

The status of humoral immunity in occult HBV infection in south-eastern Iranian patients

Mohammad Kazemi Arababadi^{1*}, Ali Akbar Pourfathollah³, Abdollah Jafarzadeh^{1, 2}, Gholamhossein Hassanshahi^{1, 2}, Ali Shamizadeh⁴, Behzad Nasiri Ahmadabadi², Derek Kennedy⁵

1. Dept. of Microbiology, Hematology and Immunology, Faculty of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan-Iran.
2. Molecular-Medicine research center, Rafsanjan University of Medical Sciences, Rafsanjan-Iran
3. Dept. of Immunology, School of Medical Sciences, Tarbiat Modares University, Tehran-Iran.
4. Dept. of Physiology, Faculty of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan-Iran.
5. School of Biomolecular and Physical Sciences, Eskitis Institute for Cell and Molecular Therapies, Griffith University Nathan, Queensland-Australia.

*Corresponding author:

Dr. Mohammad Kazemi Arababadi, Dept of Microbiology and Immunology
Faculty of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan-Iran

E-mail: kazemi24@yahoo.com

Tel: 00983915234003-5

Mobile: 0989132926113

Fax: 00983915225209

Running title: Humoral immunity in occult HBV infection

Abstract

Background: Occult hepatitis B infection (OBI) is characterized as a form of hepatitis in which, despite of absence of detectable HBsAg, HBV-DNA is present in patient's peripheral blood. The aim of this study was to investigate components of humoral immunity during OBI as a possible measure of how patients respond to Hepatitis B viral infections.

Material and methods: In this study, HBsAg-/anti-HBc+/HBV-DNA+ samples were assigned as OBI cases and SRID techniques were performed to measure levels of circulating antibodies (IgG, IgM and IgA) as well as C3, C4. In addition, complement system function was assessed by CH50.

Results: Our results showed that the serum levels of IgG and C4 were significantly lower in OBI patients, while IgM and C3 were higher in patients when compared to healthy controls. Serum levels of IgA and CH50 were not significantly different between OBI patients and controls.

Discussion: Based on these results, it could be concluded that although OBI patients produced elevated levels of IgM there may be a problem converting and progressing this response to generate enough IgG to overcome HBV infection.

Key words: Occult hepatitis B infection, Humoral immunity, HBsAg, HBV-DNA.

Introduction

Occult HBV infection (OBI) is characterized as a clinical form of hepatitis B in which, despite the absence of detectable hepatitis B surface antigen (HBsAg) in the serum of patients, hepatitis B viral DNA (HBV-DNA) is

present in the serum and liver [1-3]. This type of hepatitis is a huge problem for blood transfusion services worldwide because despite all donated blood being screened for HBsAg, some cases of post-transfusion hepatitis (PTH) have been reported [4]. As a result of these incidences, we recently reported OBI in Isfahan and Kerman, the two populated central provinces of Iran [5, 6]. The mechanisms responsible for the progression of OBI are yet to be clarified but some investigators blame the involvement of several factors for progression of OBI [7-9]. For instance low levels of HBV-DNA load may lead to reduced production of HBsAg which remain below detectable levels [10]. Mutations in the pre-S/S genome region of HBV also results in reduced production of HBsAg [10]. Apart from DNA load and mutations within the HBV genome there are other mechanisms that the virus may utilize to evade an immune response, these include but are not limited to, infection of host immune cells, such as peripheral mononuclear cells, integration into the host genome, formation of HBV-containing immune complexes and modulating the host immune response directly [4]. It would appear that host infection by other viruses may also influence that ability of the patient to clear HBV infection. [4]. Regardless of the cause, immune systems of OBI patients are unable to completely clear HBV-DNA from hepatocytes [11]. Investigators believed that the genetics and immunological parameters are different in

resistant individuals and OBI patients [2, 12]. Humoral immunity serves as an important arm of the immune response against viral infections and plays a crucial role in protection and clearance of HBV from hepatocytes [13, 14]. For example, previous studies have shown that anti-HBs antibodies play a key role in protection from HBV infection [15]. Previous studies also showed that monocytes can be infected by HBV [16], hence, it seems that humoral immunity may be important for the eradication of the virus from leucocytes by inducing the infected leucocytes to respond to HBV. For whatever reason, OBI patients are unable to completely overcome the infection by viral clearance. Therefore, to obtain a better understanding surrounding the mechanisms responsible for the etiology of OBI, this research aimed to evaluate some of the elements of humoral immunity in OBI patients.

Material and methods:

Patients

Peripheral blood samples were collected from 3700 volunteer blood donors attending the Rafsanjan Blood Transfusion Services (Kerman, Iran) and placed in 5 ml tubes without anticoagulants.. The samples were centrifuged at 370 x g for 4 minutes and the sera collected. All sera were separated within 24 hours of collection. If needed, serum samples were stored at -20°C

for a maximum of 2 months or at -70°C , where longer storage times were required, for further processing. Finally, the samples were collected from OBI (57 cases) and one hundred healthy controls. Healthy controls were selected based on the following criteria: 1. They have no significant differences in comparison to OBI patients regarding age, sex and socio-economic conditions 2. They were HBsAg/HBV-DNA negative and anti-HBc positive. The study protocol was approved by the ethical committee of the Rafsanjan University of Medical Sciences.

Prior to sample collection all participants of this study filled out and signed the informed consent form which was designed and based on the aims and objectives of the study.

Detection of serological HBV markers

HBsAg screening tests were performed by Enzyme linked immuno-sorbent assay (ELISA) (Behring, Germany). Anti-HBc screening tests were also performed by a manual microplate enzyme immunoassay using an anti-HBc commercial kit (RADIM, Italy). The present method is based on a competitive enzyme immunoassay (EIA). All of the samples were also screened by ELISA (RADIM, Italy) for possible HCV, HIV and HTLV-1 infections.

HBV- DNA Extraction from plasma samples

Viral DNA was purified from 200 μ l of plasma samples. Briefly, each plasma sample was incubated at 72°C for 10 minutes and then cooled down to 4°C for 5 minutes in 200 μ l proteinase K (200 μ g/ml). Following phenol/chloroform extraction (1:1), the viral DNA was precipitated with ethanol and the pellet was re-dissolved in DNase free, deionized water and stored at -20°C for further use.

HBV-DNA PCR and Gel Electrophoresis

PCR was carried out in a 25 μ l mixture containing 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01 % gelatin, 5 units of recombinant *Taq* DNA polymerase, 200 μ M of each dNTP, 0.6 μ M of each primer, and 5 μ l of the DNA extracted from 200 μ l of plasma. The sequences of all primers used in this study are shown in Table 1. For HBV analysis the primers are designed to amplify a 500bp amplicon of the surface antigen or S gene of HBV genome. Fast temperature cycling was performed. PCR amplification was performed using the touch-down method which included one cycle of 93°C for 60 sec, 60°C for 20 sec and 72°C for 40 sec, then 5 cycles of 93°C for 20 sec, 60°C to 56°C for 20 sec and 72°C for 40 sec followed by 30 cycles of 93°C for 20 sec, 55°C for 20 sec and 72°C for 40 sec. HBV genomic DNA provided by the Cinnagen company (Iran) was used as a positive control. For the analysis of the PCR amplification, 10 μ l of the amplified DNA were run

on a 2% agarose gel after addition of 4 μ l of loading buffer. The presence of a 500 bp fragment indicated a positive result. In parallel with samples, a 100 bp DNA ladder was also run on the gels to estimate the molecular weight of DNA fragments in the gel. The assay was able to detect less than 100 copies of HBV-DNA per ml.

Detection of IgG, IgM, IgA, C3 and C4

IgG, IgM, IgA, C3 and C4 serum levels were detected using SRID (Biogene, Iran) in patients and healthy controls immediately after blood collection. Assays were performed according to manufacturer's guide lines.

Complement system function

Complement system function was evaluated by total function assay (CH50) (DRG, Italy), according to manufacturer's guide lines.

Statistic analysis

Statistical analysis was performed using the statistical package within the GraphPad Prism software package (version 5.03 from GraphPad Software Inc.) Unpaired t-Tests and χ^2 test were performed on the data and when $P < 0.05$ the data was considered statistically significant.

Results

In this study we found that all of the 3700 examined samples were negative for HBsAg, HCV and HIV. Only 352 (9.5%) samples were positive for anti-HBc whereas HBV-DNA was detected in 57 of those 352 samples (16.1% of the HBsAg⁻/anti-HBc⁺ pool). Therefore, results of this study indicated that 16.1% of HBsAg⁻/anti-HBc⁺ samples had detectable HBV-DNA and were considered OBI patients. Overall, 1.54% of all samples donated were OBI carriers.

The mean age of patients and controls was 28±6 and 28±8, respectively and the difference was not significant between the groups regarding age (Table 2). Three (3%) members of the control group were female and 97 (97%) were male while 2 patients (3.5%) were female and 55 (96.5%) were males. Analysis of socio-economical conditions showed that there were also no significant differences between the patients and controls (Table 2). The mean serum level of aspartate aminotransferase (AST) and alanine transaminase (ALT) was also noted in the table 2.

Our results showed that average serum levels of IgG were 1308.21±68.98 and 1605±42.29 mg/dl in OBI patients and healthy controls, respectively (Figure 1 shows this data displayed as a scatter plot, Figure 3 shows the data as a distribution plot). Statistical analysis indicated that this difference was significant (P=0.0002). It should be noted that although the average in OBI

patients is reduced the absolute range of serum levels in patients is broader. In addition, the C4 levels of patients was 24.13 ± 1.084 when compared to control (50.71 ± 0.9222) showing a significant reduction ($P < 0.0001$) (see Figures 2 and 3). Unlike the IgG data, the C4 is not as broad in its range. Our results also showed that serum levels of IgM were significantly increased ($P < 0.0001$) in patients versus controls, 229.8 ± 10.42 versus 160.2 ± 84.53 respectively. This data is presented as a scatter plot in figure 2, however, it should be noted that the difference could potentially be greater because 21 of the 57 OBI patients had IgM levels that reached the maximum detectable level of the kit. The significance of this data is not apparent at this stage. C3 levels in patients were also significantly higher ($P < 0.0001$) than in controls, 76.07 ± 4.691 versus 49.33 ± 2.927 respectively (see Fig. 2). Serum levels of IgA were the same in patients and controls, $P = 0.3126$ (Figure 2). Complement function, which was measured as CH50, was 183% and 170% in patients and controls, respectively. Statistical analysis showed that the differences was not significant ($P > 0.1$).

Discussion:

In the past decades transmission of HBV through blood transfusion was a significant mechanism of infection however, due to the development of screening programs by national and international blood transfusion services,

the risk of HBV transmission has now decreased worldwide [5]. Moreover, the risk of microbial and viral infections via transfusion of blood and blood components has been greatly reduced but HBV is still the most common post-transfusion infection [6]. Despite the efforts of HBV screening programs by the Iranian blood transfusion services (IBTS), results of current studies show that 1.45% of healthy HBsAg⁻/anti-HBc⁺ donors may carry OBI in their circulating blood and are potentially a risk for transmitting HBV. Our previous study on 545 volunteer blood donors from Isfahan (a central province of Iran) [5] and 270 volunteer blood donors from Rafsanjan (Kerman-Iran) [6] showed 0.92% and 1.48% carried OBI infection, respectively. The current study which was performed on a larger scale from IBTS donors not only confirms our previous results but highlights that 1-2% of blood donors in Iran are potential OBI carriers. Based on the fact that the major blood donors in Iran are male, it can be concluded that this population of blood donors is not representative of the general population.

It is worth noting and paying attention to the observation that many other unknown factors, beyond defective immune response, may affect and lead to failure in detection of HBsAg in OBI patients. These vary from low detectable levels of HBV-DNA load [10] to the presence of mutations in the pre-S/S genome region of HBV that could affect expression of HBsAg and

its subsequent detection [10]. Our study on the S gene of HBV-DNA that was obtained from OBI patients showed that there were no mutations in this region (specifically in the α -determinant region) (unpublished data). Therefore, it seems that HBsAg has its normal structure in our cohort and should be detectable by ELISA and there were no apparent failures in the HBsAg detection used in these studies.

It is reported that in response to viral infections, antibodies and other humoral factors, such as complement factors are increased [17-19]. IgM is the first antibody that is elevated at the onset of infection and neutralizes free viruses in the serum and mucosa [20, 21]. IgG is produced sequentially after IgM and induces a vigorous and long term protection against viral infections [21]. In instances where IgG production is aberrant, the serum levels of IgM remain higher during chronic infection [22] and our data would appear to agree with that finding (Fig. 2). Our results showed that OBI patients were capable of producing IgM, IgG, IgA, C3, C4 and CH50 indicating that there were no general defects in these pathways. However, the key component potentially responsible for clearing hepatitis B infection, IgG was significantly reduced when compared to controls (Fig. 1). However, we noted that the range of IgG levels in patients appeared to be much broader than those in healthy donors, the relevance of this remains unclear and may

reflect differences in individual responses to viral infection or possibly that those patients were in the process of raising an elevated IgG response to a separate infection. In relation to the current study, we concluded that one possible reason that HBV is able to persist in OBI patients is their inability to produce appropriate levels of IgG antibodies directed towards the clearance of the viral infection. The data would suggest that the general mechanisms are in place and that the patients are capable of initiating humoral immune responses as indicated by the presence of IgM, IgA, C3 and C4 in their serum as well as a functional complement system. However, it is yet to be determined why our OBI patients fail to clear the HBV infection. To our knowledge, this is the first study to evaluate humoral immunity factors in OBI patients. There is limited information in the literature regarding OBI of particular interest is a study by, Lu CY *et al.*, showing that not all HBV vaccinated subjects produced enough IgG against HBsAg and that some of them needed a booster vaccine to gain affective immunity [23]. Martinetti M *et al.*, reported that reduced humoral response to HBV in some neonates was associated with polymorphisms in HLA class I and II genes [24]. Vranckx R *et al.*, reported that humoral immunity can also be defective in chronic HBV infected patients [25]. Other studies have also shown that some vaccinated adults are unable to produce specific IgG

against HBsAg [26]. Therefore, based on our results and those of others it can be concluded that some of the HBV infected OBI patients may have a defect in general levels of IgG production or the generation of specific IgG against HBsAg. In combination with regulatory factors of the immune system, these may be important contributing factors in the development of OBI.

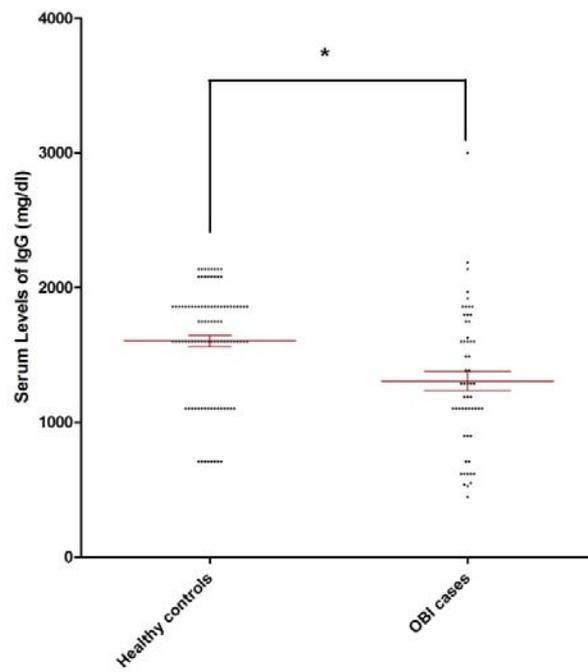
Acknowledgments:

The authors of this article would like to take this chance to thank all the OBI patients and healthy controls who contributed to this research program. In addition we would like to thank Leanne Towerzey for her help with software and George Mellick for his critical analysis of data.

References:

- [1] Lorient MA, Marcellin P, Walker F, Boyer N, Degott C, Randrianatoavina I, et al. Persistence of hepatitis B virus DNA in serum and liver from patients with chronic hepatitis B after loss of HBsAg. *J Hepatol* 1997;27(2):251-8.
- [2] Arababadi MK, Pourfathollah AA, Jafarzadeh A, Hassanshahi G, Rezvani ME. Association of exon 9 but not intron 8 VDR polymorphisms with occult HBV infection in south-eastern Iranian patients. *J Gastroenterol Hepatol* 2009;25(1):90-3.
- [3] Marcellin P, Martinot-Peignoux M, Lorient MA, Giostra E, Boyer N, Thiers V, et al. Persistence of hepatitis B virus DNA demonstrated by polymerase chain reaction in serum and liver after loss of HBsAg induced by antiviral therapy. *Ann Intern Med* 1990;112(3):227-8.
- [4] Hu KQ. Occult hepatitis B virus infection and its clinical implications. *J Viral Hepat* 2002;9(4):243-57.
- [5] pourazar.
- [6] jafarzadeh.
- [7] ArababadiL Tlabmedicine.
- [8] Arababadi MK, Hassanshahi G, Yousefi H, Zarandi ER, Moradi M, Mahmoodi M. No detected hepatitis B virus-DNA in thalassemic patients infected by hepatitis C virus in Kerman province of Iran. *Pak J Biol Sci* 2008;11(13):1738-41.

- [9] Arababadi NK lab medicine.
- [10] Katsoulidou A, Paraskevis D, Magiorkinis E, Moschidis Z, Haida C, Hatzitheodorou E, et al. Molecular characterization of occult hepatitis B cases in Greek blood donors. *J Med Virol* 2009;81(5):815-25.
- [11] Arababadi IL17 hepatitis monthly.
- [12] Arababadi FAS pollab medicine.
- [13] Alavi S, Rashidi A, Arzanian MT, Shamsian B, Nourbakhsh K. Humoral immunity against hepatitis B, tetanus, and diphtheria following chemotherapy for hematologic malignancies: a report and review of literature. *Pediatr Hematol Oncol*;27(3):188-94.
- [14] Schumann A, Lindemann M, Valentin-Gamazo C, Lu M, Elmaagacli A, Dahmen U, et al. Adoptive immune transfer of hepatitis B virus specific immunity from immunized living liver donors to liver recipients. *Transplantation* 2009;87(1):103-11.
- [15] Tsai HT, Tsai TH, Lu TM, Yang CC. Immunopathology of hepatitis B virus infection. *Int Rev Immunol* 2008;27(6):427-46.
- [16] Calmus Y, Marcellin P, Beaurain G, Chatenoud L, Brechot C. Distribution of hepatitis B virus DNA sequences in different peripheral blood mononuclear cell subsets in HBs antigen-positive and negative patients. *Eur J Clin Invest* 1994;24(8):548-52.
- [17] Tsai CC, Lin TM, You HL, Eng HL. Mannose-binding lectin in high-risk human papillomavirus infection. *Am J Obstet Gynecol* 2009;200(6):618 e1-6.
- [18] Coffey LL, Mertens E, Brehin AC, Fernandez-Garcia MD, Amara A, Despres P, et al. Human genetic determinants of dengue virus susceptibility. *Microbes Infect* 2009;11(2):143-56.
- [19] Thio CL. Host genetic factors and antiviral immune responses to hepatitis C virus. *Clin Liver Dis* 2008;12(3):713-26, xi.
- [20] Nanche D. Human immunology of measles virus infection. *Curr Top Microbiol Immunol* 2009;330:151-71.
- [21] Mateescu RB, Rimbas M, Voiosu R. The immunopathogenesis of viral hepatitis. *Rom J Intern Med* 2004;42(1):59-67.
- [22] Aghamohammadi A, Parvaneh N, Rezaei N, Moazzami K, Kashef S, Abolhassani H, et al. Clinical and Laboratory Findings in Hyper-IgM Syndrome with Novel CD40L and AICDA Mutations. *J Clin Immunol* 2009;3:3.
- [23] Lu CY, Ni YH, Chiang BL, Chen PJ, Chang MH, Chang LY, et al. Humoral and cellular immune responses to a hepatitis B vaccine booster 15-18 years after neonatal immunization. *J Infect Dis* 2008;197(10):1419-26.
- [24] Martinetti M, De Silvestri A, Belloni C, Pasi A, Tinelli C, Pistorio A, et al. Humoral response to recombinant hepatitis B virus vaccine at birth: role of HLA and beyond. *Clin Immunol* 2000;97(3):234-40.
- [25] Vranckx R. Humoral immune response in Hepatitis B virus infections. *Infection* 1983;11(2):114-7.
- [26] Alfaleh F, Alshehri S, Alansari S, Aljeffri M, Almazrou Y, Shaffi A, et al. Long-term protection of hepatitis B vaccine 18 years after vaccination. *J Infect* 2008;57(5):404-9.



IgG levels in Occult Hepatitis B and healthy patients

Figure 1. Scatter plot graphs showing serum levels of total IgG in OBI patients and healthy controls.

* Significant difference in serum level of total IgG ($P= 0.0002$), t-test, case VS control). Red bars show the Mean \pm SE.

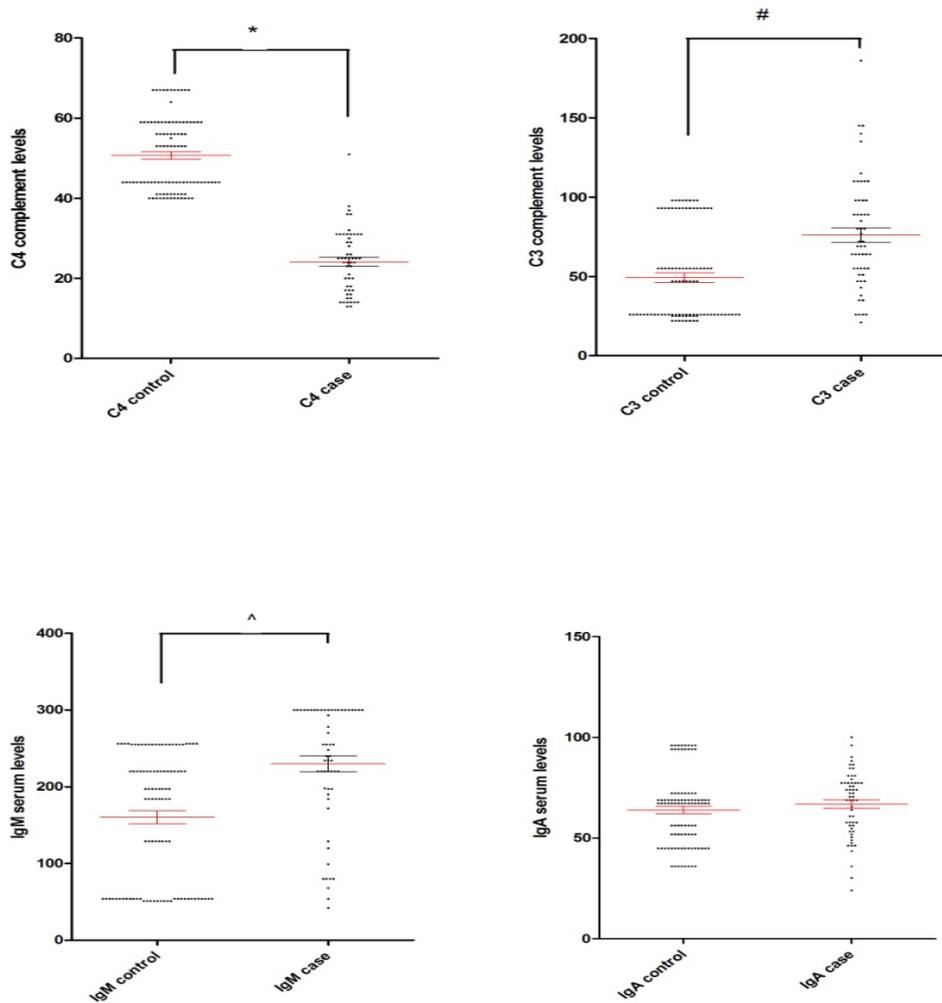


Figure 2. Scatter plot graphs showing serum levels of C4 and C3, IgM and IgA in OBI patients and healthy controls.

* Significant difference in serum level of total C4 ($P < 0.0001$, t-test, case VS control).

Significant difference in serum level of total C3 ($P < 0.0001$, t-test, case VS control).

^ Significant difference in serum level of total IgM ($P < 0.0001$, t-test, case VS control). Red bars show the Mean \pm SE for each analysis. Note that 27 out of 57 OBI cases in the IgM plot reached maximum detectable levels of the antibodies.

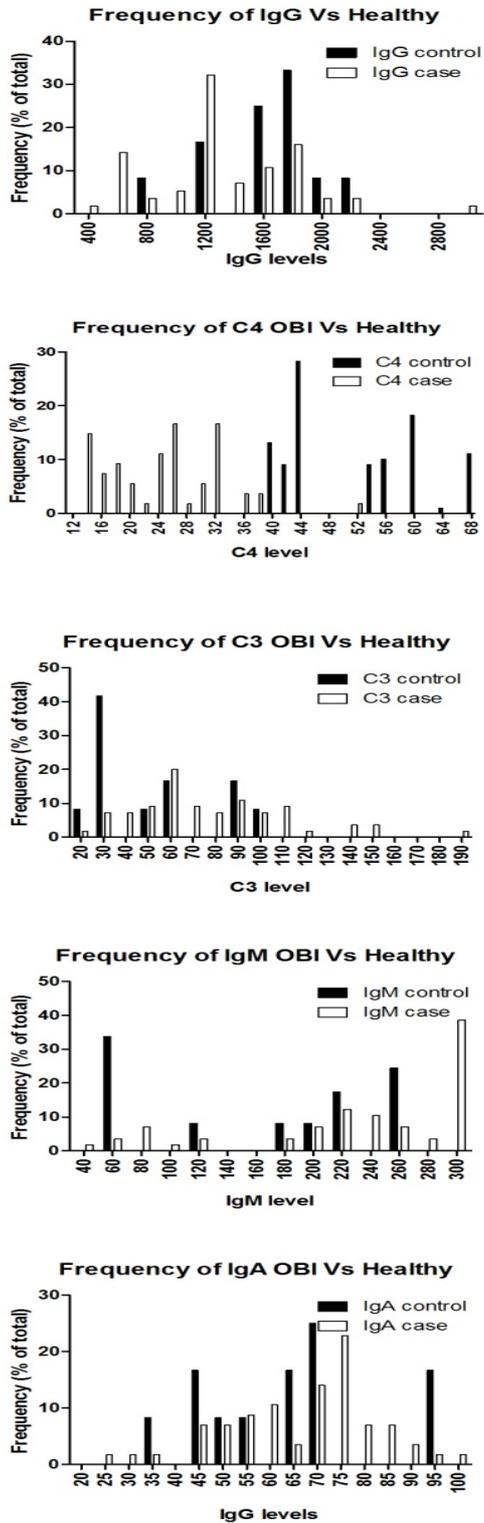


Figure 3. Frequency distribution of healthy donors and OBI patients. The data shown in Figures 1 and 2 are displayed here as a distribution plot.

Gene Name	S gene
Primers (5` to 3`)	F: TCGTGGTGGACTTCTCTC R: ACAGTGGGGGAAAGCCC
AT (°C)	55
MT (°C)	93
Nucleotides position of primers	S gene
Fragment Size	500bp

Table 1. Primers used in the study.

The table shows sequences of primers, the expected amplified fragment size and temperatures that were used in HBV S gene amplification. AT= annealing temperature; MT= melting temperature; F= forward primer; R=reverse primer

Variant	Healthy controls	Patients
Age	28 ± 8	28 ± 6
Sex		
Female	3 (3%)	2 (3.5%)
Male	97 (97.8%)	55 (96.5%)
Socio-economic status		
Weak	22 (22%)	12 (21%)
Medium	47 (47%)	28 (49%)
High	31 (31%)	17 (30%)
Liver enzymes serum levels		
<u>Mean serum levels of ALT (U/L)</u>	15 ± 8	18 ± 9
<u>Mean serum levels of AST (U/L)</u>	25 ± 4	28 ± 6

Table 2. demographic and socioeconomic conditions of occult hepatitis B virus-infected patients and controls