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Serological and Molecular Detection of Hepatitis B Virus and its Impact on Key Immunological Cytokines in Patients from Kirkuk, Iraq

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Abstract: Introduction and Objectives: Hepatitis B virus (HBV) infection is a major global health burden, and a leading cause of liver-related mortality, including hepatocellular carcinoma and cirrhosis. HBV disrupts immune regulation and metabolic pathways, affecting NK and γδ T cells, IFN-γ, and TNF-α production. The current study aimed to isolate and identify HBV using PCR techniques and to evaluate specific immunological factors, including IL-10, TNF-α, IFN-γ, and SCF/MGF, comparing their levels between HBV patients and healthy controls.

Materials and Methods: This study included 320 participants, 160 HBV patients and 160 healthy controls, recruited between January and July 2025 from Azadi Teaching Hospital, Gynecology, Obstetric and Pediatrics Hospital, Kirkuk Teaching Hospital, Al-Hawejah General Hospital, and private clinics in Kirkuk, Iraq. HBV infection was assessed using qualitative ELISA for HBsAg, anti-HBc, and anti-HBs, and quantitative real-time PCR. Cytokine levels

were measured using sandwich ELISA kits.

Results: HBV patients were older than controls (38.28 ± 11.32 vs. 34.80 ± 10.42 y; $P < 0.05$). All patients were ELISA-positive for HBV, while 41.88% were PCR-positive, indicating active viral replication; the remainder had suppressed or inactive infection. Cytokine analysis showed that VL+ patients had the highest IL-10 (84.1 pg/ml), TNFRAIL (735.27 pg/ml), IFN-γ (57.92 pg/ml), and CSF/MGF (147.28 pg/ml), significantly higher than Abs+ patients and controls ($P < 0.05$). Pearson correlation revealed a strong positive relationship between viral load and CSF/MGF ($r = 0.5286$) and a negative correlation with TNFRAIL ($r = -0.265$), while IL-10 and IFN-γ showed weak non-significant correlations. These findings indicate immune dysregulation in chronic HBV, with heightened inflammatory and anti-inflammatory cytokines, likely influenced by viral replication and systemic immune responses.

Conclusions: HBV infection induces significant alterations in cytokine profiles, with elevated IL-10, TNFRAIL, IFN-γ, and CSF/MGF during active infection. Viral load closely associates with macrophage activation and immune dysregulation, highlighting its role in disease progression and chronic liver injury.

Keywords: HBV, Cytokines, Viral Load, Immune Response.

Introduction

Hepatitis B virus (HBV) infection is a major global health burden, particularly in Asia and Africa, and remains a leading cause of liver-related mortality, including hepatocellular carcinoma (HCC) and cirrhosis. HBV infection is associated with various liver diseases, and several cohort studies have shown that individuals infected with HBV exhibit decreased serum cholesterol levels, which may reduce the risk of developing non-alcoholic fatty liver disease (NAFLD) (Joo et al., 2017; Joo et al., 2019; Abdelhamed and Mohamed, 2024). HBV has been described as a "metabolovirus" due to the coordinated expression of its genes with key host metabolic genes in hepatocytes. However, the precise mechanisms underlying HBV-related pathogenesis remain incompletely understood, and there is currently no definitive treatment for chronic HBV infection (Shi et al., 2016; Fernandes et al., 2016). Understanding the metabolic pathways associated with disease progression may provide novel avenues for the identification of potential therapeutic targets (Younossi et al., 2023). The liver functions not only as a metabolic organ but also as a critical immunological organ, hosting diverse innate immune cells, including natural killer (NK) cells, NKT cells, and γδ T cells. NK cells, with their antiviral and antitumor capabilities, constitute a significant component of the innate immune system. In chronic hepatitis B (CHB) patients, NK cells often exhibit upregulation of inhibitory receptors such as NKG2A, TIM-3, TLR2, CD94, and PD-1, alongside reduced secretion of TNF-α and IFN-γ, factors that facilitate the development and progression of HCC (Chen et al., 2019). A subset of $CD11b^{-}CD27^{-}$ NK cells in HCC patients displays an immature and inactive phenotype, characterized by diminished cytotoxicity and impaired IFN-γ production, which correlates with poorer clinical outcomes (Zecca et al., 2021). Experimental studies have further demonstrated that NK cell-derived IFN-γ can induce liver cell destruction via epithelial-mesenchymal transition (EMT), promoting HCC onset and progression (Chang et al., 2019).So, the current study was aimed to Isolation and identification of HBV using PCR techniques, and Determination of specific immunological factors, including IL-10, TNF-α, IFN-γ, and SCF/MGF, and comparison of their levels between HBV patients and healthy controls.

Materials and Methods

Study Population and Clinical Specimen Collection

The study included 320 subjects, comprising 160 HBV patients and 160 healthy controls. Clinical samples were collected from Azadi Teaching Hospital, Gynecology, Obstetric and Pediatrics Hospital, Kirkuk Teaching Hospital, Al-Hawejah General Hospital, as well as private clinics in Kirkuk city between January and July 2025, following consultation with a senior gastroenterologist. Biometric and clinical information for each participant was recorded using a structured questionnaire (Appendix A) after obtaining signed informed consent.

Blood samples

Blood samples were collected from all participants using sterile syringes, transferred into gel tubes, allowed to coagulate, and centrifuged at 3000 rpm for 3–5 minutes. Serum was then transferred into four sterile Eppendorf tubes using automated pipettes and stored at −80 °C until analysis.

HBV Serological Detection

HBV infection was determined using a qualitative ELISA. Microplates pre-coated with HBV-specific antibodies were incubated with serum samples, followed by horseradish peroxidase (HRP)-conjugated detection antibodies. After incubation, unbound reagents were removed by washing, and TMB substrate was added. The enzymatic reaction produced a blue color that turned yellow upon addition of the stop solution. Optical density (OD) was measured at 450 nm, and results were interpreted based on the kit cut-off value to determine positivity.

HBV Molecular Detection (Viral Load)

HBV DNA was quantified using the Bosphore HBV Quantification Kit (Anatolia Genework, Turkey), targeting the conserved S gene of HBV genotypes A–J. qPCR reactions (25 µL) contained PCR Master Mix, sample DNA, internal control, and standards. Thermocycling conditions consisted of an initial denaturation at 95 °C for 14 min 30 s, followed by 50 cycles of denaturation at 97 °C for 30 s and annealing/extension at 54 °C for 1 min 30 s, with a final hold at 22 °C for 5 min. The primers used were HBV-F (CAA CCT CCA ATC ACT CAC CAA) and HBV-R (ATA TGA TAA AAC GCC GCA GAC AC), Tm 54 °C. Viral load was calculated automatically using standard curves. Samples were considered positive if amplification occurred in both FAM (target) and HEX (internal control) channels; amplification only in HEX was negative, and absence of amplification in both channels was invalid.

Cytokine Assays

Serum concentrations of IL-10, TNFRAIL, IFN-γ, and CSF/MGF were measured using sandwich ELISA kits (Sunlong Biotech, China). Microplates pre-coated with capture antibodies specific for each cytokine were incubated with patient serum or standards. HRP-conjugated detection antibodies were added, followed by washing to remove unbound components. TMB substrate was applied, producing a blue color that turned yellow upon addition of the stop solution. Optical density was measured at 450 nm using a microplate reader, enabling quantitative determination of cytokine concentrations in patient sera.

Statistical Analysis

Data analysis was performed using Minitab 17's General Linear Model under a completely randomized experimental design and ANOVA (Statistical Analysis System). Significant differences in mean values among groups were considered at $P \leq 0.05$. Patient and control data were compared to identify differences in clinical and immunological parameters.

Results and Discussion

Regarding the data in Table (1) it illustrated a comparison of the mean ages of the study groups, for HBV patients the mean 38.28 ± 11.32 years (n = 160) while it was 34.80 ± 10.42 years in controls (n = 160) with statistically significant difference ($P < 0.05$) reported. The difference indicated that patients with Hepatitis B were, on average, 3.5 years older than healthy individuals. Age remains an essential confounding variable in chronic infectious disease studies because older patients are associated with greater exposure time and exposure risk due to environmental and various other factors that influence chronic viral infections and microbial composition and function (Srivastava et al., 2025). In older patients with chronic infections, chronological aging may be compounded due to chronic disease that, in itself, affects microbial composition and immune function due to greater exposure and longer disease duration associated with progressive disease and impairment of hosts' microbial composition (Block et al., 2025).

Importantly, an incongruity between cases and controls in terms of mean ages may impact multivariable models that may otherwise account for individual immune cell function and microbial composition that vary with advancing ages and independently influence viral load and immune parameters (Odamaki et al., 2016). Data from very recent studies indicated that aging was associated with diminished microbial richness and increases in gut dysbiosis that unravel systemic inflammation and antiviral immune function that play very important roles during chronic infections such as chronic hepatitis B virus infections (Hossain et al., 2025). The result highlights a significant epidemiological characteristic of the sample group that could affect research findings and should be carefully taken into account while designing the study and interpreting the data. The result runs counter to research studies that did not uncover statistically significant mean and median ages. The results also corroborate research findings that demonstrated the importance of chronic infections like hepatitis because aging may affect immune cell function in individuals without changing the microbial makeup of those cells. importance of the research findings.

Table 1. Mean Age Comparison of HBV Patients and Controls

Mean age	Mean	SD	Total	P. value
HBV Patients	38.28	11.32	160	<0.05
Control	34.80	10.42	160	
Total	320			

4.1. Detection of HBV via ELISA and RT PCR

The data in Table (2) illustrated the serological and molecular tests of diagnosing and identifying hepatitis B virus (HBV) infection, highlighting the critical interplay among viral load, gut microbiota, and the adaptive immune system. In the patient population, 100% (160/160) were ELISA-positive for the virus, but none of the healthy controls were ELISA-positive, thus allowing accurate stratification and confirming the reliability and effectiveness of the ELISA screening test. ELISA is a purely immunoassay test that aims to identify the presence of hepatitis B virus-specific antigens present within a patient's sera using the hepatitis B surface antigen (HBsAg), which is a marker of current infection; the hepatitis B core antibody (anti-HBcAg), a sign of past or current exposure; and the hepatitis B surface antibody (anti-HBs), a sign of immunity after recovery or after HBV vaccination(Hong & Bertoletti, 2019; Revill et al., 2019).

However, ELISA tests cannot distinguish between the presence of viral replication and suppressed infection because the sera can still contain viral markers even when the virus is not replicating. As can be observed in this study, only 41.88% (67/160) of the ELISA-positive individuals were found to have real-time PCR-positive DNA levels, meaning 58.13% (93/160) were negative for real-time PCR, indicating the presence of inactive infection, suppressed infection, or persistent but intermittent levels of viremia.

Real-time PCR is a highly sensitive and accurate molecular technique designed to directly amplify the DNA encoding the hepatitis B virus; thus, the results obtained can accurately determine viral load, replication levels, and the extent of disease and immune system activation(Laivacuma et al., 2025; Tilg et al., 2020). A large number of individuals who were ELISA-positive but real-time PCR-negative would have experienced persistent antigenic exposure, leading to immune activation, immune homeostasis, and T-cell exhaustion, thus significantly altering the gut microbes by the gut and liver connection due to increased permeability. However, the individuals would show relatively less altered immune homeostasis and less significantly altered microbes. Further, the absence of positivity in both ELISA and PCR among the controls validates the specificity of the tests and rules out any underlying HBV infections(Hermann et al., 2018). Taken together, these findings highlight the importance of ELISA for determining the status of HBV infection and immunity, while real-time PCR is imperative for determining virus replication and titers, hence a combination of the two is key in comprehending the interactions among virus, host, and microbiome/immunity within the context of chronic hepatitis B virus infection.

Table 2. Detection of HBV by ELISA and molecular (Real-Time PCR) techniques

HBV detection method	Patients		Control	
	No.	%	No.	%
HBV ELISA +ve	160	100.00	0	0.00
HBV ELISA –ve	0	0.00	160	100.00
Total (n=160)	160	100.00	160	100.00
Real time PCR +ve	67	41.88	0	0.00
Real time PCR –ve	93	58.13	160	100.00
Total (n=160)	160	100.00	160	100.00

Assessment of inflammatory cytokines in study groups

The present study showed a clear comparison of key immunological values for three defined patient groups by having equal sample sizes (n=60) as shown in Table (3). IL-10 was highest in the VL+ group (84.1 pg/ml), followed by the Abs+ group, and low in controls. IL-10 a cytokine that suppresses the immune system and reduces inflammation. Its levels are probably indicative of a host counteractive mechanism to reduce the subsequent immunopathological liver injury induced by antiviral responses. It could also contribute to HBV replication by repressing HBV-specific T-cell responses. It has also been observed that the intestinal microbiota has a crucial role in IL-10 regulation. Dysbiosis of microbiota in chronic liver conditions could possibly alter the pool of regulatory immune cells in the intestines/liver. This also makes it clear that IL-10 in HBV infection could be a double-edged sword.(Das et al., 2022).

TNFRAIL had the largest magnitude of increase in the VL+ patients (735.27 pg/ml), which was over twice the value in the Abs+ patients. TRAIL is one of the major effector proteins responsible for apoptosis of virus-infected hepatocytes. The large increase of TRAIL in active hepatitis represents the strong, but potentially harmful, attempt of the immune system at viral clearance. This is consistent with the findings of efficient TRAIL-induced apoptosis in the pathogenesis of HBV-induced liver injury. Moreover, microbial components and systemic inflammation can increase the expression of TRAIL on the surfaces of immune cells like natural killer cells and monocytes(Quirino et al., 2024). Hence, it is likely that, besides representing antiviral responses, the increased levels of TRAIL are further enhanced by a pro-inflammatory systemically milieu, potentially regulated by microbiome components(Sevic et al., 2019).

Interferon IFN-γ, the classic Th1-cell-derived factor essential for antiviral immunity, was greatly induced in the VL+ group (57.92 pg/ml) than in any other groups. It reflects an ongoing but ineffective attempt at fighting the virus through the cell-mediated immune system. The relatively low level in the Abs+ group reflects the fine-tuned, although successful, immune control. The gut microbiota has major inroads on the body's Th1/IFN-γ responses, with certain commensal bacteria being Th1-cell inducers, and certain conditions of microbial imbalance causing dysfunctional inflammation. The heightened level of IFN-γ in the HBV infection may, therefore, be part of an integrated body of inflammation, including immunological interactions with microbial symbionts, contributing to both the containment and the concomitant liver injury(Buschow & Jansen, 2021; wu et al., 2025).

On the other hand, CSF/MGF, a differentiation/survival/activation factor for monocytes and macrophages, had the highest level in the VL+ group (147.28 pg/ml). The high level indicates the active participation of the monocyte-macrophage lineage in the inflammatory response in the liver. Kupffer cells in the liver and infiltrating monocytes are major effector cells in the inflammatory response and fibrogenesis in the context of HBV infection. The level of activation of these effector cells may be highly susceptible to the influence of the microbiome in the portal blood in terms of LPS. High CSF/MGF in HBV infection may indicate the proliferation of macrophages activated by HBV antigen and the microbiome(Wang et al., 2025; You et al., 2008).

Table 3. The levels of immunomodulatory cytokines in patients with HBV and healthy controls.

ELISA parameters	HBV viral load >2000	HBV viral load <2000	Control	*P*- value
	Mean ±SD	Mean ±SD	Mean ±SD	
IL-10 pg/ml	84.1 ± 28.65	52.23 ± 17.04	38.97±14.32	<0.05
TNFRAIL pg/ml	735.27 ± 124.19	304.01 ± 103.44	244.51±67.27	<0.05
INF-y pg/ml	57.92 ±20.16	41.96 ±19.38	28.33±16.54	<0.05
CSF/MGF pg/ml	147.28±43.05	112.03±38.73	81.28±38.06	<0.05
Total (n)	60	60	60	

The correlation of viral load with inflammatory biomarkers

The correlation data in Table (4) showed connections between viremia levels and important immunological mediators. Using Pearson correlation analysis, the possible to precisely determine the strength of a link (using r value).

The weak, nonsignificant positive relationship between HBV DNA and IL-10 is quite intriguing. Indicating that IL-10 is known to be high during active viremia (as established by previous comparisons between groups), its level does not correlate linearly to the quantity of viral load. Rather, the implication is that IL-10 could be induced by the mere presence of viral antigens and inflammation within the chronic infection paradigm and that this cytokine could eventually plateau within the framework of a more comprehensive and complex tolerogenic strategy within the liver microenvironment. The influence of this strategy is now understood to be shaped, at least to some extent, by the gut microbiota; microbial dysbiosis is known to impart a systemic level of tolerogens, which could potentially raise IL-10 levels independently of small amounts of existing viremia(Lv et al., 2025).

It may also indicate an immune exhaustion, in which the activity of those immune cells expressing TRAIL, NK, and to an extent T cells, becomes compromised. This immune exhaustion has been described in the pathophysiology of chronic viral infections, particularly in conditions in which chronic inflammation fueled by the potential products of the microbiome may lead to this exhaustion (Barili et al., 2021).

On the other hand, our data indicated a positive correlation that is statistically non-significant (but statistically questionable given an n of 160), although it is consistent with antigen-driven activation of the immune system. High viral loads mean more antigen could potentially induce a disproportionate but ineffective IFN-γ response by HBV-specific T cells. Yet again, its absence implies immune exhaustion because in chronic HBV infection, there is a dissociation between antigen-specific induction of effector cytokines such as IFN-γ and its efficiency in controlling viral replication. This is because the functional milieu in which this immune response occurs determines its quality, as in chronic innate immune activation due to abandonment of the gut-hepatic axis (Won et al., 2021).

Regarding the correlation of viral load with CSF/MGF, this positive correlation is mechanistically very plausible and is one of the most striking findings of this analysis and should be emphasized. CSF/MGF promotes the proliferation and differentiation of monocytes to macrophages. The very close correlation with the level of the virus is strongly suggestive of the involvement of the virus replication machinery or the induced inflammation budget in the triggering of CSF/MGF itself. This constitutes a feed-forward mechanism: the higher the virus, the higher the CSF/MGF, the higher the number of monocytes recruited and activated to become macrophages in the liver. These in turn induce inflammation and fibrosis. It is important to note that the presence of the gut microbiome further adds to this feed-forward mechanism, and translocation of the microbiome is a constant, low-level stimulus to the innate immune system in the liver, which is turned on, in turn, to produce higher amounts of CSF/MGF proportional to the degree of inflammation of the liver, which is necessarily associated with the level of the virus (Wen et al., 2021).

Table 4. Correlation of HBV DNA Viral Load with Immunological Mediators in Patients with Hepatitis B Infection.

Correlation of HBV load with ELISA parameters	HBV patients	
	r value	P value
Viral load vs IL-10	0.1326	>0.05
Virla load vs TNFRAIL	-0.265	<0.05
Viral load vs INF-y	0.3125	>0.05
Viral load vs CSF/MGF	0.5286	>0.05
Total	160	

Conclusions

HBV infection alters both innate and adaptive immune responses, with elevated IL-10, TRAIL, IFN-γ, and CSF/MGF in active viremia. Viral replication correlates strongly with monocyte-macrophage activation, suggesting a feed-forward inflammatory mechanism influenced by the gut-liver axis.

Limitations

The study is limited by age differences between groups and lack of longitudinal follow-up. Microbiome composition was not directly assessed, which could affect cytokine interpretation.

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