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CYTOKINE PATTERN IN HEPATITIS - B - VIRUS POSITIVE INDIVIDUALS IN FEDERAL MEDICAL CENTRE, ASABA, DELTA STATE NIGERIA.

BY

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Abstract

Hepatitis B virus (HBV) infection is a viral infection that affects the liver causing acute as well as chronic disease. Hepatitis B virus infection has the tendency to induce immune activation and eventual release of cytokines causing an up – regulation of several genes in the human body. The study was aimed at evaluating HBV infection outcome using the pattern of expression of some cytokine genes (IFN-Y, TNF- α, IL-10, and IL-1β transcripts) and serum cytokine levels of HBV-positive individuals attending Federal Medical Centre Asaba. The objectives were to assess IFN-Y, TNF- α, IL-10, and IL-1β transcripts, and the serum cytokine levels in HBV-positive groups and compare the results with that of the control group. This was a cross-sectional study of 115 adults aged 22 – 64 years, 50 confirmed HBV-negative individuals as negative controls and 65 HBV-positive individuals. The One-step multi-test strip was used to screen all the individuals for HBV infection and ELISA and PCR were used for confirmation. Cytokine serum level was assessed using ELISA technique. Cytokine gene expression patterns was determined using Reverse Transcriptase PCR. Cytokine gene of IFN-Y, TNF- α, IL-10, and IL-1β was expressed in HBV positive group but was absent in the control group. The median serum levels of IFN-Y (P=0.001), TNF- α (P=0.001), IL-10 (P=0.001), and IL-1β (P=0.001) in the case groups were significantly higher when compared with the control group. Kruskal-Wallis test and Mann-Whitney test were used for statistical analysis. Cytokines both at gene and serum levels were overexpressed in HBV infection compared to that of HBV negative control group. Therefore, these indices could be used as markers in evaluating HBV outcome.

1. Introduction

The resurgence of hepatitis B infection is a cause for concern due to the morbidity and mortality associated with the disease. The disease could be progressive and acute or progressive but chronic in presentation. When treatment is successful the disease may go into latency or the infected individual recovers fully. However, if the treatment fails the consequences are often fatal. The study was designed to monitor the cytokine patterns in Hepatitis B-infected individuals in order to deduce possible mechanism that may be predictive of hepatitis B pathogenesis that may proffer solution for drug intervention.

Studies have shown that hepatitis B virus infection can cause up – regulation of several genes in the human body including interleukin 8 (IL -8), interleukin 6 (IL – 6), interleukin 1β (IL 1β), and tissue nechrosis factor – α (TNF - α) genes which showed increased expression patterns initially following exposure to HBV and reaching maximum expression levels 3 hours after stimulation[1]. This immunological process is orchestrated by the primary human hepatocytes (PHH) with the nuclear factor kappa B (NF –KB) and Toll–like receptor (TLR) 1- 9 as mediators[2]. HBV-induced production of inflammatory cytokines in monocytes depends on TLR2/MyD88/NFκB signaling[3]. It has been established that

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changes in cytokine secretion sequel to increased expression of some cytokines following HBV invasion of human body are directly or indirectly involved in susceptibility and progress of the disease[4].

Cytokine levels have been found to vary in some in flammat ory and immunologic diseases[5]. Studies have shown that among different hepatitis B virus-infected groups at different stages, significant differences were observed in different cytokines across those stages[6]. Distinctive cytokine profile was observed in hepatitis B virus-infected individuals on assessing pro-inflammatory cytokines [interleukin (IL)-8, tumour necrosis factor-alpha (TNF-α), IL-2, IL-1, and IL-6], anti-inflammatory cytokines [IL13, tumour growth factor-beta (TGF-β) and IL-10], immunoregulatory cytokines [IL-5, IL-15, interferongamma (IFN-γ), IL-7 and TNF- β][7]. Some cytokines like IL3 values have been found to be decreased in hepatitis B virus subjects, supplementation with external sources of these cytokines could help in improving the disease management. Pleiotrophic cytokine like IL6 values are usually elevated in hepatitis B virus infection and this is to be expected as hepatitis B virus has a pro-inflammatory effect on the liver[5]. Another study showed that hepatitis B patients expressed significantly higher levels of IL -32, IL-1, and IFN-γ transcripts as well as higher serum levels of these cytokines than healthy volunteers[8]. Interferon-γ is an antiviral cytokine often included in HBV therapy[8]. The cytokines selected for this study are IL-10, IFN- γ, TNF-α and IL - 1β. These cytokines have been widely studied. IFN- γ, TNF- α, and IL - 1β as pro-inflammatory cytokines possess strong pro-inflammatory effects whereas IL – 10 has an anti–inflammatory effect in immune response. In this study, the expression pattern of these cytokines at gene and serum levels in hepatitis B positive individuals will be assessed and the results compared with that of hepatitis B- negative control group.

2. Materials and methods

2.1 Study site. The study was carried out at Federal Medical Center Asaba.

2.2 Study Design. The study was designed such that gene Interleukin 10 transcript, Interferon-gamma transcript, tissue necrosis factor transcript, interleukin 1 β transcript) expression pattern and serum levels of the same cytokines in confirmed HBV-positive and HBV-negative individuals were evaluated.

2.3 Subject Recruitment. This is across–sectional study that employed simple random technique to recruit the participants for the study. The subjects consisted of confirmed HBV-positive subjects whereas confirmed HBV-negative individuals served as negative control. All the subjects were attending gastroenterology clinic at medical outpatient Department of Federal Medical Center Asaba.

2.3.1 Data collection. Five milliliters of blood sample was collected from each individual and dispensed into appropriate specimen bottle for the following indices; blood sample was dispensed into RNA container for cytokine RNA extraction for determination of cytokine gene, also into a plain specimen bottle for blood to clot and serum sample was obtained by centrifugation of clotted blood at 3,000rpm for 5mins for serum cytokine level estimation. Blood sample was dispensed into Ethylenediamine tetra-acetic acid (K_2-EDTA) bottle for HBV screening/ELISA. The red cells recovered after separ *Sam*ation of plasma was used for HBV DNA detection.

2.3.2 ple Analysis. Cytokine Gene Expression Pattern (interferon-gamma (IFN–γ) transcript, tissue necrosis alpha (TNF–α) transcript, interleukin–10 (IL–10) transcript, and interleukin 1beta IL–1β transcript) of HBV Positive individuals was determined using Reverse Transcriptase Polymerase Chain Reaction[9]. Total RNA was extracted using the ZR Whole–Blood RNA MiniPrep according to ZYMO RESEARCH specification. The extracted Total RNA was retro-transcribed and amplified using One Taq One-Step RT-PCR kit according to the manufacturer's specification. Selected primers were used to target cytokine gene using MJ research peltier thermal cycler polymerase chain reaction machine. The Cytokine serum level was assessed using ELISA technique.

2.4Statistical Analysis. Results obtained from this research were expressed as median and standard deviation then represented in tables and plates. Statistical Package for Social Science (SPSS) software version 26 was used in the analysis of data. Comparison among groups was analyzed using Kruskal Wallis, Mann-Whitney, and Chi-square tests. A value of $P \leq 0.05$ was considered as statistically significant.

3. Results

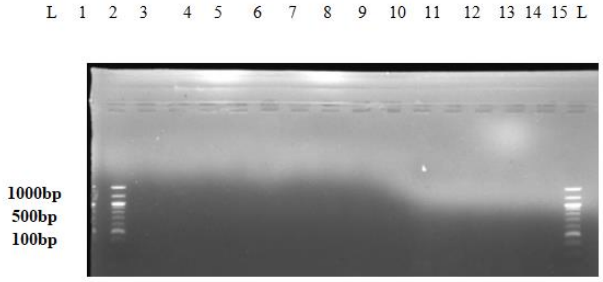

Plate 1. Reverse transcriptase PCR results for Inter Leukin-10 gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1, 2, 3, 4, 5, 6, 7,8,9,10,11,12,13,14, and 15 are negative bands for the expressed interleukin-10 genes for the control group.

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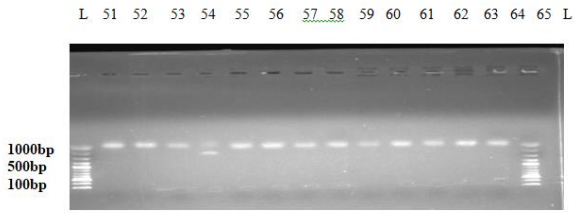

Plate 2. Reverse transcriptase PCR results for Inter Leukin-10 gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, and 65 are positive bands at 1000bp for the expressed interleukin-10 genes for the HBV-positive individuals.

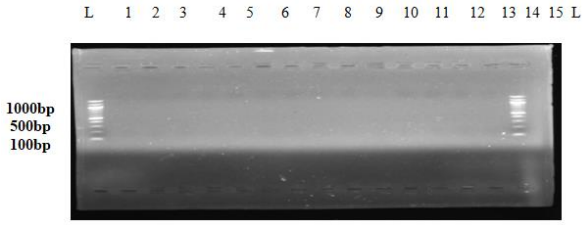

Plate 3: Reverse transcriptase PCR results for tumor necrosis factor-alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1, 2, 3, 4, 5, 6, 7,8,9,10,11,12,13,14, and 15 are negative bands for the expressed tumor necrosis factor alpha genes for the control group.

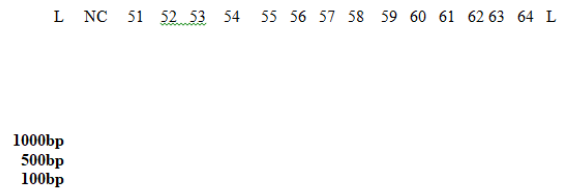

Plate 4: Reverse transcriptase PCR results for tumor necrosis factor-alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, and 64 are positive bands at 400bp for the expressed tumor necrosis factor-alpha genes for the HBV-positive individuals. NC is a no template control.

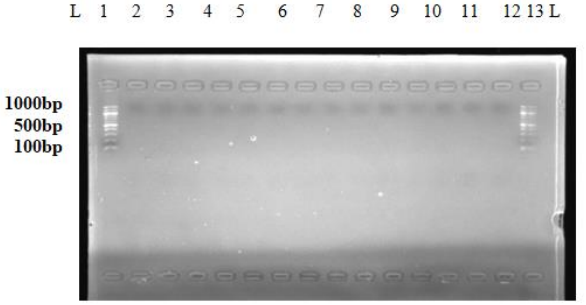

Plate 5: Reverse transcriptase PCR results for interleukin-1β gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9,10,11,12, and 13 are negative bands for the expressed interleukin-1b genes for the control group.

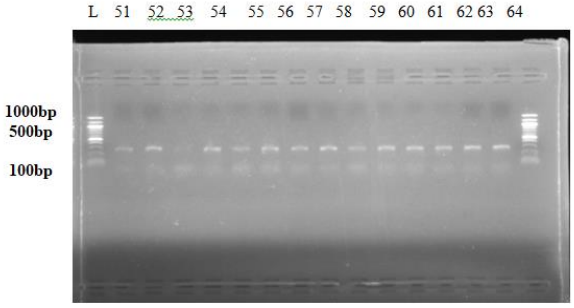

Plate 6: Reverse transcriptase PCR results for interleukin-1β gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, and 65 are positive bands at 300bp for the expressed interleukin-1β genes for the HBV-positive individuals.

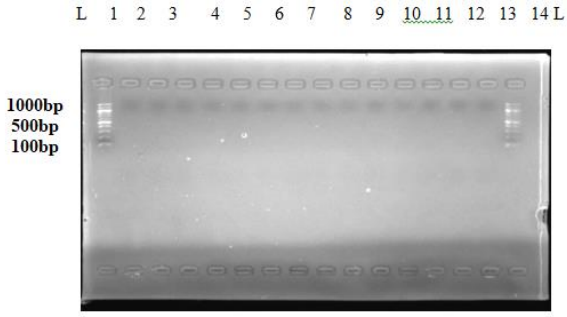

Plate7: Reverse transcriptase PCR results for interferon-gamma gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1, 2, 3, 4, 5, 6, 7,8,9,10,11,12,13,14, and 15 are negative bands for the expressed interferon-gamma genes for the control group.

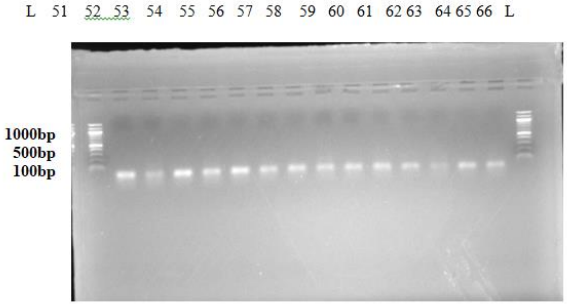

Plate 8: Reverse transcriptase PCR results for interferon-gamma gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, and 64 are positive bands at 100bp for the expressed interferon-gamma genes for the HBV-positive individuals. NC is a no template control.

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TABLE 1: Comparison of median levels of interferon-gamma, tissue necrosis factor-alpha, interleukin-10 (pg/ml), interleukin 1β (pg/ml) .0in the study population

Participants	IFN ϑ (pg/ml)	TNF α (pg/ml)	IL –)(pg/ ml)	IL -)(pg/ ml)
HBV nega tive control (A) N= 50	0.25	0.19	0.22	0.23
HBV Posi tive group (B) N= 65	745.50	114.50	549.00	244.00
Kriskal-wallis value	88.548	75.723	87.748	89.993
p-value	0.001	0.001	0.001	0.001
A vs B	0.001	0.001	0.001	0.001

In this study, some abnormalities in some cytokines both at gene and serum levels were observed.

The HBV status of all participants was confirmed using polymerase chain reaction and agarose gel electrophoresis technique for PCR product separation. The results showed that the HBV DNA gene was absent in the HBV negative control group, whereas the HBV positive case group expressed HBV DNA gene (see appendix 3-7). Furthermore, the HBV DNA gene was sequenced (see appendix 8). Interestingly, two new HBV DNA sequences were discovered and hence given accession numbers; OL419369 and OL419370.

The reverse transcriptase polymerase chain reaction results for IL – 10, TNF α, IL - 1β, and IFN γ genes revealed that the genes were expressed and up–regulated in HBV positive individuals at 1000bp 1% agar rose gel electrophoresis stained with ethidium bromide. Plates 1 and 2 show expression of interleukin 10 gene. As shown on plate 1, lanes 1 to 15 are negative bands for the expression of interleukin 10 gene for the HBV-negative control group. Plate 2 shows positive bands for expression of interleukin 10 gene on lanes 51 to 65 at 1000bp for the HBV-positive individuals.

Plates 3 shows negative bands at lanes 1 to 15 for the expression of tumor necrosis factor-alpha gene for the HBV negative control group whereas plates 4 shows positive bands for the expression of tumor necrosis factor-alpha at lanes 51 to 64 for HBV positive group at 400bp.

Plate 5 shows negative bands at lanes 1 to 13 for the expression of Interleukin - 1β gene for the HBV negative control group whereas plates 6 show positive bands for the expression of Interleukin - 1β gene at lanes 51 to 65 for HBV positive group at 300bp.

Plate 7 shows negative bands at lanes 1 to 15 for the expression of interferon-gamma gene for the HBV negative control group whereas plates 8 show positive bands for the expression of interferon gamma gene at lanes 51 to 64 for HBV positive group at 100bp.

Table 1 showed Comparison of median levels of interferon-gamma, tissue necrosis factor-alpha, interleukin-10, and interleukin 1β in the study population. The median levels of interferon gamma, tissue necrosis factor alpha, interleukin-10, and interleukin 1β in the HBV-positive individuals were significantly higher ($P < 0.05$). when compared with that of the control group.

4. Discussion

Hepatitis B virus infection (HBV) is a major cause of concern worldwide causing significant morbidity and mortality[10]. Poor diagnosis and prognostic factors remain one of the crucial factors responsible for poor management of the disease despite progress in implementing vaccination programmes and development of new treatment perspectives in the management of hepatitis B virus (HBV) infection which still remain a major health problem worldwide, contributing considerably to cirrhosis and hepatocellular carcinoma (HCC)-related mortality of 0.5 – 1 million per year[11,12]. It had been shown that among different hepatitis B virus-infected groups at different stages, significant differences were observed in different cytokines across those stages[6]. Therefore, we can postulate that monitoring cytokine genes expression and serum cytokine expression patterns of HBV positive individuals might give insight into disease progression and can result in better prognosis as well as management of HBV patients. In this study we evaluated the cytokine genes (interferon-gamma (IFN – γ) transcript, tissue necrosis alpha (TNF – α) transcript, interleukin – 10 (IL – 10) transcript and interleukin 1 beta (IL – 1 β) transcript expression pattern, cytokine serum level of IFN – γ, TNF – α, IL – 10, IL – 1 β in monitoring the disease outcome of HBV infection in Nigeria.

It could be inferred from our results that HBV infection might cause the up-regulation of some cytokine genes, hence their increased expression in the serum level in the HBV-positive individuals due to activation of cytokine production by viral invasion. The function of cytokines in host response to foreign antigen is actually aimed at controlling cellular stress and minimizing cellular damage. The failure to resolve an initial antigen invasion can provoke excessive immune cell infiltration and result to up – regulation of cytokine gene as well as persistent cytokine production. This implies that the host response to stress brings about changes in cytokine expression which affects disease advancement[13,14].

Additionally, the roles played by the cytokines such as IL-1β and IL-18 (to mention but a few) against HBV have also been well documented by several studies which include the activation of innate and adaptive immune cells, increased expression of addressing molecules on the endothelial cells of the damaged / infected tissues, up-regulation of proinflammatory cytokines, and increased number and activation of T regulatory lymphocytes15,16,17,18,19,20. Cytokine levels have been found to vary in some

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inflammatory and immunologic diseases5. Study shows that a distinctive cytokine profile for hepatitis B virus-infected individuals showed up-regulation of pro-inflammatory cytokines [interleukin (IL)-8, tumour necrosis factor-alpha (TNF-α), IL-2, IL-1, and IL-6], anti-inflammatory cytokines [IL13, tumour growth factor-beta (TGF-β) and IL-10], immunoregulatory cytokines [IL- 5, IL-15, interferon-gamma (IFN-γ), IL-7 and TNF- β] when compared with healthy individuals.7 This submission from their research is in agreement with the result of this study where the expression pattern and median levels of interferon-gamma, tissue necrosis factor, interleukin-10, and interleukin 1β in the HBV naïve, three months and six months on treatment HBV individuals were significantly higher when compared with that of the control group. It is also possible that the immune response to HBV infection is complex, and it involves the interplay between a range of cytokines such as interferon (IFN)-α/β, IFN- ϒ, and tissue necrosis factor (TNF)-α which are involved in the early phase of infection. Thus, it can be postulated that an effective antiviral response is partly mediated by the up-regulation of interferon-gamma, tissue necrosis factor, interleukin-10, and interleukin 1β genes, which are needed to control HBV replication and if possible total viral clearance which was not achieved in the study

Furthermore, for interferon response in HBV infection, $CD8^+$ T cells control viral replication through an IFN-γ dependent mechanism and not necessarily a direct cell destruction of infected hepatocytes[21,22]. A study in 2009 using a primary human hepatocyte model, revealed that soon after infection, liver macrophages recognize HBV and induce production of TNF-α, IL-6, IL-8, and IL-1β with IL-6 acting as the chief cytokine responsible for controlling viral replication[23]. It is evident from these researches that infection with HBV stimulates proliferation of immune cells, which up - regulate cytokine production to inhibit viral replication which is evident in this research.

5. Conclusion

This study concludes that gene expression of TNF-α, IFN- ϒ, IL – 10, and IL-1β were higher in HBV positive group compared to that of the HBV-negative control group, indicating a strong immune response to inhibit viral replication, thus these markers can be assessed at different stages of HBV infection to monitor disease outcome. Similarly, cytokine serum levels for TNF-α, IFN- ϒ, IL – 10, and IL-1β were again significantly higher in HBV-positive individuals when compared with that of the control group indicating an active anti–viral response. It could therefore be inferred from the outcome of our study that using cytokine gene expression and cytokine serum level can be promising in assessing outcome of HBV infection.

Consent

Both oral and written consent of each HBV-positive and control subjects were obtained before recruitment into the study.

Ethical Approval

Ethical approval was sort and obtained from the Research and Ethics Committee of Federal Medical Centre (FMC) Asaba, Delta State where the participants were recruited from. The approval letter from this committee with reference number FMC/ASB/A81 VOL. XII/119.

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Competing Interest

Authors have declared that no competing interests exist.

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