradication. They screened Saudi medical journals and Medline for reports dealing with hepatitis B, C and A, and analyzed official of blood donor screening data from the Ministry of Health (MOH) Central Blood Bank and the King Khalid University Hospital (KKUH) Blood Bank (Al-Faleh 2003).

This study aimed at evaluating the immune response to vaccination with the recombinant Hepatitis B virus surface Antigen (HBsAg) after vaccine administration of different age groups of the general population of Taif Governorate, KSA either before or after the vaccine became compulsory in 1991 (1410H).

## **Materials and Methods**

This study was done in 2011, 21 years after the start of the vaccination program in Saudi Arabia. Laboratory testing was conducted from October 2011 to January 2012 at the Serology and Molecular Virology Laboratory, Regional Laboratory of Taif, KSA, and the Research Center, King Faisal Specialist Hospital, Riyadh, KSA.

### **Collection of blood samples**

On obtaining the legal guardian consent, a number of 4213 blood samples were collected from five different locations at Taif, KSA, These locations were: Kids Hospital, Common Education School, Al-Odwani Hospital, Healthy Marital Program, and Central Blood Bank. This number was distributed as 3500, 101, 25, 221, 366, respectively. A total of 1056 serum samples from different age groups, one day post birth till 31 years, were found

to be valid for serological examination, respectively, by ELISA for detection of HBV antibodies. Distribution of study samples according to gender was comprised of 757 males and 299 females. Study samples were also divided according to HBV vaccine administration into 736 samples from the Compulsory vaccination age group and 320 samples from the non-compulsory vaccination age group.

### **Serological testing for HBV**

BIO ELISA (BIO-RAD, la-Coquette, France) kits were used to assess HBsAb, and HBcAb in collected samples.

### **ELISA detection of HBsAb**

Monolisa™ Anti-HBs PLUS from Bio-Rad company is an enzyme immunoassay (EIA) used for the detection and level determination of antibody to hepatitis-B-surface antigen (anti-HBs) in human serum or plasma.

### **ELISA detection of HBcAb**

The Monolisa™ Anti-HBc PLUS kit was used for detection of antibodies specific to nucleocapsid antigen (core) of the HBV in human serum or plasma by enzyme immunoassay. The protocol was followed Strictly. Negative and positive control sera were used for each test, in order to validate the test quality. Samples with an optical density less than the cut-off value were considered to be negative with the Monolisa™ Anti-HBc PLUS test. Samples with an optical density higher than, or equal to, the cut-off value were considered to be initially positive with the Monolisa™ Anti-HBc PLUS test and were retested in duplicate before the final interpretation.

### **ELISA detection of HBsAg**

The Monolisa™ HBs Ag ULTRA kit from Bio-Rad company was used for the detection of the surface antigen of the hepatitis B in human serum or plasma by the enzyme immunoassay technique.

### **HBV-DNA by PCR**

The **cobas**® TaqScreen MPX Test was a qualitative multiplex test that enables the screening and simultaneous detection of HIV-1 Groups M and O RNA, HIV-2 RNA, HCV RNA and HBV DNA in infected pooled and individual plasma specimen donations. The **cobas**® TaqScreen MPX Test used a generic nucleic acid preparation technique on the COBAS® Ampliprep Instrument. HIV-1 Groups M and O RNA, HIV-2 RNA, HCV RNA and HBV DNA were amplified and detected using automated, real time PCR on the COBAS® TaqMan® Analyzer. The test incorporated an Internal Control for monitoring test performance in each individual test as well as the AmpErase enzyme to reduce potential contamination by previously amplified material (amplicon). The **cobas**® TaqScreen MPX Test didn't discriminate which virus was detected in a specimen. COBAS® AmpliScreen HIV-1 Test, v1.5, COBAS® AmpliScreen HCV Test, v2.0 and COBAS® AmpliScreen HBV Test were available for the discriminatory testing of HIV-1 Group M, HCV and HBV. Discriminatory tested for HIV-1 Group O and HIV-2 were not available from Roche.

### **Partial sequencing of HBsAg gene**

QIAamp MinElute Virus Spin Kit (Catalog # 57704) was used for extraction and purification of viral DNA from serum of the selected blood sample.

Two primers specific to HBsAg (F: 5'TTC CTG CTG GTG GCT CCA G3' and R: 5'CCA ATA CAT ATC CCA TGA ACT3') were used. The reaction was conducted in a total volume of 25 µl consisted of 12.5 µl GoTaq Green Master Mix, 2X; 1.25 µl Sense Primer, 20 µM; 1.25 µl Antisense Primer, 20 µM; 2.50 µl Template and 7.50 µl Nuclease-Free water. The PCR program was 1 cycle at 95°C for 5 min, 35 cycles each of 95, 55, 72°C for 1 min for each degree and 1 cycle at 72°C for 10 min. The PCR product was visualized by gel electrophoresis as mentioned by Sambrook et al. (1989). 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. Maximum identity was taken as the genotype of the sample analyzed.

## Results and Discussion

In Saudi Arabia, viral hepatitis ranked the second most common reportable viral disease in 2007, with almost 9000 new cases diagnosed in that year. Between 2000 and 2007, a total of 14224 seropositive cases of viral hepatitis were reported to the surveillance system. The average annual incidence of seropositivity per 100000 served population was highest for HBV (104.6), followed by HCV (78.4), and lowest for HAV (13.6). Over the eight years the incidence of all three

viral hepatitis types showed a 20-30% declining trend. Despite the declining trend over the eight-year period, viral hepatitis, especially that caused by HBV and HCV, remains a major public health problem in Saudi Arabia, and had probably been underestimated in previous reports (Memish *et al.* 2009). Hepatitis B reactivation in patients infected with human immunodeficiency virus (HIV) who were positive for hepatitis B surface antigen (HBsAg) has been reported (Waite et al. 1988 and Laura et al. 2014).

## Collected blood samples and their validities

Samples were collected from 5 different locations in Taif Governorate. Data in Table (1) showed that a number of 4213 blood samples were collected from five different locations at Taif, KSA. This number was distributed as 3500, 101, 25, 221, 366, from Kids Hospital, Common Education School, Al-Odwani Hospital, Healthy Marriage Program and Central Blood Bank, respectively. In other mean, all samples of Central Blood Bank were valid for examination by ELISA followed by Common Education School, Program of Health Marriage and Al-Odwani Hospital. On the contrary, only 438 blood samples out of 3500 collected from Kids Hospitals were valid, which was attributed to the low volume of blood collected from infants and children. The average percentage of valid blood samples, from one day up to 31 years, was 25.07%. Based on the gender, numbers of 757 and 299 representing 71.7 % and 28.3% were belonging to male and female, respectively (Figure 1A). Regarding the HBV vaccine administration, data showed that 736 (69.7%) and 320 (30.3%) of the valid tested blood samples were belonging to compulsory and non-compulsory

vaccination age groups, respectively (Figure 1B).

### **ELISA detection of HBsAb**

On ELISA detection of HBsAb in the valid blood tested samples, results showed that out of the total 1056 tested individuals, 51.8% were immune while 48.2% were non-immune to HBV (Figure 2). The ELISA detection of HBsAb in the vaccinated and non-vaccinated individuals showed that in vaccinated individuals, 396 cases were immune to HBV comprising 54% of the total 736 vaccinated cases tested while 340 (46%) cases were non-immune (Figure 3A). While, in non-vaccinated individuals the number of immune cases was 151 (47.2%) cases out of 320 total samples tested (Figure 3B). The immune and non-immune cases in all tested blood samples were defined. Data in Figure (4) realized that there is not much significant difference between the immune status of vaccinated and non vaccinated individuals. This is attributed to the fact that the total number of tested cases was distributed between those born before and after year 1991.

### **ELISA detection of HBcAb**

In this study, the rate of effective protection of the administered vaccine against HBV was tested, and all blood samples were subjected to ELISA detection of the presence of HBcAb, which if present denotes previous or current infection with the virus. Results showed that 41 (3.9%) blood samples were positive HBcAb cases out of the 1056 samples tested (Figure 5A). Among the vaccinated individuals who were tested for HBcAb there was 28 positive cases out of 736 (Figure 5B), comprised 3.8% of the total tested samples. In case of non-

vaccinated individuals, 13 HBcAb positive samples were discovered among 320 samples tested, represents 4.1% (Figure 5C).

These data indicated that vaccinated individuals were better protected against infection with HBV, where the percentage of HBcAb positive cases in non-vaccinated individuals was higher (4.1%) than of vaccinated individuals which was 3.8%. However, when these data were compared to the total number of tested individuals (1056) whether vaccinated or non-vaccinated, it was found that HBcAb was more prevalent in vaccinated individuals (2.7%) while in non-vaccinated individuals it was 1.2% (Figure 6). This could be due to higher number of tested vaccinated individuals.

### **ELISA detection of HBsAg**

In order to determine whether those individuals who were having antibodies against HB core antigen (+ve-HBcAb) were truly and currently infected with HBV or not, the presence of HBsAg - which if present denotes active infection - was detected. Results showed that only 8 (0.8%) samples were positive for HBsAg among 1048 samples (Figure 7A). Among 736 vaccinated individuals there were 6 positive cases for HBsAg (0.8%) as shown in Figure (7B). In non-vaccinated individuals the numbers of positive cases was only 2 (0.6%) (Figure 7C). The number of positive and negative cases in vaccinated and non-vaccinated individuals were compared. Results in Figure (8) showed that the number of positive cases for HBsAg was higher in vaccinated individuals than that in the non-vaccinated. This is attributed to the smaller number of non-vaccinated cases compared to vaccinated cases.

### **PCR confirmation of HBsAg**

To check for the presence of hepatitis B viral DNA in HBsAg positive cases in order to confirm infection with HBV, PCR detection was performed using the DNA of the 8 positive HBsAg cases (Figure 9) as a template. Results showed that 6 (75%) out of the 8 cases had invalid results due to insufficient samples. The remaining 2 (25%) samples were found to be positive for HBV-DNA. Therefore, it was shown that HBsAg positive cases were also positive for viral DNA when detected by PCR (Figure 10).

### **Relationship between HBsAb concentration and susceptibility to HBV infection**

Successful immunity to HBV infection depends on age: in most cases, the infection of adults results in viral clearance — which depends on a potent, diverse adaptive immune response — but in infants the virus usually persists (Publicover *et al.* 2013). In this study, the immune response to HBV vaccine were analyzed and correlated the positive response to progression of age after vaccine administration. Results revealed that the best immunity to the virus was achieved in the early years of age at 1 year and then gradually declines until 17 years of age where non-immune individuals become more than immune individuals (Figure 11).

### **Immune response in all age groups of immune and non-immune cases**

Interestingly, in the age group between 24–28 years of age, the immune individuals became more than non-immune and this could be due to having the vaccine at some time before the sample

was taken for this age group. The range of HBs antibodies was determined in all age groups in immune and non-immune cases (Figure 12).

Results showed that the range of antibodies was fluctuating, being at its highest between age groups 6 months-3 years, 6-8 years, 12 years, 20 years, and 24-31 years. This could be attributed to the quality of vaccine that had been administered to these age groups as some lots and batches could be more efficient than others. The same pattern for the average amount of antibodies was noticed for immune cases of different age groups (Figure 13).

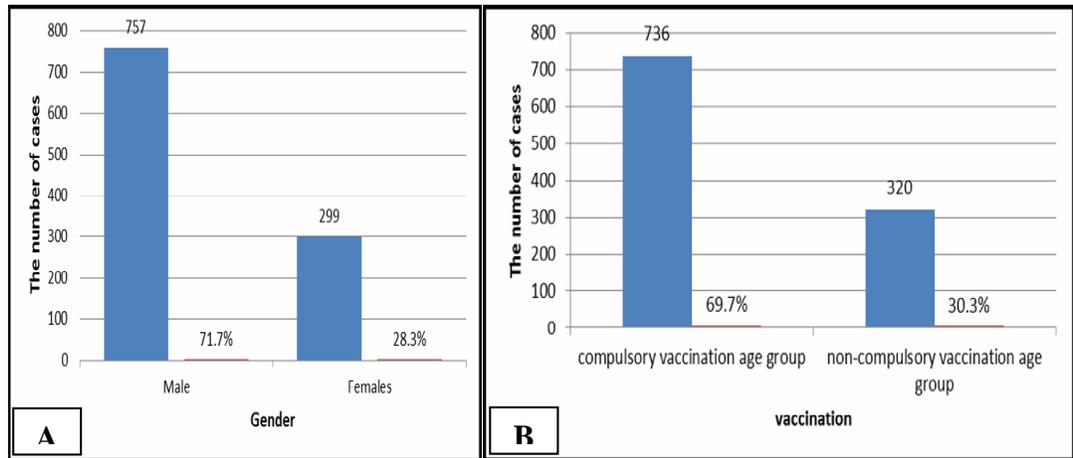
As overall, the immune status of all tested blood samples from the vaccinated age groups (736) showed a higher response to vaccine administration with 396 positive cases for HBsAb compared to 340 negative cases for HBsAb (Figure 14).

Results in Table (2) summarized the ELISA detection of HBV antibodies (HBsAb & HBcAb) in valid tested blood samples. Group A means that the persons are healthy due to lacking of immunity against the HBV. Group B It has several explanations, .i.e., i) may be recovery from acute HBV-infection and did not reach antibodies to a level that can be detected, ii) may be distantly immune and test not sensitive enough to detect very low level of anti-HBs in serum, iii) may be undetectable level of HbsAg present in the serum and person is actually a carrier and iv) may be susceptible with a false positive anti-HBc. Group C could be explained as acutely infected (Igm anti-HBc +ve) or chronically infected (Igm anti-HBc -ve). In case of group D, the persons are immune due to hepatitis B

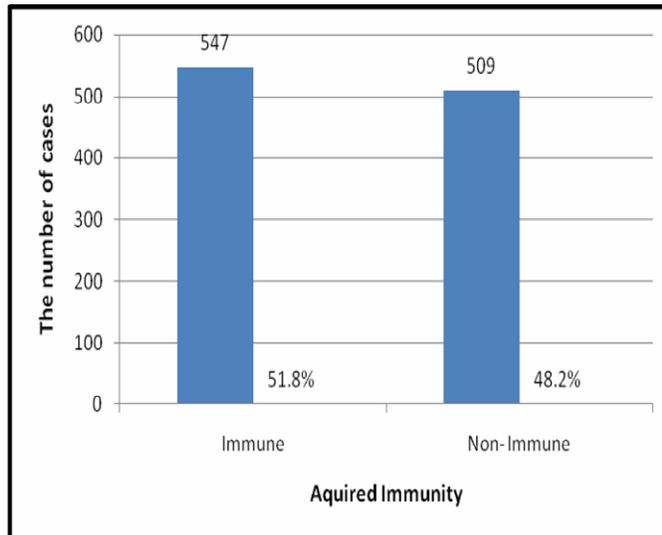
**Table.1** Total collected blood samples (TCBS) from different locations at Taif Governorate (KSA)

Source of blood samples	Age ranges	TCBS	Valid samples		Invalid samples	
			No.	%	No.	%
Kids Hospital	1day-12year	3500	438	12.51	3062	87.49
Common Education School	12-18 year	101	91	90.10	10	9.90
Al-Odwani Hospital	12-18 year	25	13	52.00	12	48.00
Healthy Marriage Program	14-19 year	221	148	66.97	73	33.03
Central Blood Bank	17-31 year	366	366	100.00	00	0.00
Total		4213	1056	25.07	3157	74.93

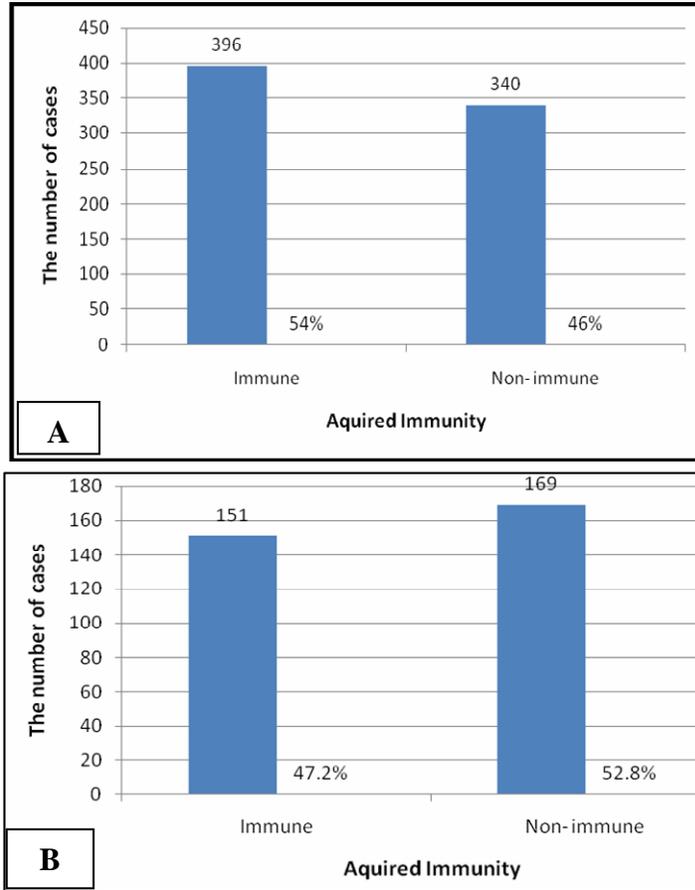
**Figure.1** Distribution of study samples according to gender (A) and HBV vaccine administration (B)



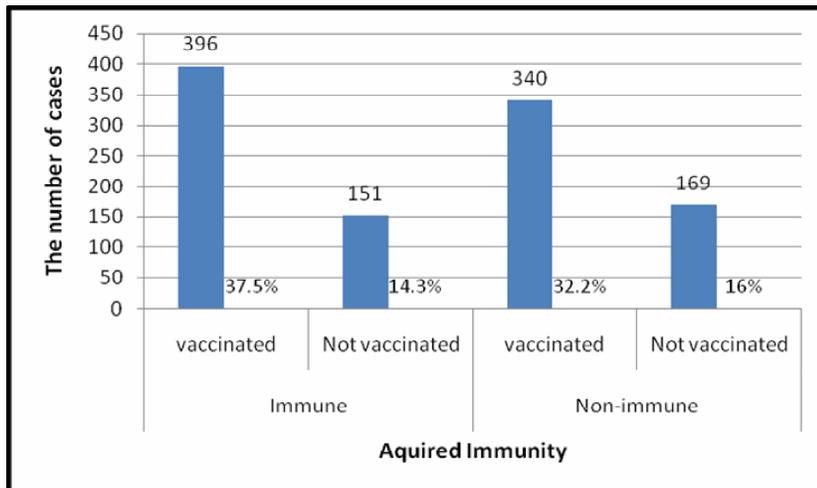
**Figure.2** ELISA detection of HBsAb in all collected blood samples



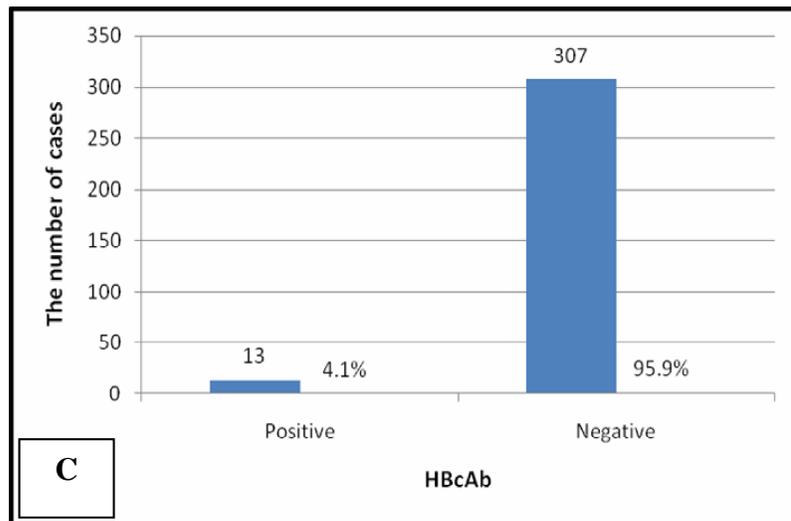
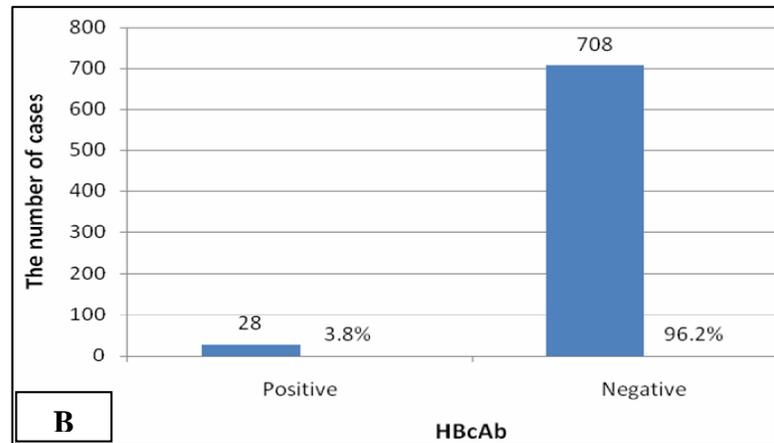
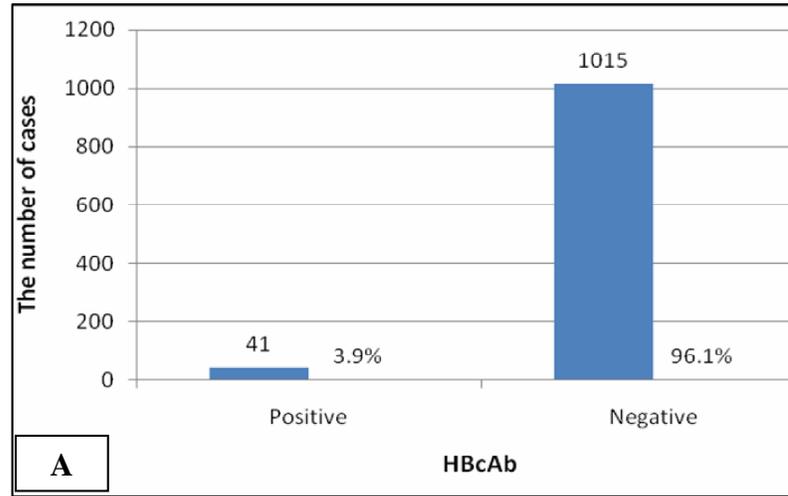
**Figure.3** ELISA detection of HBsAb in vaccinated individuals (A) and non-vaccinated individuals (B)



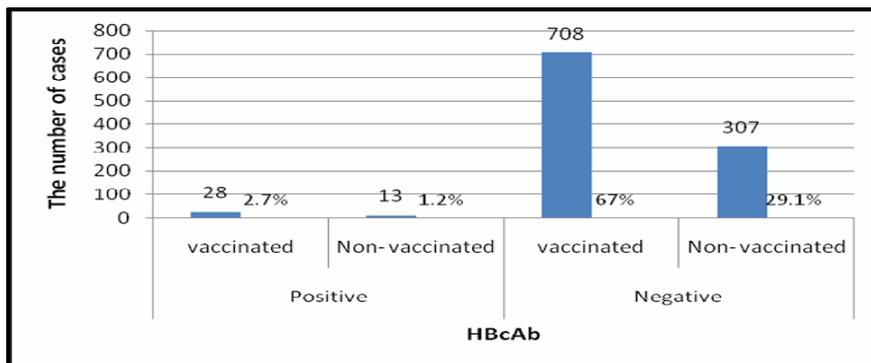
**Figure.4** Comparison between immune and non-immune tested individuals



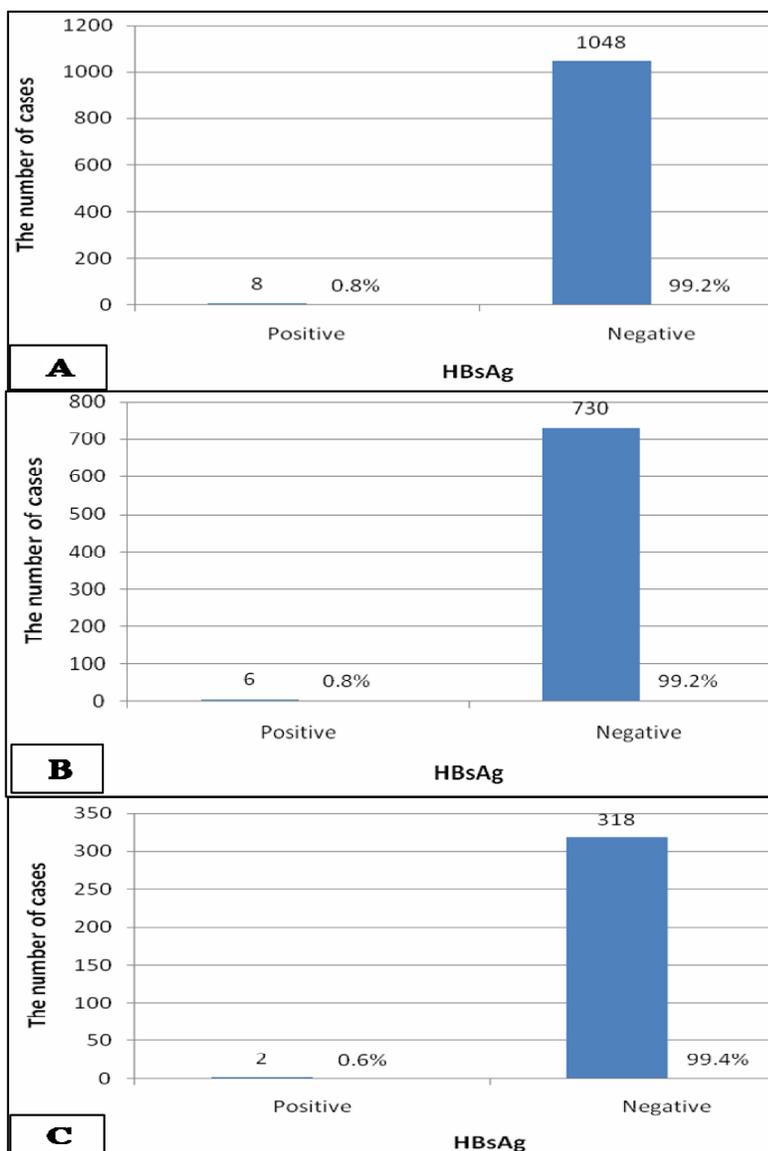
**Figure.5** ELISA detection of HBcAb in all tested blood samples (A), vaccinated individuals (B) and non-vaccinated individuals (C)



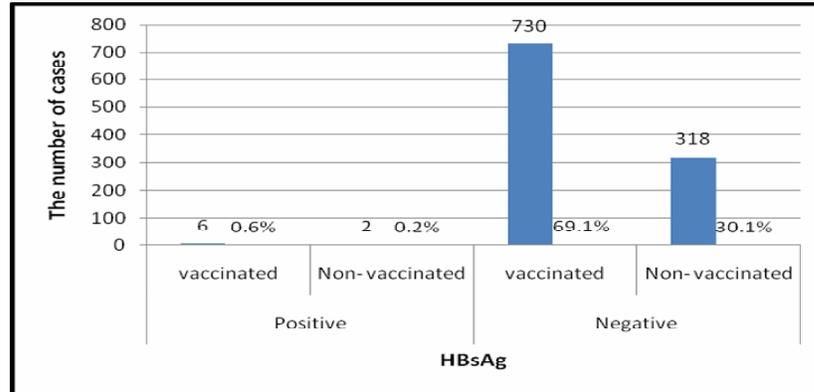
**Figure.6** Comparison between the +ve and -ve ELISA samples detected for the presence of HBcAb.



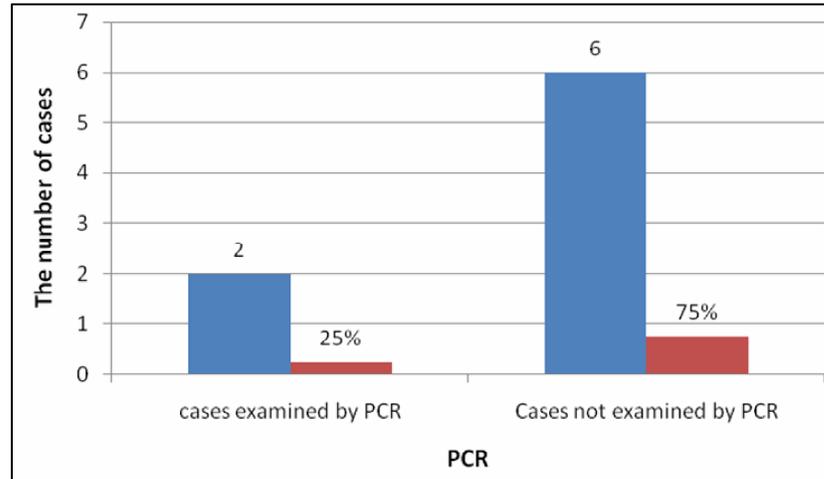
**Figure.7** ELISA detection of HBsAg in all tested blood samples (A), among vaccinated individuals (B) and non-vaccinated individuals (C)



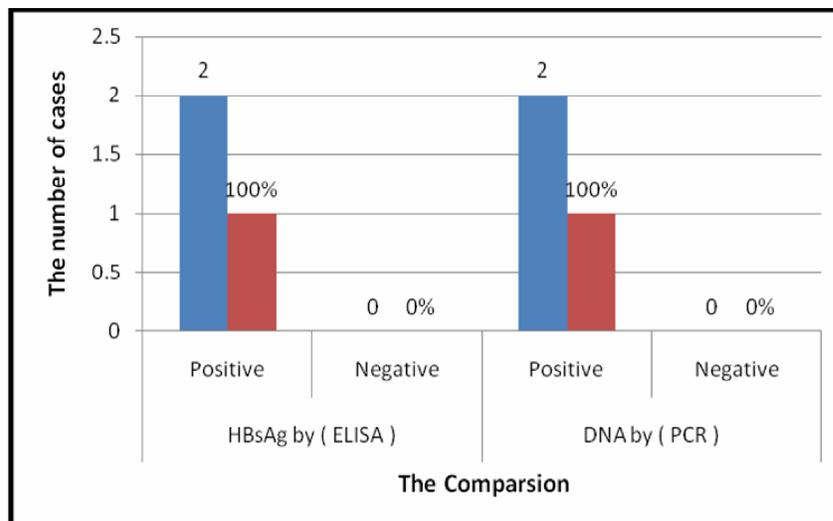
**Figure.8** Comparison between the +ve and -ve ELISA samples detected for the presence of HBsAg.



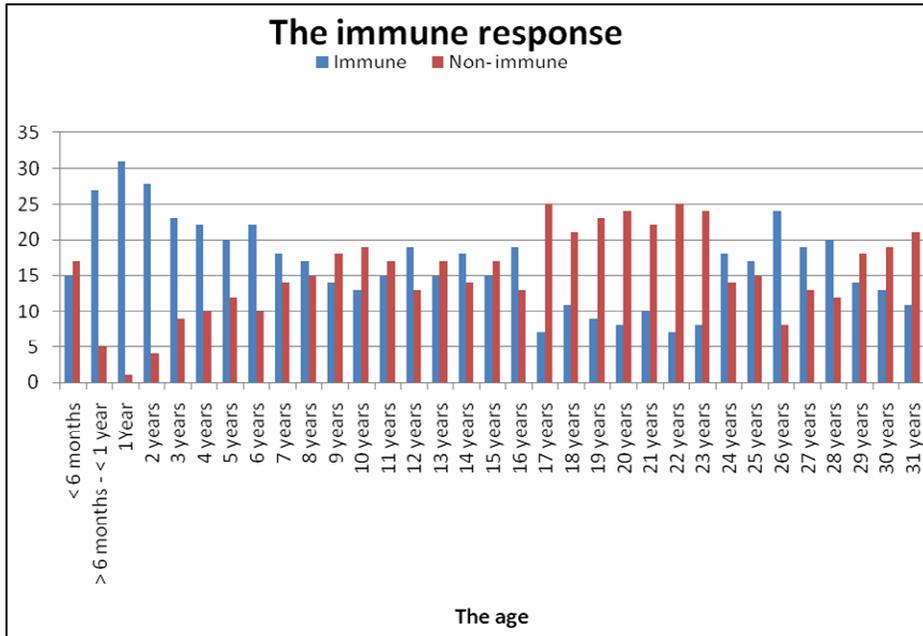
**Figure.9** Real Time PCR detection of HBV in the DNA extracted from ELISA +ve-HBsAg samples



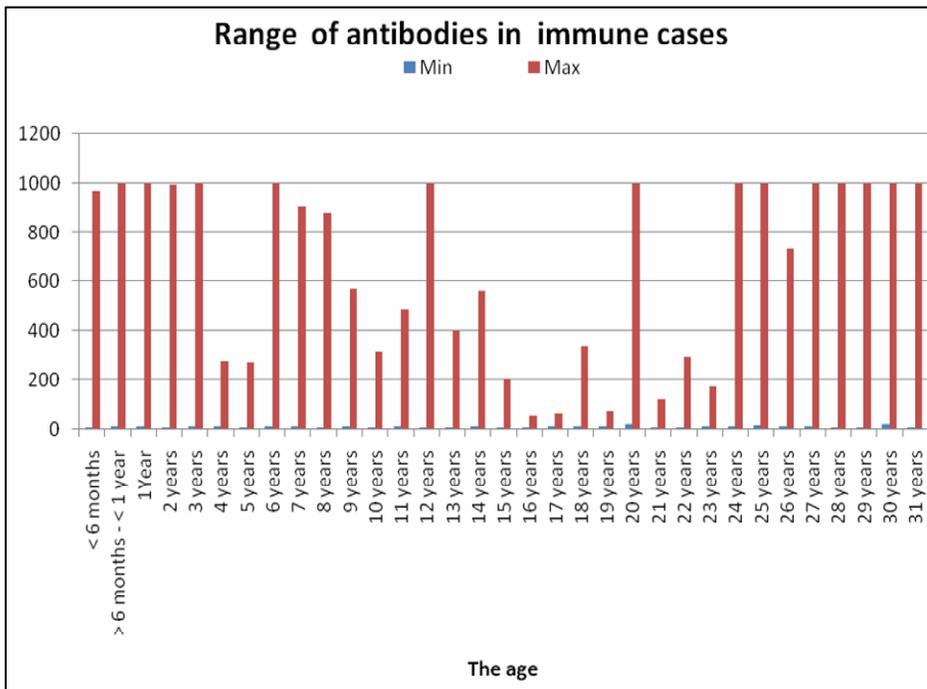
**Figure.10** Correlation between HBsAg detected by ELISA and PCR



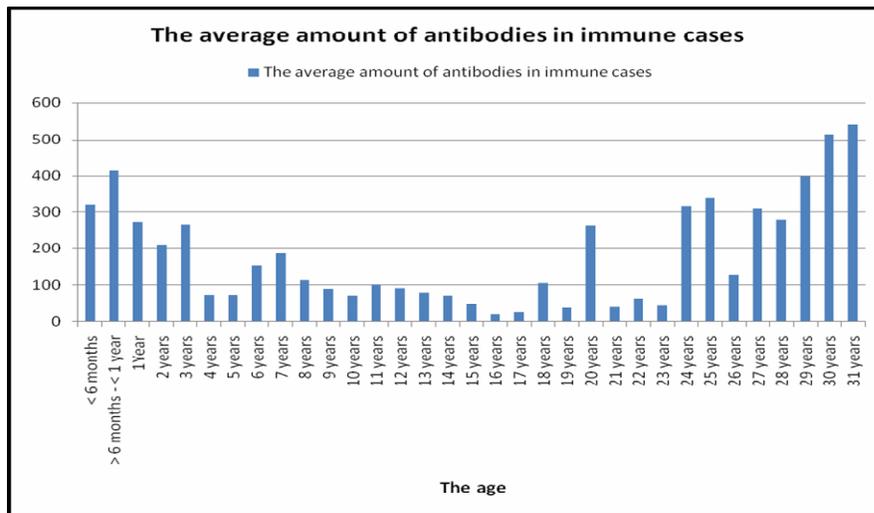
**Figure.11** No. of positive blood samples collected from immune and non-immune cases of all age groups



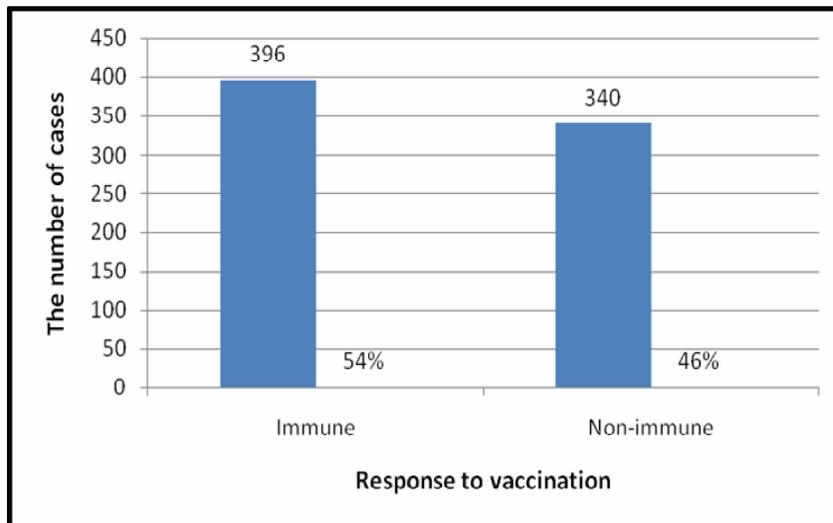
**Figure.12** Range of antibodies in immune cases based on ELISA results



**Figure.13** Average amount of antibodies in immune cases



**Figure.14** Immune status of vaccinated individuals.



**Table.2** Conclusion of ELISA detection of HBV antibodies and antigen in collected blood samples and its interpretation.

Groups	Tests			No. of samples
	HBsAb	HbcAb	HbsAg	
A	-	-	-	496
B	-	+	-	10
C	-	+	+	3
D	+	-	-	519
E	+	+	-	23
F	+	+	+	5
Total				1056

-: Negative.

+: Positive.

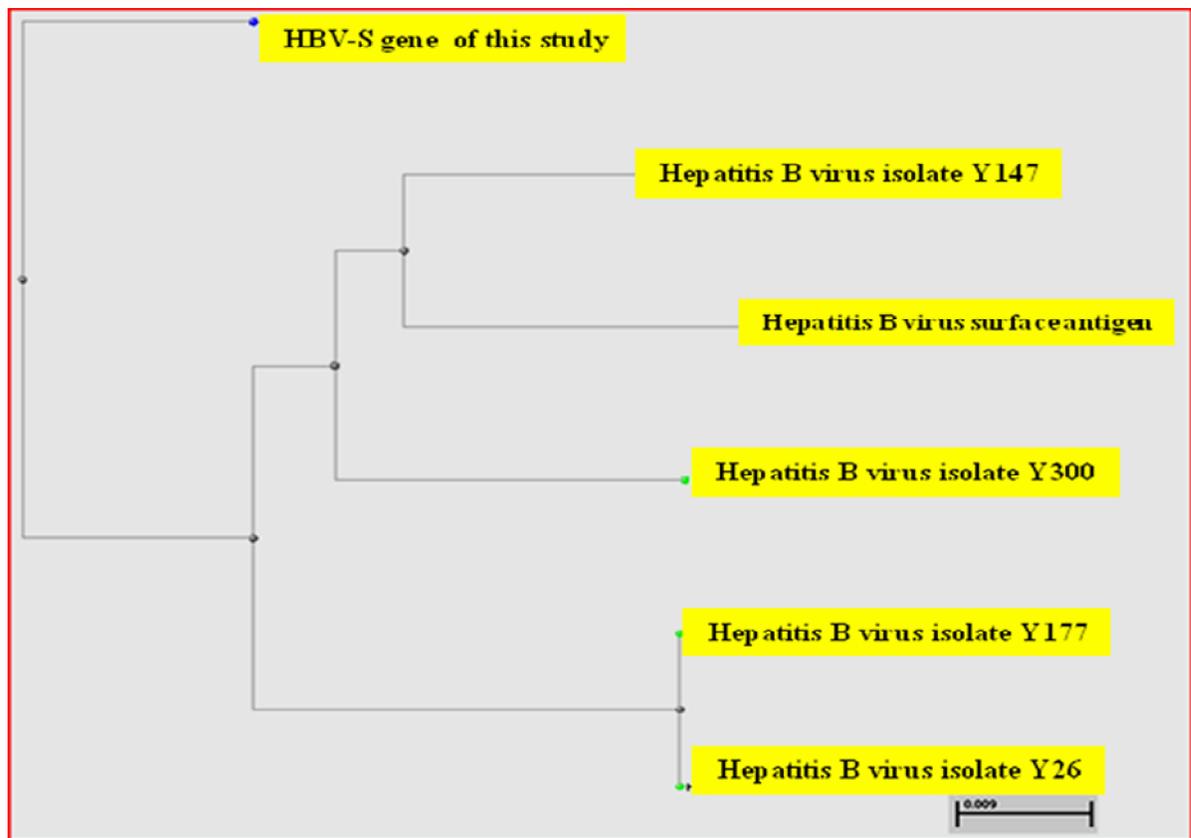
**Figure.15** Forward (892 nts) nucleotide sequence of the PCR product of HBV-S protein gene of HBV amplified from the DNA of a HBV-diseased blood sample

ATGGGGGACCGGGAACCTGCTCTGATACTGCCTCTCCCTTATCGTCATCT  
 TCACGAGGATTGTGGACCCTGCGCTGATCATGGAAAACATCACATCAGGA  
 TTCCTAGGACCCCTTCTCGTGTTACAGGCGGTGGTTTTTCTTGTTGACAA  
 TAATCCTCACTATAACGCATAGTCTAGACTCGTGGTGGACTTCTCTCAAT  
 TTTCTAGGGGAACTACCGTGTGTCTTGGCCAAAATTCGCAGTCCCAAC  
 CTCCAATCACTACCAACCTCTTGTCTCCAACCTTGTCTGGTTATCGCT  
 GGATGTGTCTGCGGCGTTTTATCATCTTCTTCTTTCATCCTGCTGCTATGC  
 CTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTG  
 TCCTCTAATTCAGGATCCTCAACAACCAGCACGGGACCATGCCGGACCT  
 GCATGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACC  
 AAACCTTCGGACGGAAATTGCACCTGTATTCCCATCCCATCATCCTGGGC  
 TTTTCGAAAATTCCTATGGGAGTGGGCCTCAGCCGTTTCTCCTGGCTCA  
 GTTTACTAGTGCCATTTGTTTCAGTGGTTCGTAGGGCTTTCCCCACTGTT  
 TGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAG  
 CATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGG  
 TATACATTTAAACCCTAACAAAACAAAGAGATGGGGTTACTCTCTAAAGT  
 TCATGGAAAATGGTATGAGGAGTTAAAA

**Table.3** Sequences producing significant alignments of HBV-S gene of this study and 5 overseas HBV isolates

<b>Accession</b>	<b>Description</b>	<b><u>Query coverage</u></b> <b>(%)</b>	<b><u>Max identity (%)</u></b>
<a href="#">JX292757.1</a>	HBV isolate Y177 small S protein gene, complete cds	82	94
<a href="#">JX292753.1</a>	HBV isolate Y26 small S protein gene, complete cds	82	94
<a href="#">JX292751.1</a>	HBV isolate Y300 small S protein gene, complete cds	82	94
<a href="#">JX292749.1</a>	HBV isolate Y147 small S protein gene, complete cds	82	95
<a href="#">J02205.1</a>	HBV surface antigen gene, complete cds	95	93

**Figure.16** Phylogenetic tree of nucleotide sequence of the PCR product of HBV-S gene of amplified from a HBV-diseased blood sample and the 5 universal HBV-S isolates



vaccination. On the other hand, persons of group **E** are immune due to natural infection. While, persons of group **F** are chronically infected.

#### Partial sequencing of HBsAg gene

In this study, the nucleotide sequences of HbsAg gene was PCR-partially determined using the DNA template of a HBV-diseased blood sample. The nucleotide sequences of the forward direction (892 nts) (Figure 15) were obtained and compared with 5 universal viral isolates ([JX292757.1](#); [JX292753.1](#); [JX292751.1](#); [JX292749.1](#) and [J02205.1](#)). The genotype of HBV was studied by determination of some HBV genes by

some investigators (Nurainy *et al.* 2008; Zhang *et al.* 2008; Li *et al.* 2010; Li *et al.* 2011 and Farazmandfar *et al.* 2012).

Results in Table (3) showed that the percent identities between the isolate of this study and the compared viral strains ranged from 94% to 95% and covered about 82% of the total score. Our isolate (Query) showed highest percentage of coverage (95%) with the Hepatitis B virus surface antigen gene, complete cds (J02205.1). On the contrary, the phylogenetic tree in Figure (16) showed the viral isolate of this study lied in a separate cluster, therefore, we suggest that it be named Hepatitis B virus isolate AAM2012 S protein gene.

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