International Journal of Medical Toxicology & Legal Medicine **Volume 27, No. 3S, 2024**
e-ISSN: 0974-4614
p-ISSN: 0972-0448

Study of IL-12 Immune Response and its impact on viral load in Hepatitis B Patients

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Received: 18.09.2024 Revised: 10.10.2024 Accepted: 23.11.2024

ABSTRACT
More than 250 million people are living with chronic hepatitis B despite the availability of highly effective vaccines and oral antivirals. Although innate and adaptive immune cells play crucial roles in controlling hepatitis B virus (HBV) infection, they are also accountable for inflammation and subsequently cause liver pathologies. IL-12 was found first and is effective in combatting a wide range of naturally occurring viral infections through the up regulation of various cytokines to clear the infected cells. Hepatitis B Virus DNA Quantification ("viral load"). Aim of study to show the importance of measuring the HBV-DNA viral load and its role for the effective diagnosis and monitoring of hepatitis B. Additionally, they help in the long-term monitoring of the patient's condition to assess their response to treatment or determine if changes to the treatment plan are necessary. The study of IL-12 in hepatitis B (HBV) patients is important for understanding the immune system's role in fighting the virus. IL-12 enhances the response of T cells and natural killer (NK) cells, which helps reduce viral replication. The result of viral load shown that the highest number of patients was found in category 1 (>2000) which was 61 (71.76%) . The result of expression of IL-12 significantly ($p=0.002$) was up-regulated in the HBV patients as compared to healthy individuals.

Keywords: HBV, IL-12, viral load , Cytokines

INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus with approximately 3200 base pairs. Approximately 350–400 million people are chronically infected with HBV and more than 3 billion people have been exposed to HBV worldwide (Lavanchy,2004; Yokosuka et al.,2011). Hepatitis B virus is one of the smallest human pathogens, encoded by a 3,200-bp genome with only four open reading frames HBV induces a variety of liver diseases, ranging from acute or fulminant hepatitis to liver cirrhosis and hepatocellular carcinoma. HBV is one of the most important causes of liver cirrhosis and hepatocellular carcinoma (Michitaka et al.,2010). On the other hand, hepatitis is self-limited in most adult patients with acute infection. Meanwhile 1-2% of patients progress to fulminant hepatic failure, and some progress to chronic infection. The rate of progression from acute to chronic HBV infection is reported to be 90% in newborns and 5–10% in adults (Yim, and Lok, 2006; Hoofnagle et al.,2007). HBV can be classified into at least 8 genotypes with a divergence of more than 8% of nucleotide sequences (Okamoto et al.,1988; Kurbanov et al.,2010). There are some differences in clinical features and routes of transmission between genotypes (Chu et al.,2003; Lin and Kao,2011). Hepatitis B Virus DNA Quantification ("viral load") – This blood test measures the amount of hepatitis B virus DNA (or viral load) in the blood of chronically infected patients. The blood is tested using a Polymerase Chain Reaction (PCR) technique that is highly sophisticated and accurate.

Class I cytokines are further classified according to their receptor subunits and one group of them are interleukin (IL)-12 family cytokines which are considered as the critical bridge between innate and adaptive immunity .Interleukin (IL)-12 family cytokines including IL-12, IL-23, IL-27, IL-35 and IL-39 are heterodimeric cytokines composed of two subunits, an α-chain (entitled p35, p19 and p28) and a β-chain(Wang et al.,2023)**.** T cell receptors, in the presence of substances that stimulate these receptors (such as CD28) and pro-inflammatory cytokines (such as IL-12), are indispensable for sending signals to trigger CD8 T cells that can specifically recognize HBV antigens. Similar to CD8 T cells, CD4 T cells are also activated when co-cultured with IL-12 and cause HBV clearance **(**Chuai et al.,2019**).** In addition, IL-12 inhibits PD-1 in this kind of infection, increases T-bet, and promotes IFN-γ and TNF-α production for immunity **(**Schurich et al.,2013**).**

MATERIAL AND METHOD

Samples collection

study included the collection of 85 HBV patients and 20 of healthy individuals as a control group for comparison , HBV patients divided to 47 males and 38 females, ranging from (20- 75) year , with median age 41 year. Patients were outpatients attending Al-Karama Teaching Hospital in AL-Kut City/ Wasit Province / Iraq. This work was carried out in the College of Science, Department of Biology, center gene x , PCR unit at AL-Karama Teaching Hospital in Wasit Province/Iraq ,from December 2022 to June 2024.

Three milliliters of peripheral blood were collected from patients. blood were used to isolate RNA and DNA .Patients co-infected with hepatitis C and HIV and other autoimmune diseases, as well as the pregnant women were excluded from our study.

Quantitative Real-Time PCR (qPCR)

The quantitative Real-Time PCR used in quantification of IL-12 gene expression analysis that normalized by housekeeping gene (GAPDH) in patient and healthy blood samples by using Real-Time PCR technique. This method included several steps :

1- Total RNA Extraction

Total RNA were extracted from blood samples by using (GENEzol™ TriRNA Pure Kit, Taiwan) and done according to company instructions as following steps Kit Contents. RNA used to measure the expression level of IL-12gene**..** The extracted RNA was checked by using Quantus™ Fluorometer (Promega. USA), using QuantiFluor® RNA Dye that check RNA concentration.

2-cDNA synthesis

RNA samples were also used in cDNA using EasyScript® One-Step cDNA Synthesis SuperMix and done according to company instructions

3- RT- qPCR Primers

The Real Time PCR primer that used in gene expression of IL12gene , and housekeeping GAPDH gene , they were designed in this study by using NCBI Genbank database and perimer.

4.Data analysis of qRT-PCR

The data results of q RT-PCR for target and housekeeping genes were analyzed by the relative quantification gene expression levels (fold change) (The ΔCT Method Using a reference gene) that described by (Livak and Schmittgen, 2001).

- **DNA Extraction from Virus**

According to the manufacturer's instructions of (gSYNC^{TM} DNA extraction kit ,USA), DNA extraction was completed

- **Viral load (Quantification assays of HBV-DNA)**

According to the manufacturer's instructions of (Sacace Biotechnologies kit , Como – Italy), Quantification assays was completed

Statistical analysis

The collected results were analyzed using SPSS (V.20, IBM); independent t-test and One Way ANOVA test were used as appropriate, by calculating least significant difference (LSD) to find p value between the studied groups. The results were presented as Mean±S.E. and the p value ≤0.05 was considered as significant result. Chi square $\varkappa^2$ test was used for obtaining p value between number and percentage

RESULTS

- **IL-12 gene relative expression level**

Furthermore, this study also measured the fold expression of IL-12 using RT-qPCR technique, and it was shown that the expression of IL-12 significantly (p=0.002) was up-regulated in the HBV patients as compared to healthy individuals (Table 1). The fold expression were (1.009±0.054) and (2.288±0.4) in control and HBV patients respectively.

Table 1: IL-12 gene relative expression level

Parameter	Groups	Gene expression (Folding) (Mean±S.E.)	P value
IL-12	**Control**	1.009±0.054	0.002**
	HBV Patients	2.288±0.4	

**** Significant differences**

In the comparison in expression of IL-12 between HBV patients and control group, the statistical results showed a significant value (p=0.002) .

The result agree with many study such as s (He et al., 2012; Wu et al., 2015), suggesting serum levels of IL-12 may be an available marker to evaluate cellular immunity for HBV infection. Elevated IL-12 rescues the anti-viral function of exhausted HBVspecific CD8+ T cells, enhances the anti-virus properties of cytotoxicity, polyfunctionality, and multispecificity of HBVspecific T cell. On the other hand , another study by Wang et al., (2015). showed that IL-12 levels in the blood and its expression in the liver were significantly elevated in patients with chronic hepatitis B (CHB) because Interleukin-12 (IL-12) is a common proinflammatory cytokine that has a crucial function in the host's immune response to infections, including the Hepatitis B Virus (HBV). Xiong et al., (2007); Wu et al. (2015); Zeng et al. (2013); Carreno et al. (2000) also explain the role of IL-12 in HBV infection it is Promotes cellular immunity and modulates the cytotoxic activity of CLTs and NK cells and Enhances the anti-virus properties of cytotoxicity, polyfunctionality, and multispecificity of HBVspecific T cells; combination treatment with IL-12 favors HBV clearence .

Heufler et al., 1996 also explain the role of IL-12 is mostly synthesized by dendritic cells (DCs), monocytes, and macrophages, with a lower contribution from B cells, which principally facilitate the development of naïve T lymphocytes. become Th1 cells, acting as a connection between innate and cellular Immunity. Rossol et al., **(**1997 **)**It additionally fosters the proliferation affects the survival of activated T cells and NK cells, while modulating the cytotoxic action of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. In the adaptive immune response, IL-12 Primed antigen-specific T-cells for elevated IFN-γ synthesis. Interleukin-12 has the capacity to further function as an adjuvant for humoral immunity by augmenting Production of antibodies (Abs) by B cells Metzger et al., (1997).

HBV-induced IL-12 expression is mediated by HBx stimulation of the PI3K-Akt pathway, resulting in the transactivation of IL-12. Promoters p35 and p40 (Wang et al., 2015). Serum concentrations of IL-12 are correlated with alanine aminotransferase (ALT) levels, and the elevated serum levels of IL-12 were associated with HBeAg or Seroconversion of HBsAg in both acute hepatitis B (AHB) and chronic hepatitis B (CHB) patients (He et al.,2012; Wu et al., 2015), indicating that serum concentrations of IL-12 may be accessible biomarker to assess cellular immunity for HBV infection. Increased IL-12 restores the antiviral function of fatigued HBV-specific CD8+ T cells and amplifies their antiviral characteristics. Cytotoxicity, polyfunctionality, and multispecificity of HBV-specific T cells. Moreover, IL-12 markedly reduces the Pro-apoptotic molecule Bim, which can facilitate premature depletion of HBV-specific CD8+ T cells (Xiong et al., 2007; Wu et al., 2015). Co-stimulation with IL-12 is observed to be considerably enhance the HBs/e/cAg-specific production of IFN-γ (Vingerhoets et al., 1998; Szkaradkiewicz et al., 2005). Furthermore, there exists a distinct agreement that CHB patients have access to currently accessible anti-HBV treatments Therapy with elevated levels of IL-12 is correlated with beneficial results (Ozkan et al., 2010)

Low levels of IL-12 may be associated with disease progression to liver fibrosis or cancer, making it a potential target for new treatments that support immunity and reduce the risk of disease advancement.

Viral Load (Quantification assays of HBV-DNA)

The current study included the determination of viral load in 85 HBV patients as it is crucial factor for detecting the HBV disease. The viral load was measured using RT-qPCR technique in the patients via detection the number of viral-DNA copies.

As shown in table (2) and figure (2), this study found that the number of viral copies was ranged from $0.8x10^1$ to $3.05x10^7$ IU/mL and the mean was $1.2x10^7$ IU/mL. According to the results of RT-qPCR results and the levels of HBV-DNA, the patients were classified into three major categories including category 1 with HBV-DNA level <2000 IU/mL, category 2 with HBV-DNA level 2000-20000 IU/mL and category 3 with HBV-DNA level >20000 IU/mL. The results were described in table (2) noticed that the highest number of patients was found in category 1 (>2000) which was 61 (71.76%) while equal number of patients was found with category 2 (2000-20000) and category 3 (>20000) where the number was 12 (14.12%) in each category. Moreover, the statistical analysis revealed that there is a significant differences ($p<0.001$) was showed between the number and percentage of three categories of HBV patients depending on the viral load (HBV-DNA) as mentioned above. In addition, only six patients were with high titer of DNA viral copy number (about more than 256020), and this may play an important role in the progression and development of HBV disease.

Table 2: Distribution of HBV patients according to viral load

HBV DNA Viral load (IU/ml)	**NO.**	**Percentage (%)**	**Chi $\varkappa^2$**	**P value**
<2000	61	71.76%	56.5	<0.001**
2000-20000	12	14.12%		
>20000	12	14.12%		
Total	85	100		
Range	9-$3.05x10^7$			

Mean (IU/mL	$1.2x10^7$		

** **Significant differences.**

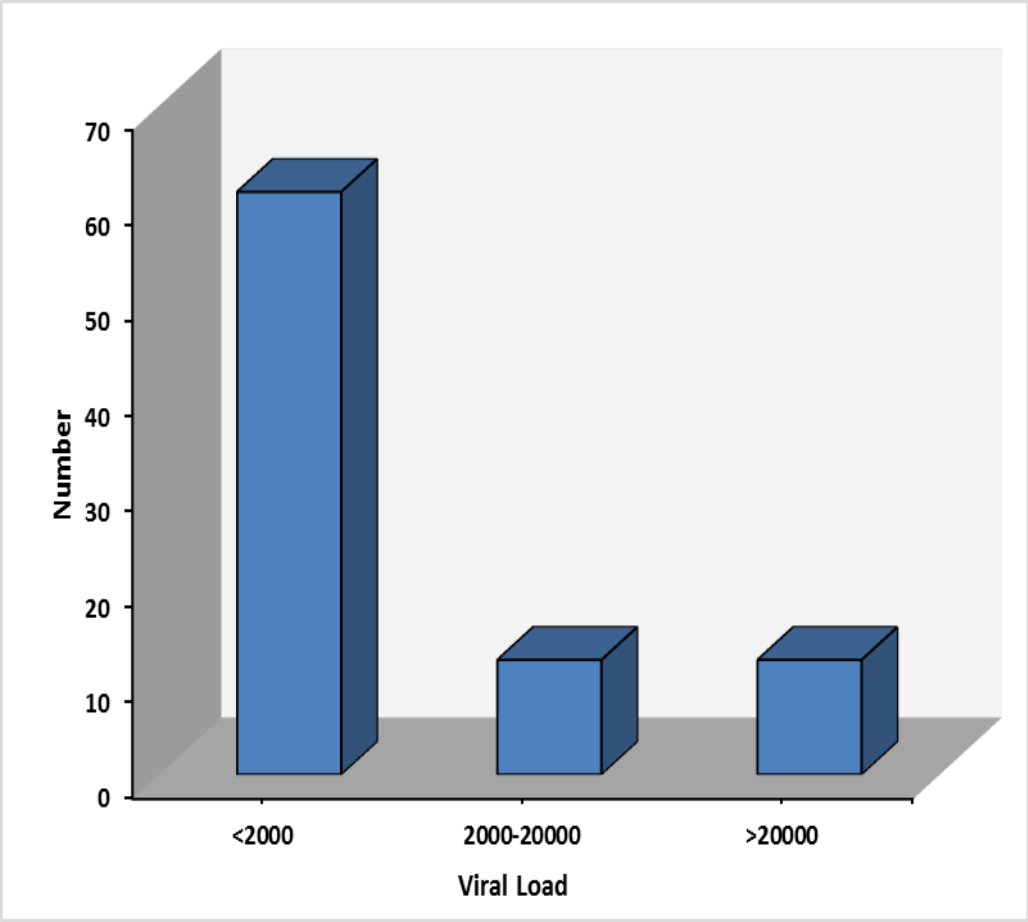

Figure 2: Distribution of HBV patients according to categories of viral load

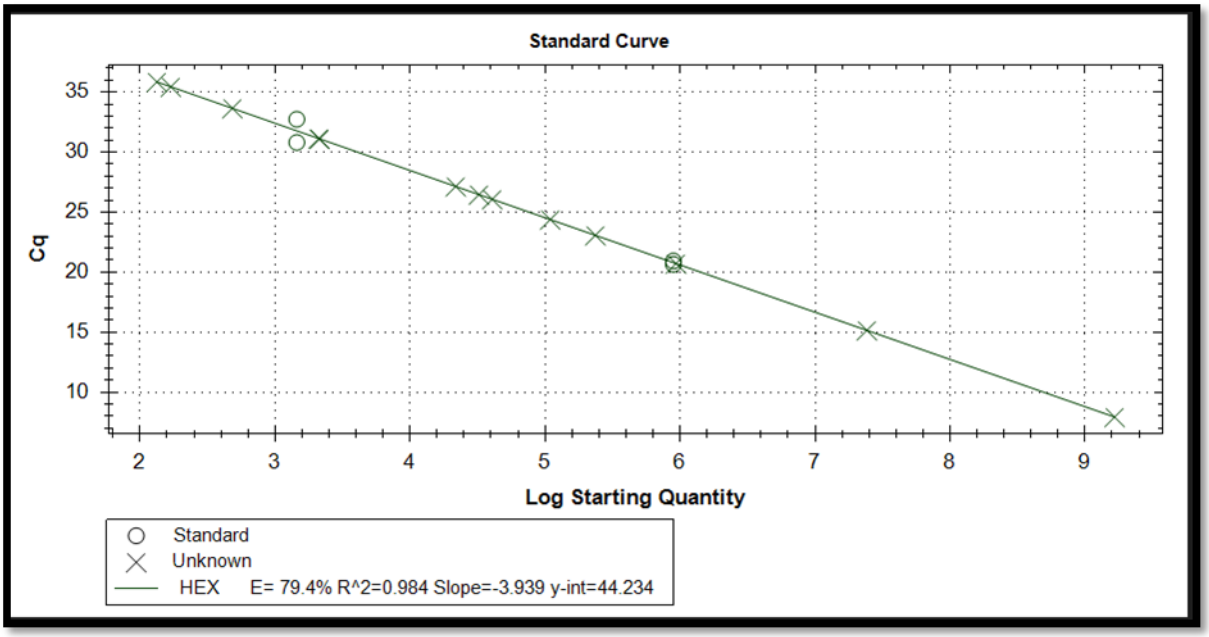

Figure 3: Standard curve of the definite 4 standards and samples by HEX filter.

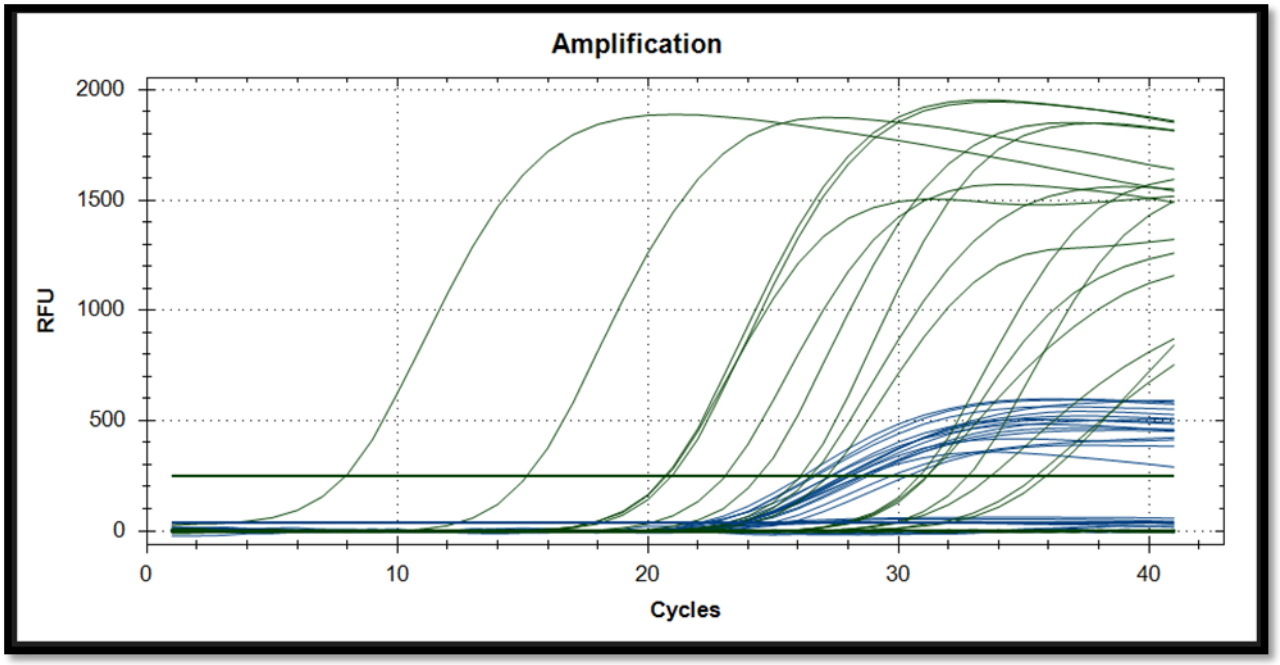

Figure 4: Amplification plot for qPCR viral load(HBV)

Chen, G etal., 2006 explain in study found a significant increase in mortality rates for HCC and CLD in individuals with high viral load compared to those with low viral load. The relative risk for HCC mortality was 1.7 in low viral load and 11.2 in high viral load groups, with high viral load risk not changing with follow-up time. Yu et al., 2005 sample with very high viral copy number may lead to have progressive liver diseases such as cirrhosis or HCC.

Ohkubo et al .,(2002) The study found that viral load is a useful prognostic marker for HBV-related HCC, with patients with a less favorable course either clearing the virus poorly or having a greater level of virus production. A low level of viremia was associated with longer survival, even in patients negative for the hepatitis B antigen.

Viral load (HBV-DNA) according to Sex

The correlation between the viral load and Sex

The HBV patients in three categories of viral load were divided according to the sex into male and female. As shown in table (3) and figure (3), which illustrated the number and percentage of male and female in each category of viral load in the patients, it was observed that there is no significant difference in the number between male and female (p=0.07). Although, the highest number of both sex was found with <2000 IU/mL category which was 36 and 24 in male and female respectively, while it was 8 and 4 in male and female respectively in category 2 (2000-20000), and was 3 and 9 in male and female respective in the category 3 (>20000) (Table 3, Fig 3).

Table 3: The correlation between viral load and sex

DNA Viral load (IU/ml)	Male No. (%)	Female No. (%)	Total	Chi χ^2	P value
<2000	36 (42.35%)	25 (29.41%)	61	5.43	0.07 NS
2000-20000	8 (9.41%)	4 (4.71%)	12		
>20000	3 (3.53%)	9 (10.59)	12		
Total	47 (55.29%)	38 (44.71%)	85		

NS= Non-significant

This result coincided with Parizad et al ., (2016) found no correlation was observed between viral DNA titer in serum samples with the sex. While Jia et al ., 2019 found a correlation between the HBV DNA level with sex, and this may be due to the opposite effects of the sex hormones androgen and estrogen (Wang et al .,2015)

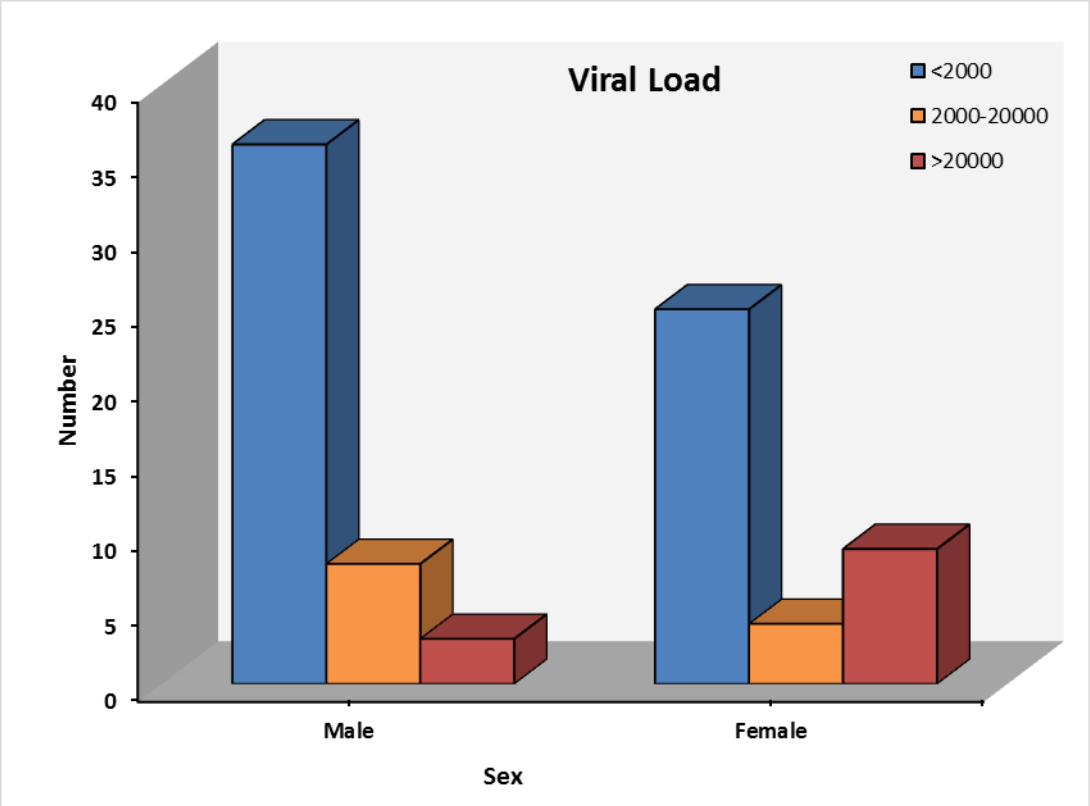

Figure 3: The correlation between viral load and sex

Viral load according to AGE

The correlation between the viral load and Age

The present study also studied the correlation among the viral load categories according to the age of HBV patients, and it was observed that there is no significant differences (p=0.09) in the three studied categories of viral load between all the age groups (Table 4, Fig 4). However, the high number of HBV patients was seen in viral load category 1 (<2000) with 20-30 age group (n=21) followed by 31-40 (n=15), 41-50 (n=10), 51-60 (n=8) and 61-75 group (n=7). While in category 2 (2000-20000) the high number was also in the age group 20-30 (n=7) followed by 61-75 group (n=2) while the number in all other age groups was (n=1). In addition in the category 3 of viral load, the high number was in the age group 41-50 (n=6) followed by 51-60 (n=2), 61-75 age groups (n=2) and the number was (n=1) in the age groups 20-30 and 31-40 (Table 4, Fig 4)

Table 4: The correlation between viral load and age groups

Viral load / Age groups	<2000	2000-20000	>20000	Total
	NO. (%)			
20-30	21 (24.71%)	7 (8.24%)	1 (1.18%)	29 (34.12%)
31-40	15 (17.65%)	1 (1.18%)	1 (1.18%)	17 (20%)
41-50	10 (11.76)	1 (1.18%)	6 (7.06%)	17 (20%)
51-60	8 (9.41%)	1 (1.18%)	2 (2.35%)	11 (12.94%)
61-75	7 (8.24%)	2 (2.35%)	2 (2.35%)	11 (12.94%)
Total	61	12	12	85
Pearson Chi $\varkappa^2$	13.98			
P value	0.09 NS			

NS= Non-significant

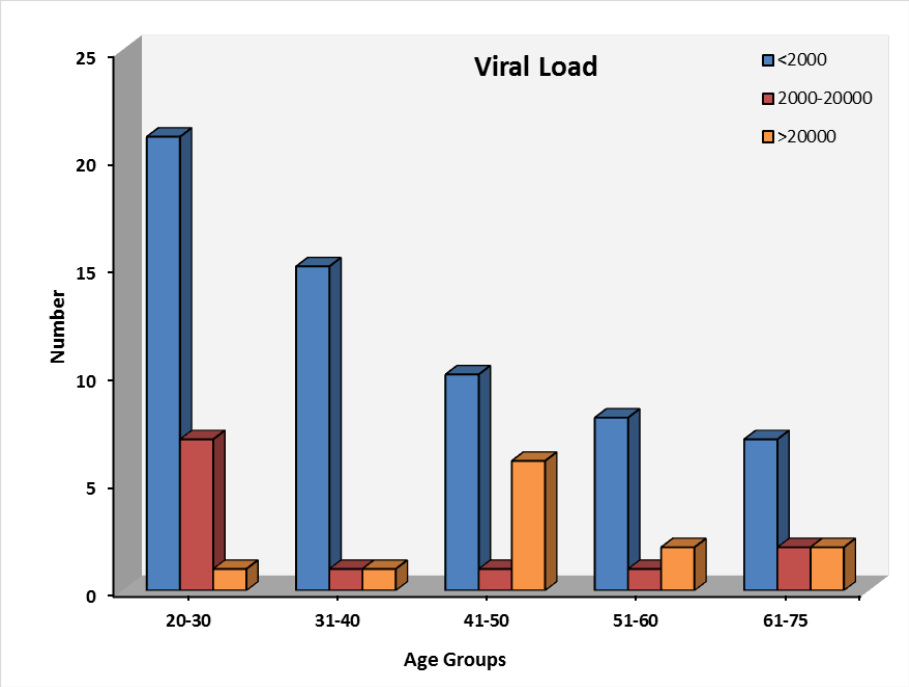

Figure 4: The correlation between viral load and age

This result agree with Hanash et al.,(2020) who showed no a significant different between viral load with age groups (P = 0.827) also Parizad et al., (2016) who found no correlation was observed between viral DNA copies in serum samples with the age of patients. Also Mendy et al., (2008) Found no substantial variation in serum HBV DNA concentrations throughout the various age demographics.
Another study's results confirmed that HBV DNA levels diminished. In elderly age (Jia et al., 2019). Tran et al. (2015) documented that younger women exhibited a markedly higher likelihood of possessing elevated HBV viral levels burden diminished with advancing age.

The correlation of viral load with the IL-12 expression.
The correlation between the viral load (HBV-DNA) and . As shown in table (5) it was found that there is no correlation between viral load and the IL-12 expression.

Table 5: The correlation of viral load with IL-12 expression

	Parameter	**IL-12 expression**
Viral Loads	**Pearson Correlation (r)**	-0.01
	Sig. (2-tailed)	0.939

Other study show the relationship between IL-12 and viral load such as Saeidi et al.,2018 & A. Rahman et al.,2023 show particularly in the context of chronic viral infections such as hepatitis B virus (HBV), is significant due to IL-12's ability to influence immune responses. IL-12, a key cytokine in the immune system, promotes the development of Th1 cells and enhances the cytotoxic activity of CD8+ T cells, which are crucial for combating viral infections. Studies indicate that IL-12 can reduce viral load by improving the functionality of exhausted T cells. It achieves this by lowering the expression of inhibitory molecules like PD-1 and increasing the levels of the transcription factor T-bet. This leads to better cytokine production, polyfunctionality, and cytotoxic responses, helping the immune system control persistent viral infections effectively. Moreover, IL-12 also reduces the expression of pro-apoptotic molecules like Bim, which helps sustain the population of virus-specific T cells essential for viral clearance.

CONCLUSION

In this study conducted on patients with hepatitis B, the results showed elevated levels of interleukin-12 (IL-12) in patients compared to the control group, while viral load levels were low in most samples. The increase in IL-12 suggests a strong immune response that may contribute to suppressing viral activity and limiting its spread, as IL-12 is one of the cytokines that stimulate the immune system.

This elevation could be part of the body's mechanism to combat the infection. On the other hand, the low viral load observed in the samples reflects the effectiveness of the immune response in these patients, which may help in controlling the virus and reducing its impact on the liver. These findings suggest that IL-12 may play an important role in combating hepatitis B, opening the door to potential therapeutic strategies focusing on cytokine modulation to enhance disease control.

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