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Evaluating the Serum CTLA-4 Levels in Patients with HBs Ag (-)/HBc IgG (+)/Hbs Ab (+): Across Sectional Study in the Najaf

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ABSTRACT

Background: *Hepatitis B virus is a virus that attacks the liver, leading to viral hepatitis, cirrhosis, and liver cancer in humans. The diagnostic markers for hepatitis B, anti-HBc IgG and anti-HBs Ab, are widely recognized. A cytotoxic T-lymphocyte antigen-4 called CTLA-4 is an immune checkpoint protein that stops the HBV infection from spreading. It accomplishes this by serving as a sort of inhibitory receptor, restricting the quantity of damage that an acute infection can cause to the hepatocyte and enhancing the infection's capacity to remain in the body throughout a chronic illness.* ***Aim of the study:*** *The study aims to evaluate serum CTLA-4 levels in individuals with HBsAg-negative, HBc IgG-positive, and HBs-positive Ab and explore the association between these findings and the existence and development of HBV infection.* ***Patients and methods:*** *A cross-sectional study was performed from July to October 2023. The serum was taken from 200 individuals, all of whom were tested by using an immunochromatographic assay for HBsAb, HBsAg, HBcAb, HBeAg, and HBeAb and also by using an ELISA technique for CTLA-4 and HBc IgG. The statistical analysis was conducted by using SPSS version 26.* ***Results:*** *Serum CTLA-4 level positively correlated with HBsAg-negative, HBc IgG-positive, and HBs-positive patient antibodies ($p = 0.000$), serum HBs Ab positivity ($P = 0.000$), and total HBc Ab positivity ($P<0.001$), all linked to the amount of CTLA-4. Serum HBe Ab negativity was not linked to CTLA-4 ($p = 0.181$).*

Conclusions: *Elevated serum CTLA-4 level in patients with HBs Ag-negative, HBc IgG-positive, and HBs-positive Ab.*

Keywords: Anti-HBs, Anti-HBc IgG, CTLA-4, ELISA, Hepatitis B virus.

Article Information

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INTRUDUCTION

Hepatitis B virus continues to be a persistent global health concern despite the success of immunization strategies and the decline in HBsAg seroprevalence since 2000 due to the ongoing complications associated with chronic infection, which continue to contribute significantly to morbidity and mortality. The annual number of deaths from HBV-induced hepatocellular carcinoma and liver cirrhosis is approximately 820,000 (1). The hepatitis B virus releases numerous antigens, such as hepatitis B core, surface, and envelope antigens. Antigens exhibit the ability to induce an immune response as a result of their immunogenic characteristics (2). Approximately two billion people globally have been exposed to HBV, which is confirmed by the existence of antibodies to the hepatitis B core antigen (3). The markers can be successfully applied to aid in the selection of therapy options, predict illness severity in the

future, and facilitate the clinical care of CHB (4). The non-existence of HBsAg in the blood does not necessarily indicate the absence of HBV infection (5). Numerous mutations occur in the HBV genes due to a lack of a proofreading mechanism throughout replication (6).

The majority of investigations related to HBV reactivation have depended on the absence of detectable HBs Ag in individuals with occult hepatitis B infection and the existence of HBc Ab as a basis to identify occult hepatitis B infection (7). Individuals with seropositive occult hepatitis B infection (OBI) reveal antibodies in their blood circulation that are directed against the core antigen of the hepatitis B virus (anti-HBc) and/or antibodies that attack the surface antigen of the hepatitis B virus (anti-HBs). This particular kind of OBI represents roughly eighty percent of all OBI circumstances (8). The hepatitis B core antigen triggers the production of a significant amount of IgM, followed by IgG anti-HBc, due to its strong immunogenicity. Experts widely regard antiHBc as a highly sensitive and dependable marker for exposure to the hepatitis B virus. The levels of anti-HBc IgM rise fast after acute infection. Therefore, the presence of anti-HBc IgM is included in the initial diagnostic tests for HBsAg-positive patients who have elevated ALT levels, despite the existence or lack of a liver disorder (9). This diminished immune response makes the host's immune system incapable of eradicating HBV, resulting in a persistent infection (10). After a long-term HBV infection, the host's immune system frequently exhibits deficiencies or lacks the reactivity of T-cells specific to the virus. T-cell depletion is a state in which T cells exhibit impaired immune responses, like reduced production of cytokines, a decline in the ability to eliminate cells, and elevated levels of suppressive molecules like cytotoxic lymphocyte-associated antigen-4 (CTLA-4), programmed cell death-1 (PD-1), cytotoxic T, and lymphocyte activation gene-3 (11).

A co-inhibitory molecule that may be expressed on T cells is CTLA-4 (10). CTLA-4 (CD152) is an additional receptor that suppresses T cell function during prolonged viral infections (12). The CTLA-4 gene belongs to the immunoglobulin supergene family and resides on chromosome 2q33 (2). The upregulated inhibitory receptors on CD8 T lymphocytes in chronic hepatitis B individuals limit their immune defense process, leading to an exhausted phenotype (13). Elevated levels of CTLA-4 hinder the body's ability to resist certain kinds of infections. This is especially true for people who have HIV, where the virus temporarily weakens the immune system and makes the disease worse (14).

Immune checkpoint molecules have a significant impact on the progression of HBV infection. They help to minimize liver damage during acute infection and may contribute to the establishment of persistent infection in chronic HBV cases (15). There is a lack of studies that demonstrate an association between serum CTLA-4 levels and HBV infection in patients with HBs Ag (-)/ HBc IgG (+)/Hbs Ab (+). As far as our knowledge goes, this study is the first to evaluate the CTLA-4 immune checkpoint levels in relation to clinical parameters of the hepatitis B virus (HBV) infection in patients with HBs Ag (-)/HBc IgG (+)/Hbs Ab (+). The study aims to assess serum CTLA-4 levels in patients with HBsAg-negative, HBc IgG-positive, and HBs-positive Ab and investigate the correlation between these values and the presence and progression of HBV infection in the Najaf Government.

PATIENTS AND METHODS

The current study is a cross-sectional research project performed in Najaf- Iraq from July to October 2023. The study comprised a total of two hundred subjects and included exclusively Iraqi individuals who were recruited as patients from As-Sader Teaching Hospital in Najaf city. Among the overall sample, there

were 64 male and 136 female participants who varied from 18 to 80 years old. Every individual underwent screening for serum levels of CTLA-4, HBs Ag, HBs Ab, anti-HBc IgG, HBeAg, HBeAb, and HbcAb. The serum samples that confirmed negative for HBsAg but positive for HBc IgG and HBs Ab were chosen for inclusion whereas those that showed positive for HBsAg had not been included. The individual's data were acquired by giving a questionnaire and drawing a blood sample. Prior to being investigated, all participants were provided with an extensive description of the study's nature and aims and subsequently a permission was obtained for their participation.

The investigation comprised serum samples that screened negative for HBsAg, as well as individuals varying in age from 18 to 80 years old. Exclusion Criteria individuals who have the following were excluded:

1. a medical record of autoimmune illnesses, including systemic lupus erythematosus (SLE) and chronic inflammatory arthritis, and others.
2. a hepatic failure, persistent inflammatory disorders, or autoimmune illnesses.
3. a diagnosis of the hepatitis C virus (HCV), COVID-19, and CMV.
4. an HIV-related immunodeficiency and cancer.
5. a recent adoption of the HBV vaccine for immunization.

Five milliliters of venous blood were collected from every patient and transferred into a gel tube. After allowing the blood to clot, centrifugation separated the serum sample. Finally, the serum sample was placed in a plain tube to complete the necessary assays.

Presently, the techniques employed for clinical laboratory detection of HBV serological Hepatitis C virus rapid chromatographic immunoassay qualitative test, Human immunodeficiency virus rapid markers primarily consist of enzyme-linked immunosorbent assays (ELISA) and immunochromatographic assays. A fraction of the serum was used for a human hepatitis B virus screen test (immunochromaticographic assay): HBc Ab, HBs Ag, HBs Ab, HBe Ag, HBe Ab (Eugene Biotech/China), Human immunochromatographic test (Wondfo/China). The remaining portion was then divided into 1.5 ml Eppendorf tubes and stored in a refrigerator at -80°C for immunological analysis. After that, an ELISA method (Sun Long Biotech, China) was used to find a cytotoxic T lymphocyte antigen (CTLA)-4 and a qualitative HBc IgG. The procedures for all tests were conducted according to the instructions outlined in the kit's manual.

STATISTICAL ANALYSIS

The statistical analysis in this study was conducted with Version 26 of the Statistical Package for the Social Sciences (SPSS). The relationship between category and numerical data was illustrated by using the Mann-Whitney test. The findings are displayed in tables and figures, accompanied by a descriptive narrative, utilizing MS Word and Excel 2016.

The Ethics Council of the University of Kufa/Faculty of Medicine gave its approval before starting this research project. All participants gave their consent, and the As-Sader Teaching Hospital in Najaf also gave its approval.

RESULTS

The present study included a sample of 200 patients, who had undergone testing for HBV infection between July and October 2023, and were screened to detect the presence of HBc Ab, HBsAg, HBsAb, HBeAg, HBeAb, Hbc IgG, and CTLA-4. Fig 1 presents an overview of the general scheme followed in this investigation. Patients who were tested positive

for HBsAg, patients with hepatitis C infection patients with HIV, and patients with inadequate clinical data were excluded. This study has found that 10.5% of patients who tested negative for HBsAg, but positive for HBc IgG were also positive for HBs Ab. The study has also revealed that 75.5% of patients had HBc IgG, 77% had HBc Ab, 10.5% had HBs Ab, and 16.5% had Hbe Ab.

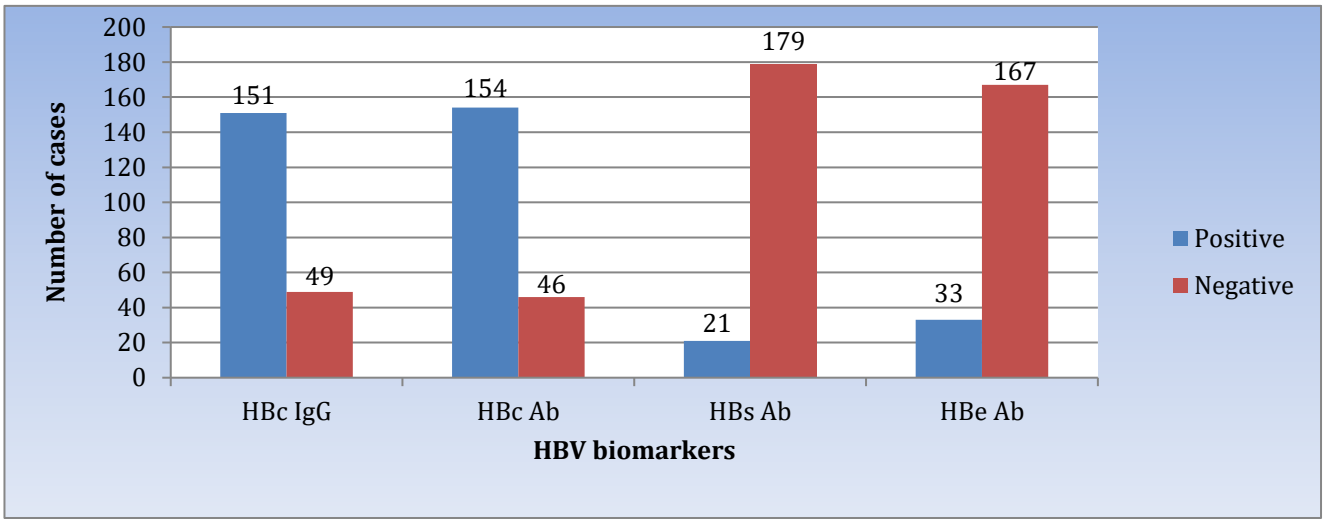

Figure (1): Count of HBV indicators in the study group.

A statistically significant association was observed between CTLA-4 levels and HBc IgG antibodies (p value<0.05, Z = -8.770). Specifically, individuals with positive HBc IgG (N = 151, M = 14.8131pg/ml) were more likely to have higher CTLA-4 levels than those without HBc IgG antibodies (N = 49, M = 4.5637pg/ml). As seen in Table 1., There was actually a statistically significant link between being positive for HBc Ab and CTLA-4. The p-value was less than 0.05, and Z was -8.292. Specifically, people who were tested positive for HBc Ab (N = 154, M = 14.5960 pg/ml) had a higher likelihood of producing higher levels of CTLA-4 than those who were tested negative for HBc Ab (N = 46, M = 4.6222 pg/ml). As seen in Table 1., A statistically significant association was found between CTLA-4 and the presence of HBs Ab (p value<0.05, Z = -5.076). Patients who were tested positive for HBs Ab (N = 21, M = 39.1348 pg/ml) had a higher likelihood of producing higher levels of CTLA-4 compared to patients who were tested negative for HBs Ab (N = 179, M = 9.1540 pg/ml). As illustrated in Table 1. However, there was no statistically significant link observed between the presence of HBe Ab and the production of CTLA-4 (p > 0.05). A strong association was found between individuals who have been evaluated for a positive result for HBsAg (-)/HBc IgG(+)/HBs Ab (+) and the production of CTLA-4 (p value<0.05, Z=-5.076-). Specifically, individuals with positive results (N = 21, M = 39.1348 pg/ml) had a significantly higher likelihood of producing higher levels of CTLA-4 compared to individuals with negative results (N = 179, M = 9.1540 pg/ml). As indicated in table 2.

Table (1): The relation between CTLA-4 and viral markers by Mann-Whitney test.

Test Result type	HBc IgG (+)	HBc Ab (+)	HBs Ab (+)	Hbe Ab (+)
Mann-Whitney U	612.500	686.000	606.000	2349.000
Z	-8.770-	-8.292-	-5.076-	-1.338-
P value	0.000	0.000	0.000	0.181
p value=probability value (level of significance at <0.05)				

Table (2): The relation between CTLA-4 level and HBsAg (-)/HBc IgG/HBs Ab (+) group by Mann-Whitney test

Test Result type	HBs Ab (+)/HBc IgG/HBsAg (-)
Mann-Whitney U	606.000
Z	-5.076-
P value	0.000
p value= probability value (level of significance at <0.05)	

DISCUSSION

The current study has demonstrated that anti-HBc (+) had the highest detection rate at 77% (154/200), followed by anti-HBc IgG (+) at 75%, and anti-HBs (+)/anti-HBc (+) at 10.5% (21/200). These results were in line with the findings of a study conducted by Cai *et al*. (2022), which found that the pattern of HBV serologic markers was found in anti-HBc(+) (3.73%, 10/268) and anti-HBs(+)/anti-HBc(+) (1.87%, 13/695) (16). The generation of anti-HBc is intimately associated with the synthesis of cccDNA and HBcAg (17). The variations in antibody frequencies can originate from variations in various geographical regions with various sample sizes, and the serologic window in the incubation period after infection, can be responsible for the differences in HBV infection rates worldwide.

Regarding the HBsAg-negative/anti-HBc-positive state as an OBI stage in the HBV infection's natural course. Over 90% of people who are anti-HBc positive could have OBI. The "alternative" antiHBc test is considered the most acceptable and practicable marker for occult hepatitis B infection diagnosis (18). The overall frequency of occult hepatitis B infection (OBI) was 6.2% among blood donors who tested negative for HBsAg but positive for anti-Hbc (19). They found that the HBsAg-negative/anti-HBc-positive state is a stage of occult hepatitis B virus (HBV) infection that happens naturally as the disease gets worse (20). HBsAg clearance often indicates a previous HBV infection solely through the presence of anti-HBc. People who do not have HBsAg during the last stage of either resolved or occult HBV infections, on the other hand, usually have 1000 times lower anti-HBc levels than people who do have HBsAg (21).

Furthermore, in the present study, 16.5% of HbeAb seroprevalence is less than the 33.4% HbeAb-positive Chinese population (22). Ndako *et al*. (2021) found a 45.8% positive rate among participants with HbeAb (23), differing

from the results of the current study. HBV infection naturally triggers HBeAg seroconversion, a crucial event in the disease progression. This is when you lose HbeAg and make anti-HBeAg antibodies. This usually happens years after the replicative phase and marks a shift to a low/non-replicative phase where the liver's necro-inflammation can decrease and the infection may be resolved (24). However, the correlation between the proteins of HBV and the production of CTLA-4 after CHB infection is still unclear (22). The current investigation revealed a notable association between increased levels of CTLA-4 and markers of HBV in patients with HBs Ag (-)/HBc IgG (+)/Hbs Ab (+). Dharma (2023) found a strong association between the amount of CTLA-4 in the blood serum and the progression rate of chronic hepatitis B ($P<0.001$), which aligns with the current study's findings. Patients with chronic hepatitis B, hepatocellular cancer, and liver cirrhosis exhibited elevated serum levels of CTLA-4. The researchers found a positive correlation between the serum level of CTLA-4 and the progression of chronic hepatitis B (25).

The present study discovered a significant association between increased levels of CTLA-4 and positive markers of HBV. Tang *et al.* (2016) discovered that CD4+ T cells from people with chronic hepatitis B had more CD28 family receptors, like PD-1 and CTLA-4, than CD4+ T cells from healthy people (26). These results are very similar to what this study has found. A new study by Peng *et al.* (2011) showed that looking at the presence of CTLA-4 on CD8+ T cells in people who had a CHB infection gave different outcomes. However, the overall trend suggests an increase in CTLA-4 levels on CD8+ T cells. People who have HBeAg-positive chronic hepatitis B experience this effect, and having HBeAg around makes CD8+ T cells make more CTLA-4. While these discoveries hold the possibility of major progress, the underlying mechanism of these events remains unknown (27). Although Wang *et al.* (2018) found something similar, the new results do not support earlier research that said HBV infection lowers the expression of CTLA-4 on T cells (28). This reduction may affect their capacity to regulate immunological responses.

The importance of CTLA-4 levels in patients with HBV has significant clinical consequences. High levels of CTLA-4 can serve as an indicator of more severe liver inflammation and worse outcomes. Additionally, using immunotherapy to target CTLA-4 appears to be a possible way to manage HBV infection. One study found that blocking CTLA-4 increased T cells that are specific to HBV and decreased inflammation in the livers of people who had HBV (29).

CONCLUSIONS

1. Patients who tested negative for HBs Ag, positive for HBc IgG, and positive for Hbs Ab exhibited elevated levels of CTLA-4 in the serum. CTLA-4 levels may serve as indications of the immune response to HBV infection or the severity of a disease.
2. The study found a strong correlation between serum CTLA-4 levels and virus-specific proteins such as HBs Ab, HBc Ab, and HBc IgG, but did not find a significant correlation between anti-HBe and CTLA-4 levels.
3. Researchers need to conduct additional multicenter investigations to determine the role of CTLA-4 in the intensity and progression of HBV infection.
4. Elevated levels of CTLA-4 in individuals exposed to HBV infection may provide a chance to explore novel therapeutic approaches. These strategies could involve targeting the regulation of CTLA-4 levels to minimize the adverse development of HBV and enhance patient response to therapy.

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