Some of immunogenetic status of HBsAg negative, HBcAb positive Blood donors in Basra province-Iraq

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ABSTRACT

Occult HBV infection is characterized as a clinical form of hepatitis B in which, despite the absence of detectable HBsAg in the serum of patients, HBV-DNA is present in the serum or liver. In this study we aimed to selecting of HBsAg negative, HBcAb positive patients among plasma samples of healthy blood donors and evaluate some of the elements of humoral immunity. The results from this study showed that all examined 350 Blood donors were divided by ELISA screening tests into four studied groups, HBsAg−/anti-HBc+ 65(18.58%) and Recovery 85(24.28%), 100 (28.57%) patients and 100 (28.57%) healthy. Our results showed that the serum levels of IgG and C3 were significantly higher in patients, while IgM and C4 were not significant among the studied groups. Based on these results, it could be concluded that one possible reason that HBV was able to persist in patients in HBsAg−/anti-HBc+ group was their inability to produce appropriate levels of IgG antibodies directed towards the clearance of the viral infection.

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INTRODUCTION

Hepatitis B virus (HBV) is one of the major diseases of mankind and is a serious global public health problem [1]. It is estimated that 40% of the world's population have had contact with or are carriers of the HBV. This corresponds to an estimated 350 million HBV carriers and around one million persons die of HBV-related causes annually [2]. HBV belongs to the family Hepadnaviridae and has some unique properties. It is highly species specific [3]. HBV is a partially double-stranded circular DNA virus [4]. The genes of HBV comprise genetic codes that create numerous protein products including Hepatitis B surface Antigen (HBsAg), Hepatitis B core Antigen (HBcAg),Hepatitis B e Antigen (HBeAg) and DNA polymerase [5]. These four proteins are of vital significance as they are measured in blood tests and aid in the diagnosis of the virus [6]. Both molecular and serologic testing methods are useful for interpreting the HBV status of a patient. However, because there are many different HBV markers, it is critical to understand how the appearance of a marker relates to a patient's disease or infection state. Research on HBV has clarified the clinical utility of specific HBV markers and has improved their diagnostic use [7]. The safety of blood products is one of the major issues in the area of transfusion medicine [8].Occult HBV infection (OBI) is characterized as a clinical form of hepatitis B in which, despite the absence of detectable HBsAg in the serum of patients, HBV-DNA is present in the serum or liver [9]. 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 [10]. Investigators believed that the genetics and immunological parameters are different in resistant individuals and OBI patients [11].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 [12-13].So,the aim of the present study were to selecting and detecting of HBsAg negative, HBcAb positive patients among plasma samples of healthy blood donors in the Central Blood Bank of Basra and evaluate some of the elements of humoral immunity in HBsAg −/anti-HBc + donors.

MATERIALS AND METHODS

A cross sectional study was conducting on the following groups at period February 2013 to August 2014. A total of 350 Blood donors of both sex, comprising 150 individuals as HBsAg negative, HBcAb positive group, 145 of them were males and 5 females with age range 18-59 years .Furthermore, 100 individuals as patients group, 98 of them were males and 2 females with age range 20-50 years and 100 individuals randomly selected as healthy groups, all of them was male with rang 21-46. Serological testing for HBsAg, Total anti-HBc were performed on Blood donors by using third generation enzyme linked immunosorbent assay (ELISA) kits(Plasmatec, USA and Foresight, USA), respectively.   Consecutively, HBsAg –/anti-HBc + donors were tested with anti-HBs by using ELISA (Dialab,Austria) kit . All ELISAs were performed accordingto the manufacturers' instructions. Then HBV-DNA was detected by using (Sacace, Italy) kit. The concentration of Ig′s(IgG, IgM) and complement components (C3, C4) were measured by single radial immunodiffusion(SRID) assayby using (LTA, Italy) kit.Analysis of the data obtained was made by using SSPS software. Calculation of mean values and standard deviation (SD) were made for humeral immunity and Biochemical parameters. The statistical significance of difference in mean of variables between more than two groups was assessed by ANOVA - LSD test. P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Characterization of Study Population
Of 350 blood donors, the present results showed that 343(98%) were males with significantly elevated (P<0.001) than7 (2%) females. These may be because males have activities in the community especially as blood donors and may be due to the behaviors of males that might be associated with transmission routes of HBV infection. Based on the fact that the major blood donors in Basra / Iraq are male, it can be concluded that this population of blood donors is not representative of the general population. The mean age of Blood donors was 29±6 years. This can be attributed to the fact that these age ranges people are the most active individuals in the society and the more likely to be blood donors.

Serological Tests
[14] show that after inoculation, HBV does not immediately start to replicate efficiently. HBV-DNA and HBV antigens are not detectable in serum or the liver until 4-7 weeks post-infection. Analysis of early events following HBV infection has revealed that HBV fails to activate early immunological responses, which are delayed until the exponential phase of replication [15].The results from this study showed that all examined 350 Blood donors were divided by screening tests, ELISA, in four studied groups gives up the follows percentages on the basis of the presence of HBsAg, HBsAb , and anti-HBc antibodies (Table ,1). A 150(42.86%) HBsAg negative , HBcAb positive group, which can be divided also in to two subgroups, HBsAg–/anti-HBc+ 65(18.58%) which negative ofHBsAb as a first groups, and Recovery 85(24.28%) which positive of HBsAb as a second groups .The third groups were 100 (28.57%)Patients which positive of HBsAg and HBcAb, and negative of HBsAb. The fourth groups were 100 (28.57%) healthy which negative of all HBV markers.

Table (1) Serological and molecular tests of the studied groups

Studied groups	HBsAg	HBsAb	HBcAb	HBV DNA
HBsAg–/anti-HBc+	-	-	+	-
Recovery	-	+	+	-
Patients	+	-	+	+
Healthy	-	-	-	-

In this study, during the use a wide range of HBV biomarkers as shown in table (1), in fact all viral markers were important in the diagnosis of the disease and in giving a clear indicated of the cases tested.So using HBsAg marker only for detection of the virus in the medical institutions were not enough to detecting all HBV infections.HBcAg is a unique immunogen and functions as both a T cell-independent and a T cell-dependent antigen in mice and humans [16]. and is ~1000 fold more immunogenic than the HBeAg[17]. The response of T cells to HBcAg has been reported to contribute to the resolution and seroconversion in chronic hepatitis B [18].IgG anti-HBc remains positive for life following exposure to HBV, also it persists for many years. However, unlike anti-HBs, anti-HBc is not a protective antibody [19]. The present study showed the vast majority of patients were Recovery group 85(24.28%). These cases can be considered as a complete recovery from acute infections due to the formation of anti-HBs. Also, in HBsAg–/anti-HBc+ group, in acute stage, the isolated anti-HBc can beconsidered a false positive test and the patient considered non-immune and vaccinated with the 3-vaccine series, or the patients most likely has resolved infection with gradually waning anti-HBs titers as incomplete recovery caseswithin an anti-HBc window, where the absence of anti-HBs can be due to its undetectable quanity.[20] shown that anti-HBs antibodies play a key role in protection from HBV infection. The loss of HBsAg or the presence of detectable anti-HBs indicates resolution from

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acute HBV infection. Both HBsAg and HBV DNA are detected in the majority of chronically infected patients [21]. With acute hepatitis, antibody production is critical for the neutralization of free HBV particles, the interference with virus entry into the host cells and contributes to limit cell to cell spread of viral particles, but anti-envelope antibodies are not detectable because they complexed with the excess of envelope antigens produced during virus replication [22]. Ultimately, the eventual outcomes of acute HBV infection are associated with distinctly different adaptive immune response profiles. Self-limited resolution is associated with a vigorous, multispecific antiviral CD4+ and CD8+ response involving adequate anti-envelope antibodies[23]. In chronic infections, the annual rate of HBsAg clearance has been estimated to be less than 2% in Western patients and even lower (0.1 - 0.8%) in patients of Asian origin [24].HBsAg–/anti-HBc+ group also can be as Occult HBV infection like in chronic case because we enable to detect the HBsAg and HBV-DNA in the serum.Many patients with occult HBV will have anti-HBc as the only serologic marker to suggest HBV infection. In this scenario, an HBV DNA level should be checked and the infection was strongly suggests the diagnosis of chronic HBV infection with an HBsAg-negative variant, since HBV was actively produced and detectable in serum or liver.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 [10]. 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, to the presence of mutations in the pre-S/S genome region of HBV that could affect expression of HBsAg andits subsequent detection [25].Regardless of the cause, immune systems of OBI patients are unable to completely clear HBV-DNA from hepatocytes [26]. Occult HBV infection is an entity with world-wide diffusion, and the available data show that its prevalence varies in different countries because of different prevalence of HBV infection and different sensitivity and specificity of the methods used for its detection in many studies [27-28]. Prevalence of occult hepatitis B virus infection in Zekri study from Saudi Arabia in blood units with isolated anti-HBc was 1.25% [29]. One report from Germany showed that 1.6% of first-time blood donors with antibodies to hepatitis B core antigen had occult hepatitis[30]. Higher rate of occult hepatitis B among anti-HBc only positive individuals has been reported [27].A Swedian study showed that 10% of isolated anti-HBc individuals were HBV-DNA positive [31].
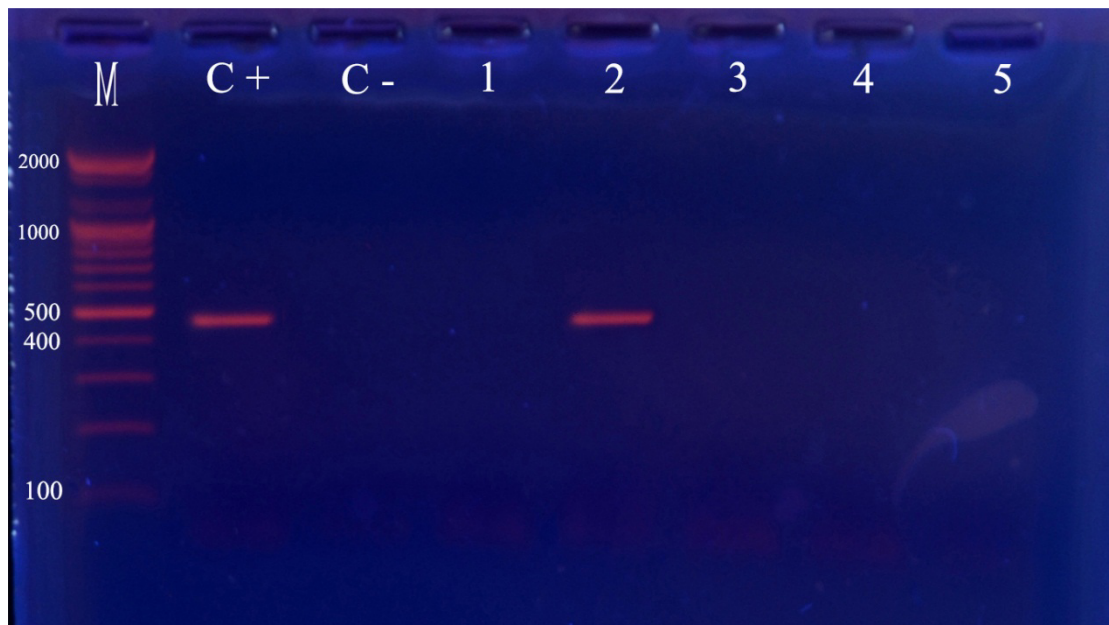

Figure (1) Electrophoresis of amplified HBV DNA Material
Lane 1, 3=HBsAg–/anti-HBc+, Lane 2=Patient, Lane, 4= Recovery 5=Healthy Lane M=PCR Marker, C+= positive control, C- negative control

Molecular Tests
Regarding the results, HBV-DNA was detected in patients group only (Table, 1; Fagure, 1). Although nucleic acid amplification assays detect HBV DNA in up to 14% of patients with isolated anti-HBc, the detectable HBV DNA generally occurs at relatively low levels [32]. In addition, several reports have documented HBV transmission from blood and organ donors who had isolated anti-HBc [33-34]. but other relatively large studies have shown no risk of HBV transmission from donors with isolated anti-HBc to kidney allograft recipients [35-36]. Nevertheless, based on available data, it appears that most persons with isolated anti-HBc have very low risk of transmitting HBV, except in settings involving potential transfer to susceptible individuals of substantial quantities of virus, such as with blood transfusion or liver transplantation [37]. In the latter setting, the practice of preoperative administration of hepatitis

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B immune globulin and postoperative prophylactic antiviral therapy has reduced the risk of HBV transmission or reactivation [38].The clearance of the covalently closed circular form of HBV DNA (cccDNA) has been difficult, and clearance of hepatitis B surface antigen   rarely occurs after 1 year of treatment[39].Thus, itwassuggestingthatHBV- DNAwouldbepositiveinpatients withhighertiterof anti-HBc than others would.

Humoralimmunity tests
Serum levels of IgG and IgM

Because induction of the virus-specific immune response usually requires a number of days, the initial protection against HBV is believed to be afforded by nonspecific mechanisms, among these mechanisms, killing of virus infected cells without HLA restriction or apparent specificity for viral antigens and secretion of antiviral cytokines by NK cells [40]. The initial lag phase of HBV replication does not appear to be a consequence of HBV inhibition by elements of innate and adaptive immunity[41].Our results ofIgG levels (Mean± SD) showed significantly elevated(P< 0.001) in Patients group (1382.06±891.82) and significant differences (P< 0.05) in Recovery group (954.67±336.55) in comparison with other studied groups, Whilethe statistical analysis of levels IgM showed that the differences were not significant among the studied groups as shown in table (2), figures (2,3).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]**.**IgM is the first antibody that is elevated at the onset of infection and neutralizes free viruses in the serum and mucosa [42]. It is found in smaller concentrations in human than IgG and IgA[43]IgM is the most efficient of all immunoglobulins at triggering the classical complement pathway and the main functions of IgM are include agglutination, complement fixation, opsonization, and toxin neutralization.IgM also serves as a surface receptor for antigen [44].

Table (2) humeral immunity tests (IgM and IgG mg/dl)of the studied groups

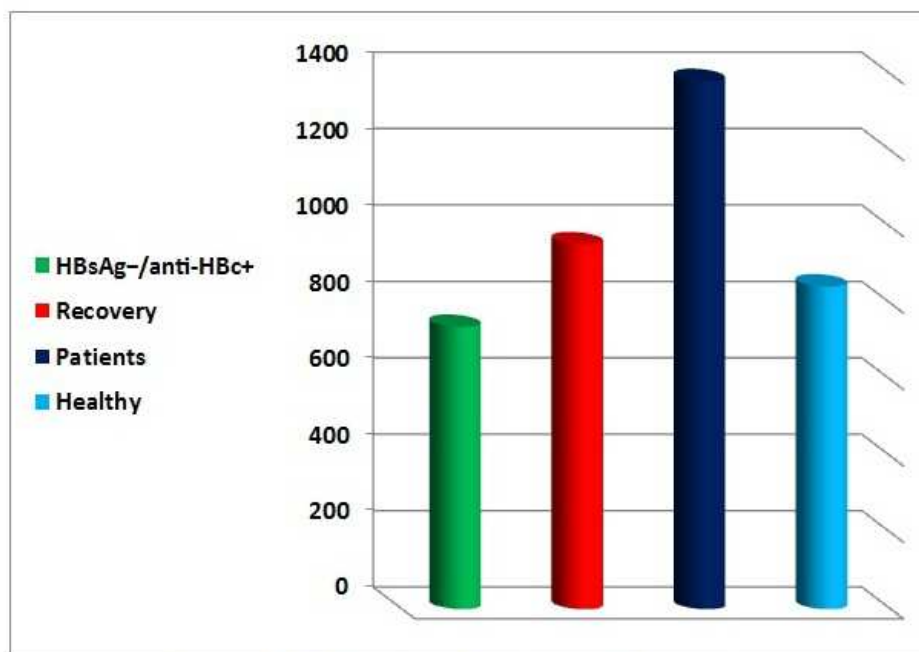| Studied groups	IgM Min.-Max.(Mean ±SD)	IgG Min.-Max.(Mean ±SD)
HBsAg–/anti-HBc+	162.7-1628.4 (817.9±407.3)	162.7-1628.4(738.797±416.66)
Recovery	162.7-1628.4(730.16±439.02)	437.5-1628.4(954.67±336.55)
Patients	437.5- 1628.4(820.23±340.4)	437.5- 3368.8 (1382.06±891.82)
Healthy	162.7-1628.4(769.34±439.62)	162.7-1628.4(844.653±490.57)
P<0.05		

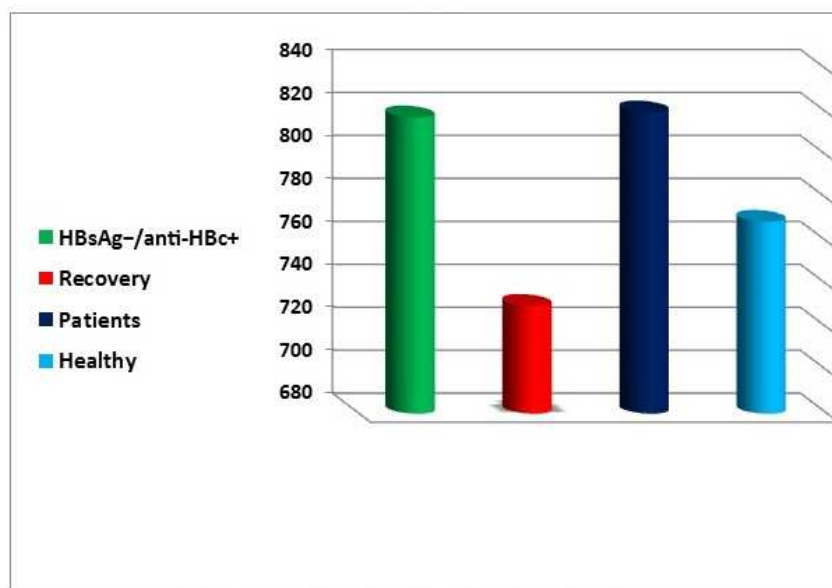Figure (2) the levels of IgG among studied groups

Figure (3) the levels of IgM among studied groups

IgG is produced sequentially after IgM and induces a vigorous and long term protection against viral infections [45]. Antibody production is critical for the neutralization of free HBV particles, the interference with virus entry into the host cells and contributes to limit cell to cell spread of viral particles, but anti-envelope antibodies are not detectable because they complexed with the excess of envelope antigens produced during virus replication [22].HBV envelope antigens represent an exception to the rule that CTL activation is selectively induced by recognition of endogenously synthesized antigen, because also exogenous forms of HBV envelope proteins can gain access to the class I pathway of antigen processing and presentation, CTL activation by this mechanism has been suggested to cause selective killing of HBV envelope-specific B cells acting as antigen presenting cells [46].The contribution of B cells and antibody are also important in HBV control, although these components of adaptive immunity have attracted less scientific attention in comparison to T cells. Nevertheless, HBV clearance is associated with the production of anti-envelope antibodies and sera with high levels of anti-viral antibodies (specific for the viral envelope) can control HBV infection [47].Study by [48] 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. [49] reported that reduced humoral response to HBV in some neonates was associated with polymorphisms in HLA class I and II genes. Therefore, based on our results and those of others it can be concluded that some of the HBV infected HBsAg−/anti-HBc+ patients may have a defect in general levels of IgG production or the generation of specific IgG against HBsAg.These results were in disagreement with Iraqi researchers, whom found the increased levels of IgM in patients with HBV infection [50], also a study done by Japanese [51] and in Indian researcher [52].Indeed all studies [50-51-52]that found the increased levels of IgG in patients with HBV infection, these results were in agreement with our study.

Serum levels of complements components (C3 and C4)
In addition, the C3 levels (Mean± SD) of Healthy group (70.78±22.69) and HBsAg−/anti-HBc+ group (72.09±28.49) showing a significant reduction (P< 0.05) when compared to Patients group(101.93±33.06). Furthermore, the C4 levelsshowed that the differences were not significant among the studied groups (Table, 3; figuers (4,5). Regarding the results within each group, the level of IgG was showed high significant (P<0.001) than IgM in Patients and Recovery groups. Interestingly the level of C3 had elevated mean valuesthan C4 among the studied groups.

Table (3) humeral immunity tests (C3and C4)of the studied groups

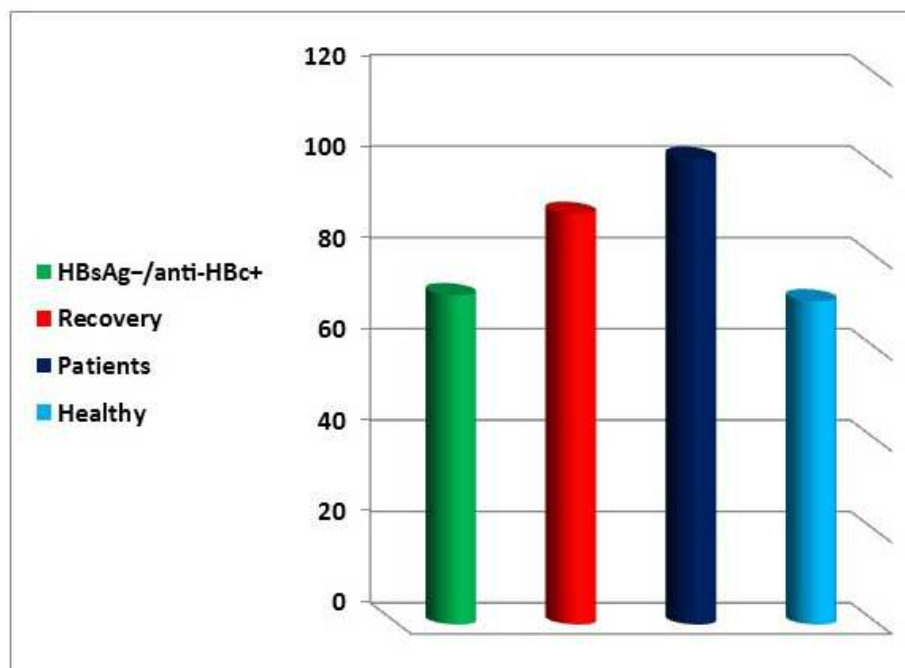| Studied groups	C3 Min.-Max.(Mean ±SD)	C4 Min.-Max.(Mean ±SD)
HBsAg−/anti-HBc+	39.1-84.4(72.09±28.49)	5-52.7(19.51±13.12)
Recovery	44.4-141.5(89.91±27.81)	5-37.8(17.84±7.54)
Patients	68-186.1(101.93±33.06)	5-52.7(23.28±14.81)
Healthy	39.1-102.1(70.78±22.69)	4-52.7(22.09±4.269)
P<0.05		

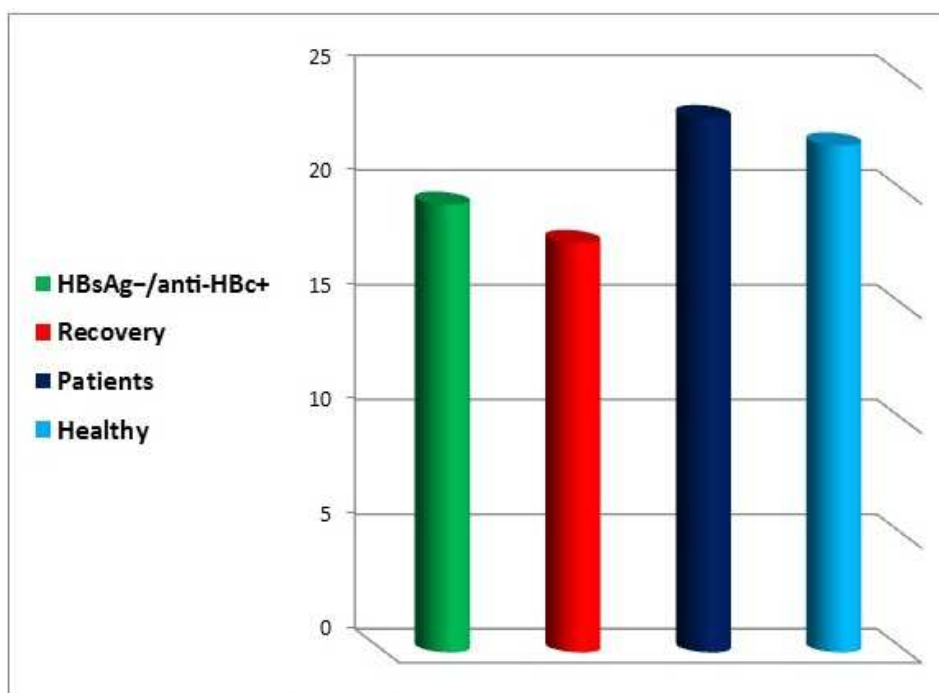Figure (4) the levels of C3 among studied groups

Figure (5) the levels of C4 among studied groups

Complement activation is one of the earliest responses to infection including viral hepatitis and its level has been shown to be reduced in viral hepatitis disease [53]. The decrease of complement levels in liver diseases has been assumed to be the result of failure of components synthesis in the liver [54]. Most of the complement components are synthesized in the liver by hepatocytes, but some of it is also produced by other cell types, including tissue macrophages, blood monocytes, fibroblasts, and epithelial cells of the gastrointestinal and genitourinary tracts [55]. The most important of the complement system proteins are C3 and C4 [56]. The third component of complement protein C3 plays important role in both classical and alternative pathways of complement activation [57],whereas the C4 is make up in bone and the lung tissue and its play important role in classical pathway, the

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deficiencies of C4 result in increased susceptibility to the development of autoimmune disease and associated immune complexdisease [58]However, the role of innate immunity has been more difficult to analyze, as HBV infection is usually diagnosed several weeks after the onset of infection when viremia is already high; thus the role of innate immunity in defense against HBV remains controversial [59] In 1998, Potter *et al*. reported a decrease levels in C3 and C4, and in 2003, Sinniah and Yadav reported a same results. In[60] study,the results showed that the levels of the three types of Igs (IgA, IgG and IgM) were significantly higher in chronic patients than inactive HBsAg carrier group; on the other hand, the serum complement components (C3 and C4) levels were significantly decrease among patients group compared to control group. Alsoin [26] study, of the 3700 samples donated from southeastern Iranian patients were 1.54% of HBsAg-/anti-HBc+ had detectable HBV-DNA and were considered OBI patients. This study 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.To our knowledge, this was the first study in Iraq to evaluate humoral immunity factors in patients in HBsAg–/anti-HBc+ group. The data would suggest that the general mechanisms were in place and that the patients were capably of initiating humoral immune responses as indicated by the presence of IgM, C3 and C4 in their serum. However, it was yet to be determined why our patients in HBsAg–/anti-HBc+ group failed to clear the HBV infections.The raised immunoglobulin levels may be due toover production of Ig's through HBV infection mediated immunoreactions and there is a much evidence that CHB infection is associated with the accelerated host immunoresponses. Our results showed that HBsAg–/anti-HBc+ were capable of producing IgM, IgG, IgA, C3 and C4 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 in HBsAg–/anti-HBc+ group when compared to others studied groups. Therefore, based on our results it can be concluded that some of the HBV infected patients in HBsAg–/anti-HBc+ group may have a defect in general levels of IgG production or the generation of specific IgG against HBsAg. However, we noted that the range of IgG levels in patients appeared to be much broader than those in healthy donors, for this reason we did not consider them as a complete recovery cases.

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