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EVALUATION OF CYTOKINE PATTERN IN HBV POSITIVE SUBJECTS TREATED WITH TENOVOFIR AT FEDERAL MEDICAL CENTER ASABA, DELTA STATE.

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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 treatment naïve, three and six months on treatment individuals attending Federal Medical Centre Asaba. The objectives were to assess IFN-Y, TNF- α, IL-10, and IL-1β transcripts, the serum cytokine levels in HBV positive groups and compare the results with that of the control group. This was a cross-sectional longitudinal study on 115 adults aged 22 – 64 years. The cross-sectional study consists of 50 confirmed HBV negative individuals as negative controls whereas, the follow-up study consists of 65 treatment naïve HBV positive individuals who were followed-up at three and six months on treatment with tenovofir respectively. 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 treatment naïve, three and six months on treatment individuals 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.

Keywords: Cytokines, HBV positive subjects, tenovofir, interleukin

Introduction

Hepatitis B virus (HBV) infection is a viral infection that affects the liver causing acute as well as chronic disease [1]. The resurgence of hepatitis B infection is a cause for concern due to the morbidity and mortality associated with the disease. 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 necrosis factor – α (TNF - α) genes which showed increased expression patterns initially following exposure to HBV and reaching maximum expression levels 3 hours after stimulation [2]. 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 [2,3]. Furthermore, [1], reported that immune responses significantly increased with the loss of infectivity suggesting a very early event of immune control. Another study demonstrated that HBV-induced production of inflammatory cytokines in monocytes depends on TLR2/MyD88/NFκB signaling [4]. Cytokine levels have been found to vary in some inflammatory 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]. [7], reported distinctive cytokine profile for hepatitis B virus infected individuals in their study assessing pro-inflammatory cytokines [interleukin (IL)-8, tumor necrosis factor-alpha (TNF-α), IL-2, IL-1 and IL-6], anti-inflammatory cytokines [IL13, tumor growth factor-beta (TGF-β) and IL-10], immunoregulatory cytokines [IL-5, IL-15, interferongamma (IFN-γ), IL-7 and TNF- β]. Comparing healthy individuals and those who recovered from the infection to those individuals who are still infected their results shows an increase in some cytokines such as IL2, Tissue growth factor β (TGF β), and TNF - α and decrease in some cytokines like IL15, IL 8, IL 10 and TNF - β. 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. Pleiotropic 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]. A study by [8], 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. Nonetheless, little is known about the relationship between these three cytokines and HBV infection in hepatitis B patients. Interferon-γ is an antiviral cytokine often included in HBV therapy [8].

Research Method

The study was carried out at Federal Medical Center Asaba. The study was designed such that 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 individuals were evaluated prior to commencement of medication, three months and six months on treatment as well as in

HBV negative control individuals. This was a cross-sectional longitudinal study on 115 adults aged 22 – 64 years. The cross-sectional study consists of 50 confirmed HBV negative individuals as negative controls whereas, the follow-up study consists of 65 treatment naïve HBV positive individuals who were followed-up at three and six months on treatment with tenovofir respectively. All the subjects were attending gastro – enterology clinic at medical out – patient Department of Federal Medical Center Asaba. 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 Ethylene diamine tetra-acetic acid (K2-EDTA) bottle for HBV screening/ELISA. The red cells recovered after separation of plasma was used for HBV DNA detection. 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 Reaction9. 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.

Presentation of Results

The reverse transcriptase PCR results for IL – 10, TNF α, IL - 1β, and IFN γ genes revealed that the genes were expressed and up – regulated in HBV treatment naïve, three and six months on treatment individuals at 1000bp, 400bp, 300bp and 100bp 1% agar rose gel electrophoresis stained with ethidium bromide respectively. Plates 1 – 4 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 treatment naïve individuals. Plate 3 shows positive bands for expression of interleukin 10 gene on lanes 51 to 67 at 1000bp for the HBV positive treatment at three months individuals while plate 4 shows positive bands for expression of interleukin 10 gene on lanes 51 to 67 at 1000bp for the HBV positive treatment at six months individuals. Plates 5 to 8 show expression of expression of tumor necrosis factor alpha gene in the study groups. Plates 5 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 6, 7 and 8 show positive bands for the expression of tumor necrosis factor alpha at lanes 51 to 64 for treatment naïve HBV positive group, lanes 51 to 65 for both three and six months on treatment HBV positive groups, all at 400bp. Plates 9 to 12

show expression of Interleukin - 1β gene in the study groups. Plate 9 shows negative bands at lanes 1 to 13 for the expression of Interleukin - 1β gene for the HBV negative control group whereas plates 10, 11 and 12 show positive bands for the expression of Interleukin - 1β gene at lanes 51 to 65 for treatment naïve, three and six months on treatment HBV positive group respectively, all at 300bp. Furthermore, plates 13 to 16 show expression of interferon gamma gene in the study groups. Plate 13 shows negative bands at lanes 1 to 15 for the expression of interferon gamma gene for the HBV negative control group whereas plates 13, 15 and 16 show positive bands for the expression of interferon gamma gene at lanes 51 to 64 for treatment naïve, three and six months on treatment HBV positive groups respectively, all at 100bp.

Additionally, Table 1 show 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 naïve, three and six months on treatment individuals were significantly higher ($P < 0.05$), when compared with that of the control group.

L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 L

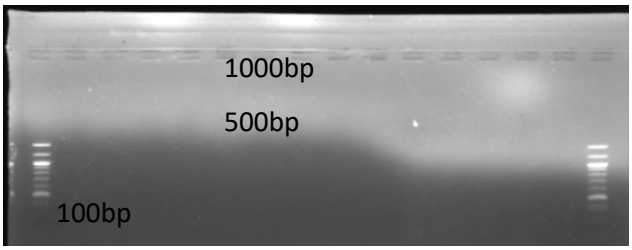


Plate 1. Reverse transcriptase PCR results for Inter Leukin-10 gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1, 2, 3, 4, 5, 6, 7,8,9,10,11,12,13,14 and 15 are negative bands for the expressed interleukin-10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

L 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 L

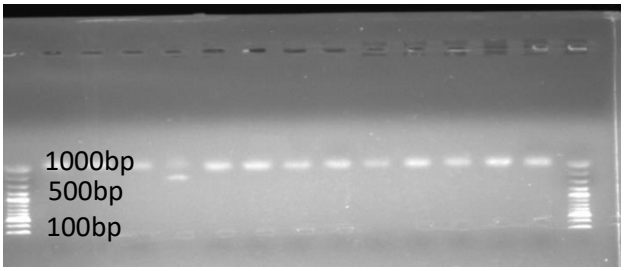


Plate 2. Reverse transcriptase PCR results for Inter Leukin-10 gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 and 65 are positive

bands at 1000bp for the expressed interleukin-10 genes for the HBV naïve treatment individuals

L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 L

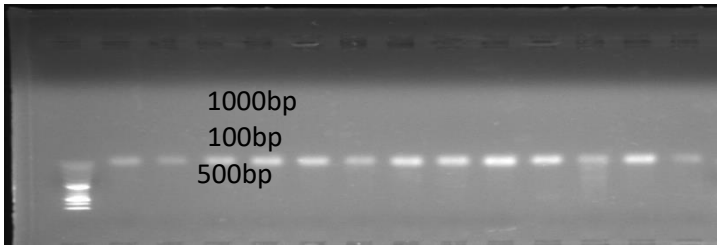


Plate 3. Reverse transcriptase PCR results for Inter Leukin-10 gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66 and 67 are positive bands at 1000bp for the expressed interleukin-10 genes for the HBV individuals at three months on treatment.

L 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 L

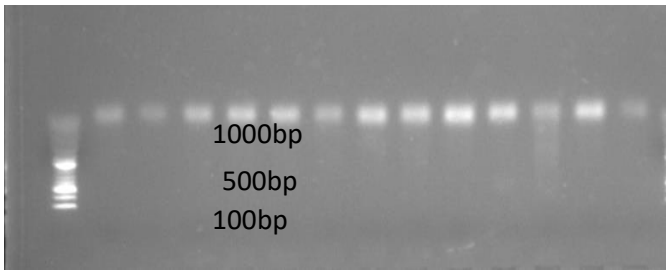


Plate 4. Reverse transcriptase PCR results for Inter Leukin-10 gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66 and 67 are positive bands at 1000bp for the expressed interleukin-10 genes for the HBV individuals at six months on treatment

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L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 L

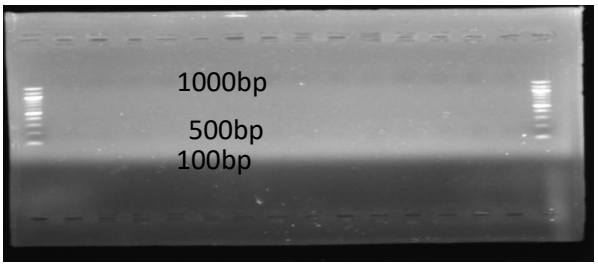


Plate 5. Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1, 2, 3, 4, 5, 6, 7,8,9,10,11,12,13,14 and 15 are negative bands for the expressed tumor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

L NC 51 52 53 54 55 56 57 58 59 60 61 62 63 64 L

1000bp
500bp
100bp

Plate 6. 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 treatment naïve individuals. NC is a no template control

L 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 L

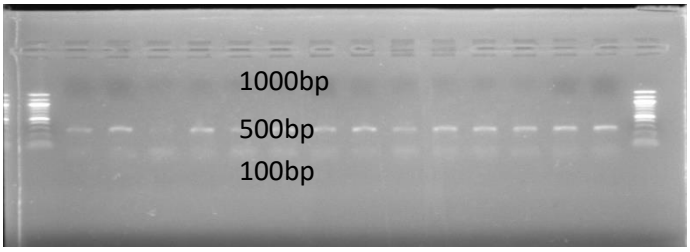


Plate 7. Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 and 65 are positive bands at 400bp for the expressed tumor necrosis factor alpha genes for the HBV three month on treatment individuals.

L 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66

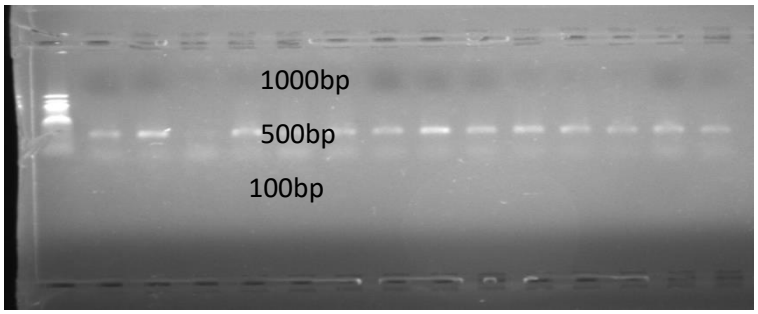


Plate 8. Reverse transcriptase PCR results for tumor necrosis factor alpha genes analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 and 65 are positive bands at 400bp for the expressed tumor necrosis factor alpha genes for the HBV six month on treatment individuals.

L 1 2 3 4 5 6 7 8 9 10 11 12 13 L

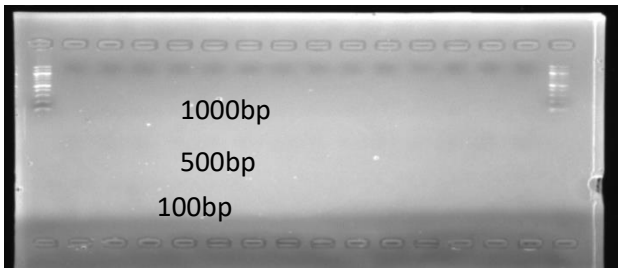


Plate 9. Reverse transcriptase PCR results for interleukin-1β gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9,10,11,12 and 13 are negative bands for the expressed interleukin-1b genes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

L 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 L

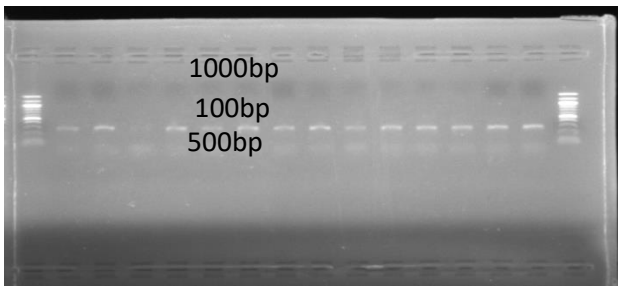


Plate 10. Reverse transcriptase PCR results for interleukin-1β gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 and 65 are positive bands at 300bp for the expressed interleukin-1β genes for the HBV treatment naïve individuals.

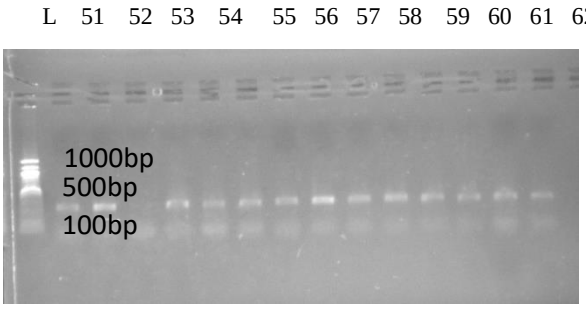


Plate 11. Reverse transcriptase PCR results for interleukin-1β gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 and 65 are positive bands at 300bp for the expressed interleukin-1β genes for the HBV three months on treatment individuals.

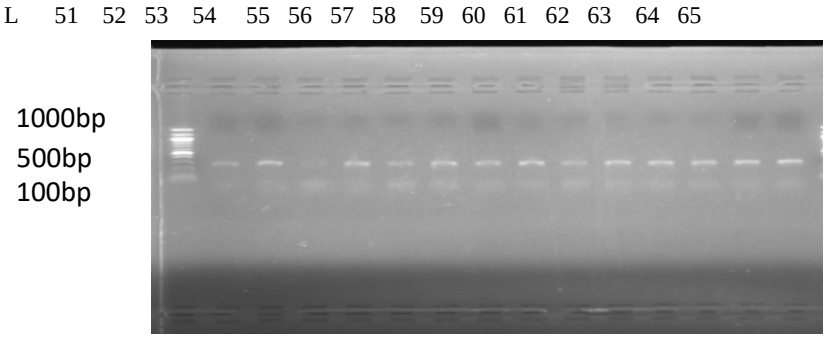


Plate 12. Reverse transcriptase PCR results for interleukin-1β gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 and 65 are positive bands at 300bp for the expressed interleukin-1β genes for the HBV six months on treatment individuals.

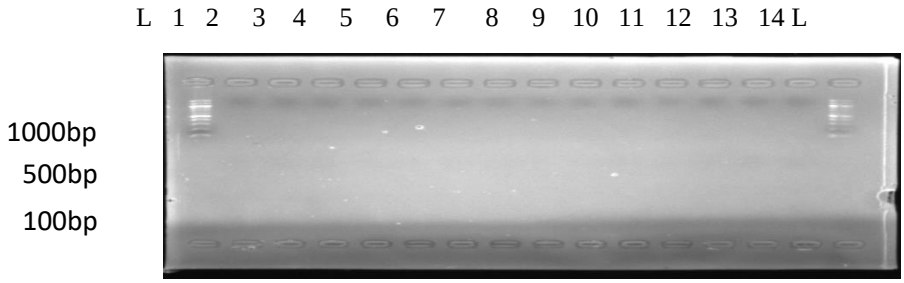


Plate 13. Reverse transcriptase PCR results for interferon gamma gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 1, 2, 3, 4, 5, 6, 7,8,9,10,11,12,13,14 and 15 are negative bands

for the expressed interferon gamma genes for the control group.

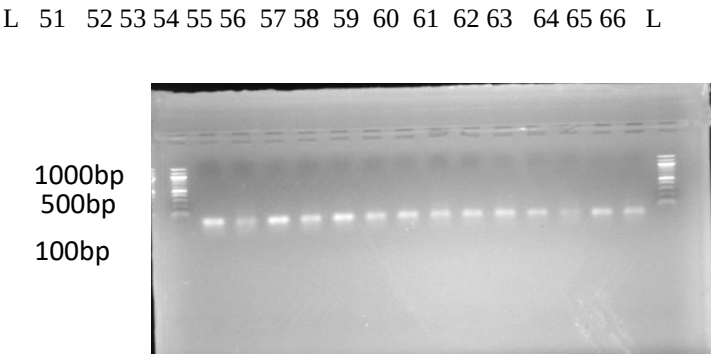


Plate 14. Reverse transcriptase PCR results for interferon gamma gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63 and 64 are positive bands at 100bp for the expressed interferon gamma genes for the HBV treatment naïve individuals. NC is a no template control

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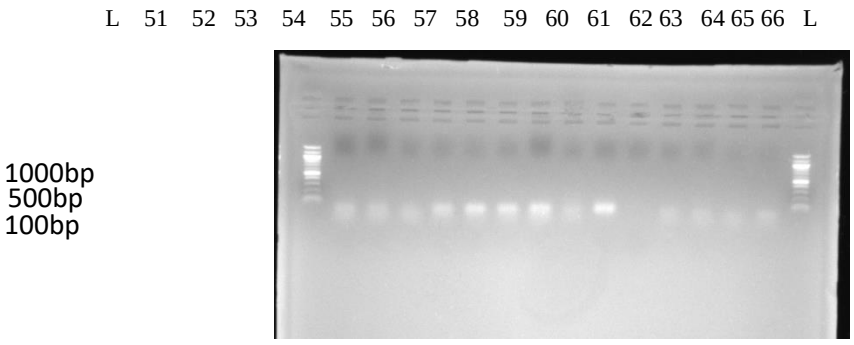


Plate 15. Reverse transcriptase PCR results for interferon gamma gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63 and 64 are positive bands at 100bp for the expressed interferon gamma genes for the HBV three months on treatment individuals.

L 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69

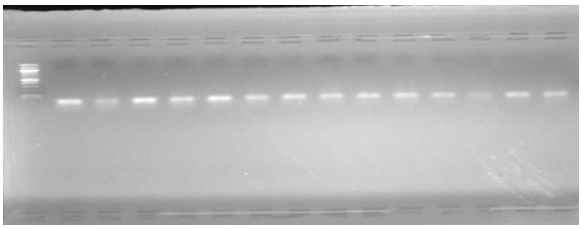


Plate 16. Reverse transcriptase PCR results for interferon gamma gene analyzed on a 1.0 % agarose gel electrophoresis stained with ethidium bromide. L is a 100bp-1000bp DNA ladder (molecular marker). Lanes 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63 and 64 are positive bands at 100bp for the expressed interferon gamma genes for the HBV six months on treatment individuals

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

Participants	IFN 8 (pg/ml)	TNF α (pg/ml)	IL – 10(pg/ml)	IL - 1β(pg/ml)
HBV negative control (A) N= 50	0.25	0.19	0.22	0.23
HBV treatment naïve (B) N= 65	745.50	114.50	549.00	244.00
Three months post treatment (C) N= 63	904.00	97.50	457.00	275.50
Six months post treatment (D) N= 61	1140.00	90.00	369.00	392.00
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
A vs C	0.001	0.001	0.001	0.001
A vs D	0.001	0.001	0.001	0.001
B vs C	0.007	0.001	0.007	0.011
B vs D	0.001	0.001	0.001	0.001
C vs D	0.001	0.090	0.001	0.001

Discussion of Findings

Hepatitis B virus infection (HBV) is a major cause of concern worldwide causing significant morbidity and mortality [9]. Poor diagnosis and prognostic factors remain one of the crucial factors responsible for poor management of the disease despite progress in implementing vaccination programs 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 [10,11]. 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 cytokine genes expression, serum cytokine expression patterns, changes in haemostatic and other hematological parameters of HBV positive treatment might be related to various HBV infection stages and can result in better prognosis and management of HBV patients.

In this study, the expression pattern and median levels of interferon gamma, tissue necrosis factor, interleukin-10 and interleukin 1β in the HBV naïve individuals were significantly higher when compared with that of the control group. Also, the expression pattern and median levels of interferon gamma, tissue necrosis factor, interleukin-10, and interleukin 1β in the HBV three and six months on treatment individuals were significantly higher when compared with that of the control group. It could therefore be inferred from these results that HBV infection might cause the up-regulation of these cytokine genes, hence their increased expression in the serum level in the HBV naïve, three and six months on treatment 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 [12,13]. 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 includes 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 pro-inflammatory cytokines, and increased number and activation of T regulatory lymphocytes [14,15,16,17,18,19]. Cytokine levels have been found to vary in some inflammatory and immunologic diseases [5]. [7] reported distinctive cytokine profile for hepatitis B virus infected individuals in their study which showed up-regulation of pro-inflammatory cytokines [interleukin (IL)-8, tumor necrosis factor-alpha (TNF-α), IL-2, IL-1 and IL-6], anti-inflammatory cytokines [IL13, tumor 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. 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- Ɣ, 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.

Conclusion

This study concludes that gene expression of TNF-α, IFN- Ɣ, IL – 10 and IL-1β were higher in HBV groups 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 progression and predict treatment outcome. Similarly, cytokine serum levels for TNF-α, IFN- Ɣ, IL – 10 and IL-1β were again significantly higher in HBV positive treatment naïve, on treatment at three and six months individuals when compared with that of the control group indicating an active anti – viral response.

Recommendation

These indices (cytokine gene expression pattern, cytokine serum level) could be used as makers in the management and monitoring of treatment and disease progression in HBV subjects which will accord clinicians a better understanding of the virus for improved patient care. Only selected cytokines were included in this study. More work can be done in this area but studying other important cytokines of the immune system.

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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