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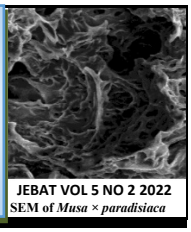

Determination of the Relationship Between C-Reactive Protein (CRP) Levels and Hepatitis B Surface Antigen

Maryam Buhari Gidado[1*], Ali Ahmed Haroun[1], Yakubu Magaji[1], Zaharaddeen Abdullahi[1] and Abdulsalami Muhammad Sani[1]

[1]Deparment of Biotechnology, Faculty of Science, Nigerian Defence Academy, PMB 2109, Kaduna State, Nigeria.

*Corresponding author:
Maryam Buhari Gidado,
Deparment of Biotechnology,
Faculty of Science,
Nigerian Defence Academy,
PMB 2109,
Kaduna State,
Nigeria.
Email: Mbg18teen@gmail.com

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ABSTRACT

The relationship between hepatitis B virus (HBV) infection and C-reactive protein (CRP), which is an inflammatory biomarker, is limited in studies on the general population. Thus, this study aimed at determining the relationship between CRP levels and Hepatitis B surface antigen in patients with hepatitis B. A total of 70 samples were screened for the presence of hepatitis surface antigen by one step hepatitis B Surface antigen test strip (serum/plasma) package insert. The samples were further subjected to ELISA test and quantitative real time PCR to determine the viral load. The performance of the assay on the 70 samples showed 17 (24.29%) patients were positive and 53 (75.71%) patients were negative for serological test. Out of the 17 samples which were positive for HBV, CRP was positive in 5 patients while 12 patients were negative for CRP. While out of 53 patients who were negative for HBV, 9 were positive for CRP and 44 were negative for CRP. For the significance of viral load for clinical monitoring, three titer groups were presented. Among the 70 samples tested for viral load of HBV, 50% (35/70) of samples showed low titer by the Ct<30, while only 15.71% (11/70) of samples were detected with high viral load by Ct>30. Statistical analysis showed insignificant relationship between CRP and HBV. Positive predictive value of CRP was lower;it is revealed that the presence of HBV infection cannot be predicted on the basis of CRP analysis only. The reason behind lower CRP concentration in HBV positive cases remains unclear but there is a perception that high CRP levels in the blood can be a marker of inflammation.

INTRODUCTION

C-reactive protein (CRP) is an acute allergic protein that is exclusive to the liver. Cytokines including interleukin-1, interleukin-6, and tumour necrosis factor alpha control the production of C-reactive protein in hepatocytes. It is created by the liver's cells. The relationship between CRP expression and disease development in chronic viral infections is still unclear, despite the fact that many modifications have been discovered in people with chronic viral hepatitis [1]. One of the acute-phase proteins, CRP sees an increase in serum or plasma levels during a broad, non-specific response to a range of disorders. Infections caused by gram-positive and gram-negative organisms, the acute stage of rheumatoid arthritis, abdominal abscesses, and bile duct inflammation fall under this category [2]. Additionally, CRP may be seen in people with Guillain-Barre syndrome, multiple sclerosis, some viral infections, TB, acute infectious hepatitis, several other necrotic and inflammatory disorders, burn victims, and in the aftermath of surgical trauma [3]. The presence of high CRP levels in the serum is a valuable diagnostic of inflammatory processes even if it is not specific for any one illness. Within 24 to 48 hours of severe tissue injury, CRP levels increase in serum or plasma, peaking during the acute period (about 1000x constitutive level), and then decline as the inflammation or trauma subsides. Before returning to normal levels, the rise in CRP concentration in human serum or plasma might remain for many days [4].

A more accurate and sensitive biomarker of the inflammatory process than the erythrocyte sedimentation rate is the measurement of CRP, which can also be impacted by physiological changes unrelated to an inflammatory process. While enzyme-linked immunosorbent assays (ELISA) offer the best sensitivity and specificity, current testing techniques such as latex agglutination, nephelometry, and radial immunodiffusion (RID) often have these drawbacks [5].

The CRP test offers helpful information for the diagnosis, management, and monitoring of inflammatory processes and related diseases since increased CRP readings are invariably linked to pathological alterations. The predictive value of other cardiac indicators (myoglobin, creatine-kinase-MB, troponin I and T), which are used to determine the risk of cardiovascular and peripheral vascular disease, may also be increased by measuring CRP using high-sensitivity CRP tests. Increases in CRP readings should not be interpreted without a thorough review of the patient's medical history, and tests of CRP should be compared to earlier results because they are non-specific [6, 7]. Each person's body produces a different quantity of CRP, and this variation is influenced by that person's genetic makeup and way of life [8]. Blood from healthy individuals often does not contain CRP. It arises following an injury or inflammation and goes away as the damage heals, the infection goes away, or the inflammation goes down [9].

Infections and numerous chronic conditions can raise CRP levels. CRP cannot be used to diagnose any ailment, but it may be used to assess a person for an acute or chronic inflammatory disorder in conjunction with signs, symptoms, and other tests. The CRP test is a blood test that assesses our body's overall levels of inflammation. The CRP test is not precise though. For many years, a common diagnostic procedure for assessing the state of recognized inflammatory illnesses has been the use of CRP [10, 11]. When a person is suspected of having a severe viral or bacterial infection based on their medical history, signs, and symptoms, CRP may be necessary [12].

The Hepatitis B virus is responsible for the deadly liver illness known as hepatitis B. Through blood, semen, or other bodily fluids, the virus is transferred from one person to another. Acute Hepatitis B can go away in less than six months (when the immune system is likely to do so) [13]. Hepatitis B chronic persists for at least six months (when immune system cannot fight off the acute infection). Serious conditions including liver cancer, liver failure, and liver scarring are brought on by hepatitis B. (cirrhosis). Hepatitis B symptoms include nausea, vomiting, lack of appetite, dark urine, fever, joint pain, and stomach discomfort [14].

An acute inflammatory protein called C-reactive Protein (CRP) is produced at sites of infection or inflammation and can rise up to 1,000-fold there. It is a pattern recognition molecule that binds to certain chemical configurations that are frequently exposed after cell death or present on pathogen surfaces. Despite the fact that there is a connection between HBV infection and CRP in the patient population. Except in the context of another continuing infection or inflammatory condition, HBV does not cause an inflammatory reaction in dialysis patients [15]. In most patient population investigations, it was shown that those with HBV positivity had lower CRP values [16].

CRP is a predictor of how quickly the patients' chronic HBV levels would decline [17]. The predictive factor for the chronicity of the infection is the HBV genotype. In various human malignancies, including hepatocellular carcinoma, a prevalent form of primary liver cancer that most frequently affects persons with chronic liver disorders, the predictive value of CRP blood level has been demonstrated [18]. Prognostic indices are created by combining CRP-based risk assessment algorithms with other variables [19-21]. With peripheral blood CRP concentration and overexpression in hepatocellular cells having been shown to be the biomarkers used to measure the progression of the disease in the patient's samples, more needs to be done to improve hepatocellular outcomes in order to lower the global burden of HBV, which is currently affecting millions of people worldwide (prognostic markers).

MATERIALS AND METHODS

Sample Collection

3ml Blood samples were collected from 70 patients via venepuncture into EDTA bottles and transferred to the Department of Biochemistry, Kaduna State University (KASU) for further analysis.

Sample Processing Techniques

With the use of centrifuge equipment operating at 3000rpm for five (5) minutes, the blood samples were centrifuged to separate the serum from the other blood components. The serum from each patient was transferred to new vials, appropriately labeled, and then kept at -40C. Blood was analyzed using a rapid test strip for Hepatitis B surface antigen in accordance with the directions provided by the manufacturer in the handbook (Swe-Care Rapid Diagnostic Test).

Screening Assay Procedure for HBsAg Detection

The package insert for the one-step hepatitis B Surface antigen test strip (serum/plasma) was used to check the sera for the presence of hepatitis surface antigen. Prior to testing, the test strip and each blood sample were given time to acclimate to room temperature. Each blood (serum) sample was tested for HBsAg by removing the test strip from the sealed pouch and placing it on a non-absorbent flat surface. This was done by pipetting a small amount (2.5 mm) of the serum and dropping it on the test strip while making sure the maximum line on the test strip was not exceeded. It was then left for duration of 15 minutes, and the result was read.

C-Reactive Protein Test

A transferred pipette was used to extract 5µl of the serum from the sample and introduce it to the detection buffer tube. The material was fully mixed after being shaken for around 10 times with the detection buffer tube's lid closed. 75µl of the sample mixture were pipetted into the test cartridge's sample well. The FincareTM FIA System's "test cartridge holder" was used to hold the test cartridge after the sample mixture had been introduced to the sample well. A person pressed the "Test" button. The outcomes seen on the primary screen were written down.

Determination of HBV Viral Load

Virus Nucleic Acid Extraction

200µl of serum was placed in a clean 1.5ml centrifuge tube and 200µl Lysis solution was added into the centrifuge tube, vortex and shakes for 10 seconds. It was then allowed to stand for 10 minutes at room temperature, then 200µl of absolute ethanol was added to the centrifuge tube, vortex and shakes for 10 seconds.

The adsorption column was put into the collection tube and all the liquid above was transferred into the adsorption column, centrifuged at 8000rpm for 1 minute and the filtrate was discarded. 600µl of washing buffer I was added to the adsorption column (to confirmed that absolute ethanol was added to washing solution I before use), centrifuged at 8000rpm for 1 minute and the filtrate was discarded. 600µl of washing buffer Il to the adsorption column (to confirmed that absolute ethanol was added to washing solution II before use), centrifuged at 8000rpm for 1 minute and the filtrate discarded. The adsorption column was then returned to the collection tube and centrifuged at 8000rpm for 2 minutes, the filtrate and the collection tube were discarded. The adsorption column was placed in a RNase/DNase-free 1.5ml centrifuge tube, and 50µl elution solution was added in the centre

of the adsorption column membrane, and kept at room temperature for 1 minute. It was then centrifuged at 8000rpm for 1 minute, the Adsorption column was discarded. The nucleic acids were collected in the 1.5 ml centrifuge tube and stored at -20ºC.

Quantitative Real-Time PCR

The molecular test was based on the genotype specific target of HBV surface region as described by Paraskevis *et al.* [22]. The assay was carried out into a 7500 Applied Biosystems. The reaction mixture contained 1X Go-Taq Flexi buffer (Promega, Germany), 1.25 mM $MgCl_2$, Rox dye (Invitrogen, California), 1Unit Go-Taq DNA polymerase, 0.25mM dNTPs (Promega, Germany), 0.375 µM each primer (hbv 305, hbv 460), 0.25 µM Probe labeled with carboxyfluorescein (FAM) at the 5'end and with N,N,N,N-tetramethylrhodamine (TAMRA) at the 3'end. The final reaction volume was 20µl containing 5µl of extracted viral DNA and 15 µl of PCR-mix. Negative controls were performed with 5µl of sterile RNase free water.

The amplification profile was as follows:initial denaturation:95°C for 10 min;followed by 40 cycles of amplification at 95°C for 15 sec, 55°C for 30 sec. Fluorescence of FAM liberated from the probe by TaqMan was measured to determine the amplification threshold cycle (Ct), which was the first cycle at which fluorescent emission was 10-fold higher than the standard deviation of the mean baseline emission (BIONEER, AccuPower@ HBV Quantitative PCR Kit, V4.3/2020-03-10).

Statistical Analysis

Data analysis was carried out using Analysis of variance (ANOVA) and visualising data in SPSS version 23.0 and values with $p \leq 0.05$ was used for statistical significance difference. The correlation between the Ct values of RT-PCR and viral load was analyzed with the Spearman correlation test.

RESULTS

Screening Assay for HBsAg Detection

A total number of 70 patients were considered for this study. All patients were diagnosed with HBV antibodies in their sera. Out of these 70 patients, 17 (24.29%) patients were confirmed positive and 53 (75.71%) patients were negative for serological test (**Fig. 1**).

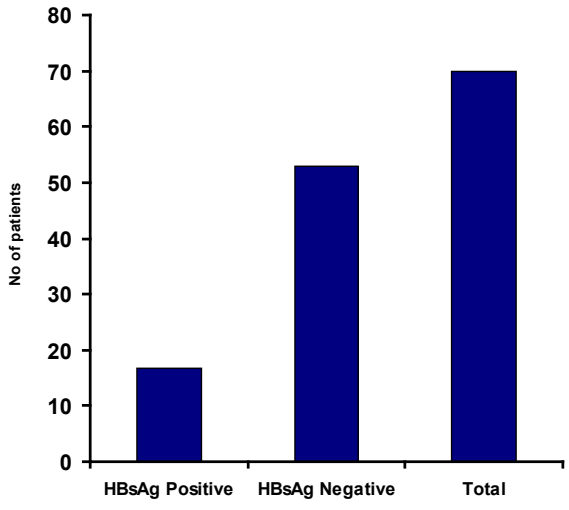

Fig. 1. Prevalence of HBsAg among the study population.

Determination of Crp Level

All samples which were tested for the HBV were also tested for the presence or absence of CRP. In total of 17 patients which were positive for HBV, CRP was positive in 5 patients while 12 patients were negative for CRP. While out of 53 patients who were negative for HBV, 9 were positive for CRP and 44 were negative for CRP (**Table 1**).

Table 1. CRP HBV ELISA cross tabulation.

	C-Reactive Protein		
HBV Detection by Elisa	Positive	Negative	Total
Detected	5	12	17
Non-detected	9	44	53
Total	14	56	70

Performance of the Real-Time PCR for Clinical Samples

Table 2 shows the performance of the assay was tested on 70 samples;24.29% (17/70) samples were previously positive in serological tests. 20% (14/70) serum samples resulted positive for CRP. For the significance of viral load for clinical monitoring, we have presented three titer groups. Among the 70 samples tested for viral load of HBV, 50% (35/70) of the negative samples have showed high titre of Ct>30 indicating low viral load, while only 15.71% (11/70) of the positive samples were detected with high viral load indicated by low titer Ct<30.

The high viral load detected for clinical was higher than 5,000 IU/ml corresponding for 1400 copies/ml. The corresponding of clinical samples results in log copies/ml ranged from 4 to 1 that indicated the sensitivity of the quantitative assay. 34.29% (24/70) of the tested serum samples resulted negative and showed Ct>37 or undetectable amplification signal.

Table 2. Viral load detection of clinical samples by real time PCR.

	Viral Titer detected by real-time PCR		
Positive	11 (15.71%)	35 (50%)	24(34.29%)
Negative	59 (84.29%)	35 (50%)	46(65.71%)
Total	70 (100%)	70 (100%)	70 (100%)

Note:Ct = threshold cycle

DISCUSSION

Monitoring the effectiveness of the medication and identifying occult hepatitis depend greatly on the molecular measurement of the HBV viral load [23]. The novel test demonstrated better sensitivity with a reading of 5000 IU/mL, or 1400 copies/ml. The test's detection limit is lower than the prior assay by 16 to 22 IU/ml [22]. The viral load was measured using linear regression with the parameter 1IU/ml = 2.8 copies/ml. The remarkable sensitivity of the real-time PCR can be explained by the DNA extraction magnetic technique for all clinical samples. By utilizing a different DNA extraction procedure on some samples, threshold cycles (Ct) were discovered to be lower.

Assessment of the patient's response to treatment, as well as the diagnosis and monitoring of HBV infection, depends heavily on the detection and quantification of circulating HBV in plasma or serum [24, 25]. The development of the illness, the appearance of drug resistance, and the identification of occult hepatitis may all be predicted based on the HBV-DNA viral load. Quantitative real-time PCR has grown in significance in the diagnostic laboratory and has been utilized for HBV detection, genotyping, and quantification due to its high sensitivity, specificity, and large dynamic range. In this work, the effectiveness of molecular real-time detection and quantification of HBV viral load in clinical samples is assessed.

The variations in CRP serum levels among different HBV-infected individuals were contrasted. The findings demonstrated that the CRP content steadily rose as the condition worsened. This data suggests that the CRP level may, to some extent, represent disease progression. HBV doesn't seem to be hepatotoxic on its own. The outcome of an HBV infection is heavily influenced by host immune variables. HBV causes immune-mediated liver damage by invoking innate and adaptive immunological responses in the host. Chronic hepatitis, LC, and HCC are among the clinical symptoms [26-30]. Initiating and controlling immunological responses depend heavily on cytokines, which link innate and adaptive immunity. Interleukins (ILs) such as IL-6 [31, 32], IL-27 [33], and IL-35 have all been linked to HBV infection and pathogenesis [34].

Among these interleukins, IL-6 is not only a crucial pro-inflammatory cytokine but also the main factor controlling the expression of the CRP gene. It was hypothesized that HBV may indirectly increase the expression of the CRP gene by increasing IL-1 or IL6 levels. According to Kao *et al.,* [32], those with HBV infections had significantly higher blood levels of IL-6 than people in the control group. According to Hao et al. [35], the serum levels of inflammatory factors, particularly IL-6, have a substantial impact on the serum CRP level in patients with HBV infections. The reported serum levels of IL-6 are consistent with the serum levels of CRP in the different individuals who were studied.

It's interesting to note that while persons with hepatitis C virus (HCV) infection have much higher blood IL-6 levels, their serum CRP levels are noticeably lower [6, 9]. High HCV RNA loads among HCV-infected people are associated with significantly lower blood CRP levels. It is thought that the replication of HCV prevents IL-6 from stimulating CRP [6, 9]. Contrarily, in those with HBV infection, the HBV DNA load is unrelated to the serum CRP level [36]. Following infection with the two different virus types, the serum CRP level exhibits opposing responses, which may be connected to variations in viral reproduction and pathogenesis. The precise method, though, is still unknown. A highly conserved host defense molecule throughout evolution is CRP. It has opsonic and anti-inflammatory properties because it may bind to phosphocholine ligands and further activate the complement system [37, 38]. According to prior research, complements C3 and C4 levels are much lower in HBV-infected people and this decline is connected with the disease's severity [39].

Clinically, there are wide variations in HBV-DNA titers, ranging from levels as high as 1010 copies/ml during acute HBV infection to extremely low levels in HBV antigen-negative chronic carriers, patients receiving antiviral medication, and people with occult HBV infection [40]. Occult hepatitis infection samples with low viral loads were found, and serological testing proved them to be negative. In this investigation, 24.29% of samples that had previously tested positive for serological testing had real-time PCR results that were also positive (65.71%).

Because there is evidence of inhibitors and co-infection with other blood pathogens in tropical areas, it is crucial to utilize DNA samples from a variety of clinical pathogens to ensure the test's specificity. For viral quantitation, 34.29% of samples were determined to be negative. In a tropical setting, the storage and transportation of the sera, the extraction technique, the technical staff's training to prevent false-positive results or cross-contamination, and the incorporation of an external quality control network can all affect the molecular assay's quality [41]. The findings of this study confirmed earlier research on the circulating HBV genotype in West Africa and showed the high performance for accuracy and sensitivity of real-time HBV to identify clinical samples [42, 43].

A statistical analysis found no connection between CRP and HBV. It is clear that the presence of HBV infection cannot be anticipated based on CRP analysis since the positive predictive value of CRP was lower. Although the cause of the reduced CRP concentration in HBV positive patients is yet unknown, there is speculation that it may be related to hepatocellular damage, which may have an impact on CRP production [44]. In the present study, results observed that higher CRP level found in HBV negative patients are similar to the findings, observed in another literature related to present study by Chen *et al.,* [45]. The findings of this study also coincide with a study by Gedik *et al.,* [36] which showed lack of correlation between CRP and hepatitis viral load in the serum of patients with chronic HBV.

CONCLUSION

The results of this study demonstrate that, while being statistically non-significant, CRP levels were lower in the presence of HBV infection. Furthermore, the CRP test is not helpful as a diagnostic indicator of hepatitis B virus, proving that there is no connection between CRP and HBV infection. However, it must be ignored that a high CRP level is a reliable indicator of several disorders. As a result, a credible link between CRP and HBV infection may exist. Further studies are warranted to explicate the causal rapport between CRP and hepatitis with a larger sample size and liver biopsy should be included to assess the extent of liver disease and its association with HBV infection status. CRP level and its relationship with several inflammatory diseases may provide more insight on its importance and relevance.

COMPETING INTEREST

The authors declare that they have no known competing personal relationships or financial interests that could have appeared to influence the work reported in this paper.

LIST OF ABBREVIATION

CRP:C-reactive protein
HBsAg:Hepatitis B surface antigen
ELISA:Enzyme-linked immunosorbent assay
PCR:Polymerase chain reaction
HBV:Hepatitis B virus
HCV:Hepatitis C virus
ILs:Interleukins
HCC:Hepatocellular carcinoma
RNA:Ribonucleic acid
DNA:Deoxyribonucleic acid

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