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Disease Pattern And Diagnostic Accuracy Of Th 1 And Th 2 Cytokines In Hepatitis B Virus Infected Patients: A Case Control Study In Ghana.

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Abstract: Background: The host immune response to hepatitis B viral (HBV) infection affect the clinical outcome. Cytokines inhibit HBV replication and influences the clinical outcome of HBV infection. This study sought to determine the disease pattern of Th1, Th2 and immunosuppressive cytokines and their diagnostic accuracy in classifying or staging HBV infection.
Method: In a case control study, patients with HBV infections and healthy blood donors were screened for HBsAg profile, HCV and HIV. Those positive with HCV and HIV were excluded. Finally, 120 HBV infected patients and 105 healthy blood donors were recruited as cases and control. Th1 cytokines (IL-12p70, IFN-γ and TNF-α), Th2 cytokines (IL-4, IL-6) and immunosuppressive cytokine (IL-10) were assayed by ELISA. Their diagnostic performance were determined using the ROC curve.
Results: The HBsAg serological profile results gave 3 acute infections, 12 HBV recovery, 5 positive chronic hepatitis B (CHB) and 100 negative CHB. Median levels of IL-12p70, TNF-α and IFN-γ were elevated in HBV infections compared to controls (p>0.05). Median levels of IL-4 was significantly elevated in HBeAg negative CHB and HBV recovery compared to controls (p=0.0196) whilst IL-10 was significantly elevated in HBeAg negative CHB infection (p=0.0253). At 95% confidence interval, the best diagnostic markers with AUC, sensitivity and specificity were IL-10 (0.72, 66.67% and 86.67%) for acute infection and 0.66, 67.0% and 60.0% for HBeAg negative CHB. IL-4 (0.72, 66.67% and 84.44%) for HBV recovery and IL-6 (0.75, 80.0% and 71.1%) respectively for HBeAg positive CHB respectively.
Conclusion: Th2 cytokines (IL-4, IL-6) and immunosuppressive cytokine (IL-10) are better diagnostic markers for classifying the various stage of HBV infections. Increased IL-4 is associated with both HBV recovery and HBeAg negative CHB whilst increased IL-10 is related to HBeAg negative CHB. The measurement of these cytokines may lead to improvements in the clinical management of HBV infected patients based on their defined cytokine profiles.

Keywords: Hepatitis B viral infection Th 1&2 cytokines, interferon gamma, cytokine disease pattern, cytokines in diagnosis, HBV Disease classification.

Introduction

Hepatitis B Viral (HBV) infection is a public health problem due to its high morbidity and mortality. Globally, about two billion people have been infected with 350 million developing chronic infection(Merrill and Hunter, 2011). Furthermore, an estimated 600,000 people die from HBV

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associated diseases annually (Ott *et al.*, 2012). Sub-Saharan Africa and East Asia have the highest prevalence of HBV with 5 to 10% of the adult population being infected(Ott *et al.*, 2012).

The clinical outcome of HBV infection is strongly linked with the host immune response to the infection (Khan *et al.*, 2011). Cytokines, soluble short lived proteins secreted by T lymphocytes and other immune and non-immune cells play important roles in HBV pathogenesis and the host response (Koziel, 1999). Th1 cytokines such as interleukin-12 (IL-12), tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) have been directly or indirectly shown to inhibit HBV replication and hence viral clearance whilst Th2 cytokine interleukin-4(IL-4) and immune suppressive cytokine, interleukin-10 (IL-10) are thought to facilitate viral persistence and development of chronic infection (Falasca *et al.*, 2006).

Traditionally, the diagnosis and monitoring of HBV infections are based on virological markers of HBV and liver function tests (Das *et al.*, 2012). However, mutations that occur in the HBV genome may result in non-secretion of HBsAg and HBeAg. Hence the use of these viral markers as diagnostic markers may result in discordant results at certain times (Pan and Zhang, 2005; Chevaliez and Pawlotsky, 2008) therefore the need for alternative diagnostic markers for classifying the various stage of HBV infections.

Cytokines have been shown to play major role in HBV associated liver damage (Yan *et al.*, 1999; Blankson *et al.*, 2005), cancers (Wong *et al.*, 2009) and the treatment response to various anti-viral agents (Hall and Cash, 2012). However, the diagnostic performance and disease pattern of Th1 and Th2 cytokines in acute HBV, HBV recovery, HBeAg positive CHB and HBeAg negative CHB have not been examined extensively. In addition, variations in host genetic immune response make it imperative to study the balance between immune suppressive, Th1 and Th2 cytokines among the Ghanaian populace. It is against this background that this study for the first time was conducted to determine the diagnostic accuracy and disease pattern of cytokines in HBV infection among a Ghanaian population.

Materials and Method

Study design and site

This cross-sectional case control study was carried out at the Serology and Virology Unit of Komfo Anokye Teaching Hospital (KATH). KATH is a 1000 bed capacity tertiary referral hospital that serves the Kumasi metropolis, surrounding towns and northern sector of Ghana and it is located in Kumasi in the Ashanti region.

Study population and recruitment

Patients with HBV infection or history of HBV infections were selected as cases and healthy blood donors as controls. They were screened for HBsAg profile, HCV and HIV. Those positive with HCV and HIV were excluded. Finally, 120 HBV infected patients and 105 healthy blood donors were recruited as cases and control respectively. Structured questionnaires were used to obtain socio-demographic information of study participants. Information pertaining to clinical care and other conditions were inferred from folders and their hospital biodata.

Inclusion and exclusion criteria

Patients with HBV or past history of HBV were included in the study while those positive for hepatitis C and human immunodeficiency viral, pregnant women, those on herbal medication, co-existence of other chronic liver disease, alcohol consumption and history of immunosuppressive/antiviral therapy were excluded from the study.

Ethical consideration

The study protocol was reviewed and ethical clearance granted by Committee for Human Research, Publication and Ethics (CHRPE) of the Kwame Nkrumah University of Science &

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Technology (KNUST) and the Research and Development Unit of KATH (CHRPE/AP/076/15).

Sample collection

Five millilitres (5ml) of blood was taken into BD Vacutainer® tubes with clot activator gel. Samples were then centrifuged using Hettich Universal 320 centrifuge (DJB Labcare Ltd, UK) at 1000 rpm for 10minutes. Serum was immediately separated into two Eppendorf vials and stored at -80^{o}C freezer (Thermo Fisher Scientific, NC, USA) until assayed.

Serological Screening

Screening for HBV serological markers including hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B enveloped antigen (HBeAg), antibody to e antigen (HBeAb) and total antibody to core antigen (HBcAb) were screened using DiaSpot Rapid Diagnostic Test kit (USA) while immunoglobulin M core antibody (HBcIgM) was performed using Advance Quality ^{TM} One Step HBcIgM test kit (InTec Products, INC., China). Anti HIV 1&2 antibody screening was performed using First Response HIV 1-2 rapid test kit (Premier Medical Corporation, Kachigan, India). Screening for HCV was performed using Advance Quality Rapid Anti-HCV test kit (InTec Products, INC., China).

Biochemical estimation of cytokines

Measurement of the various cytokines levels was done using Human ELISA MAX^{TM} Standard set available from BioLegend (San Diego, CA) according to the manufacturer's instruction. Briefly, 100µL of diluted Capture Antibody solution was added to each of the 96 wells (NuncMaxisorp, Roskilda, Denmark) and incubated between 2°C and 8°C overnight. Four cycles of washing was performed after which 200µL Assay Diluent was added to block, incubated for 1 hour at room temperature with shaking. Another four cycles of washing was done after which 100µL of samples and diluted standards were added to the appropriate wells, incubated at room temperature for 2 hours with shaking. Washing cycles were repeated 4 times and 100µL diluted Detection Antibody solution was added to each well, incubated for 1 hour at room temperature with shaking. The washing cycles were repeated and 100µL diluted Avidin-horseradish peroxidase (Avidin-HRP) solution was added to each well, incubate for 30 minutes at room temperature with shaking. Washing was repeated 5 times with soaking for 30 seconds per cycle. 10 µL of TMB Substrate Solution was added to each well, incubated in the dark for 15-30 minutes after which 100µL Stop Solution was added to each well. The optical densities (ODs) were read at 450 nm using ELx 800 Absorbance Microplate Reader (Bio-Tek Instruments. Inc., Winooski, VT, USA). The cytokine levels were then calculated from standard curve from derived from the standard concentration of the respective cytokines.

Classification of HBV infections

HBV infections were classified as acute infection, HBV recovery, HBeAg positive CHB and HBeAg negative CHB as described by Krajden *et al.* (2005) and (Shepard *et al.*, 2006) in table 1 below:

Table 1 Classification of HBV infections

HBsAg	HBsAb	HBeAg	HBeAb	HBcAb	HBcIgM	Classification
Positive	Negative	Positive	Negative	Positive	Positive	Acute infection
Negative	Positive	Negative	Negative	Positive	Negative	HBV recovery
Positive	Negative	Positive	Negative	Positive	Negative	HBeAg positive CHB
Positive	Negative	Negative	Positive	Positive	Negative	HBeAg negative CHB
Negative	Negative	Negative	Negative	Negative	Negative	Not exposed/control

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Statistical analysis

Data was entered into excel worksheet (Microsoft Office, 2013 version) and was analysed using GraphPad Prism version 6.01 (GraphPad Software, Inc., CA, USA). Data was expressed as mean ± SD, median (inter quartile range) and frequency (proportion) where appropriate. Kruskal-Wallis test followed by Dunn's multiple comparisons test were used to analyse the various stages of HBV infection and control. Area under the curve (AUC) and receiver operating characteristic (ROC) were used to determine diagnostic accuracy of the various cytokines. *P* value of <0.05 was considered significant

Results

Table 2 is a summary of the demographic characteristics of the study population. The mean age of the cases 32.1 years and controls 30.5 years were not different (p = 0.3867). Majority of the participants were males, 53.3 % (119/225), compared to females, 46.7% (105/225).

Table 2. Population demographics

Parameters	CASES(n=120)	CONTROL(n=105)	Total(n=225)	P-value
Age(years)Mean ± SD	32.1±10.8	30.5 ±10.3	31.7±10.6	0.3867
Male, %(n/total)	47.5%(57/120)	68.8%(72/105)	53.3%(119/225)	
Female, %(n/total)	52.5%(63/120)	31.1%(33/105)	46.7%(105/225)	

Age expressed as mean ± SD (standard deviation). Cases are represents acute, recovery, HBeAg positive CHB and HBeAg negative CHB

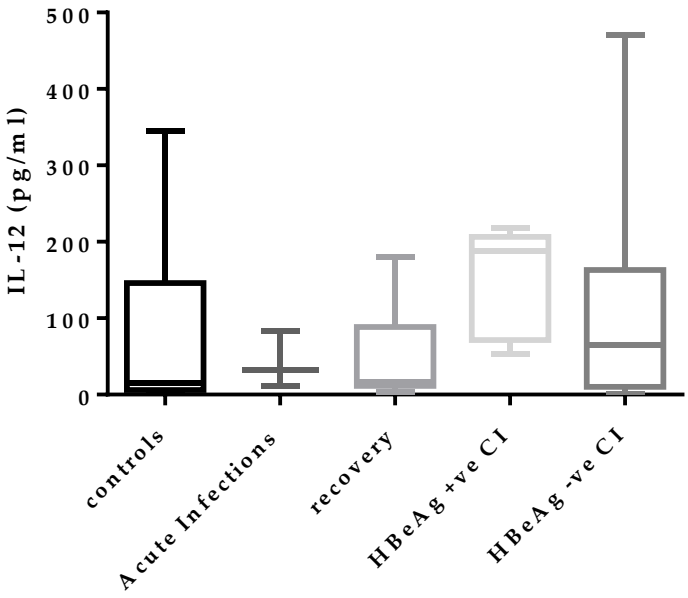

Figure 1. IL-12 concentration in the various stages of HBV infection

The median concentration of IL-12p70 was elevated in the acute infection, HBV recovery, HBeAg positive CHB and HBeAg negative CHB groups compared to the control group (p=0.1036)

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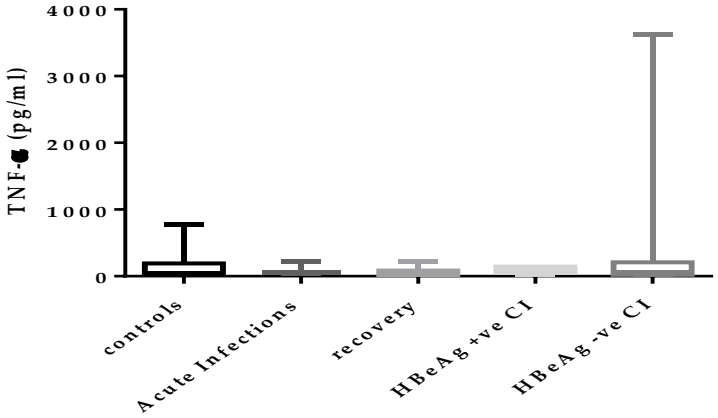

Figure 2. TNF-α concentration in the various stages of HBV infection

Median TNF-α levels were also elevated in acute infection, HBeAg positive CHB and HBeAg negative CHB groups but reduced in HBV recovery compared to the control group (p=0.3984) **(Figure 2).**

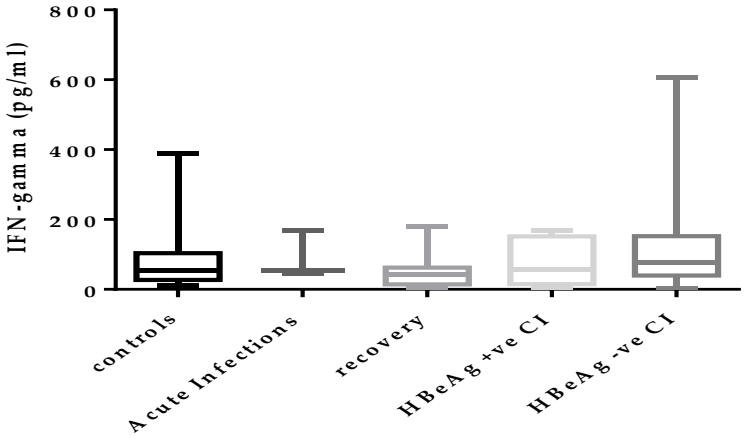

Figure 3. IFN-gamma concentration in the various stages of HBV infection

Median INF-γ concentration was not significantly different in acute infection compared to control group but was reduced in HBV recovery and elevated in both HBeAg positive CHB and HBeAg negative CHB groups compared to the control group (p=0.0954) **(Figure 3).**

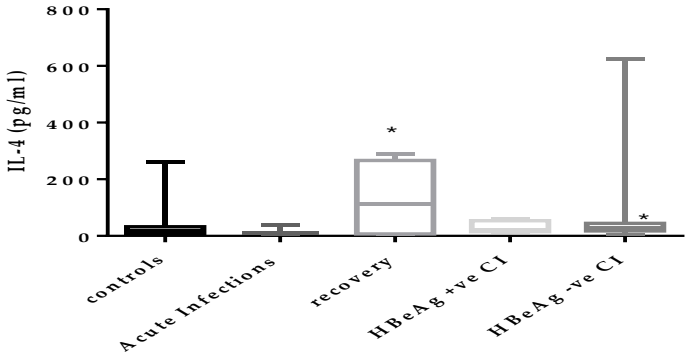

Figure 4. IL-4 concentration in the various stages of HBV infection. * indicate significant different compared to control.

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Median IL-4 levels were reduced in acute infection but elevated in HBV recovery ($p<0.05$), HBeAg positive CHB and HBeAg negative CHB ($p<0.05$) groups compared to control **(Figure 4).**

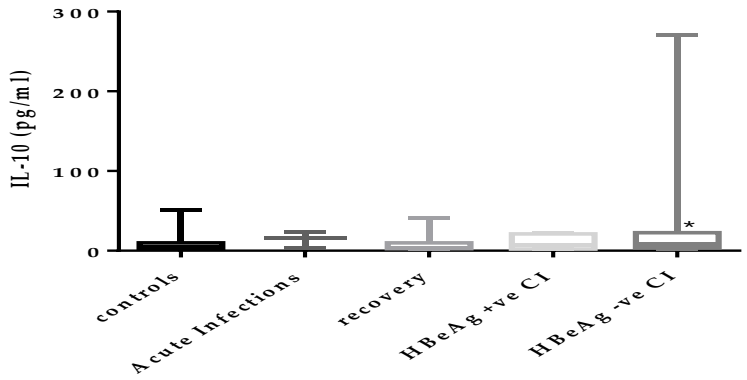

Figure 5. IL-10 concentration in the various stages of HBV infection. * indicate significant different compared to control.

Median levels of IL-10 were reduced in HBV recovery but elevated in acute infection, HBeAg positive CHB group and significantly elevated in the HBeAg negative CHB group ($p=0.0253$) compared to control group **(Figure 5).**

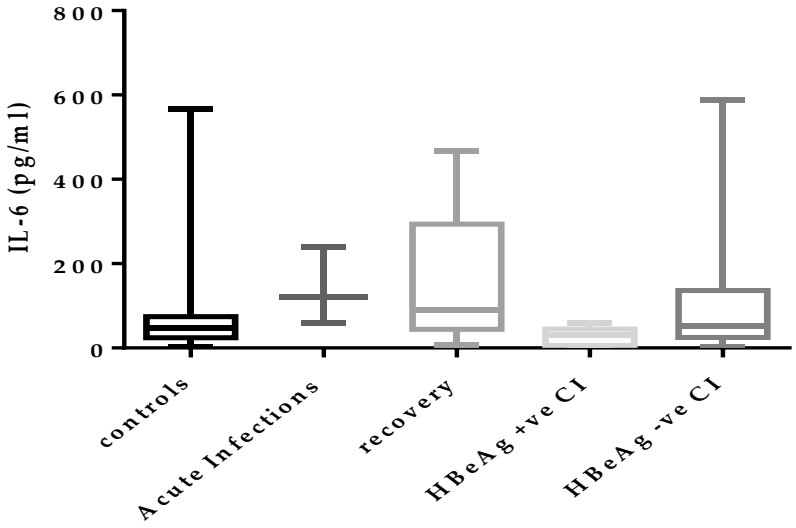

Figure 6. IL-6 concentration in the various stages of HBV infection

Median levels of IL-6 were elevated in acute infection, HBV recovery and in HBeAg negative CHB groups but reduced in HBeAg positive CHB compared to control group ($p=0.0702$) **(Figure 6).**

Table 3 summarize the diagnostic capacities of the various cytokines in acute HBV infection and HBV recovery. In the acute infection group, IL-10 had an AUC of 0.71 and at a threshold of >15pg/ml and at 95% confidence interval had a sensitivity of 66.7% the highest specificity of 86.7% which was significantly different from those of IL-12p70,TNF-α, IFN-γ and IL-6. Although IL-6 had the highest AUC 0.774(95% CI, 0.630 to 0.882), it was not significantly different from other cytokines. In the HBV recovery group, IL-4 had the highest AUC of 0.716and at a threshold value of >37.5pg/ml and 95% confidence interval had a sensitivity of 66.7% and a specificity of 84.4% which was significantly different from the specificities of IL-12p70, TNF-α and IFN-γ.

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Table 3. Diagnostic performance of cytokines for Acute HBV infections and HBV Recovery

Parameters (pg/ml)	Threshold value (pg/mL)	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%)	NPV (%)
		Acute infections				
IL-12p70	>10	0.56(0.41 - 0.70)	100(29.2 - 100.0)	44.44(29.6 - 60.0)	10.7	100
TNF-α	>39	0.65(0.50 - 0.78)	100(29.2 - 100.0)	55.56(40.0 - 70.4)	13.0	100
IFN-γ	>42	0.59(0.43 - 0.73)	100(29.2-100.0)	40.00(25.7 - 55.7)	10.0	100
IL-4	≤11.3	0.56 (0.41 - 0.70)	66.67(9.4 - 99.2)	60.00(44.3 - 74.3)	11.1	94.9
IL-6	>58.8	0.77(0.63 - 0.88)	100(29.2-100.0)	55.56(40.0 - 70.4)	13.0	100
IL-10	**>15**	**0.72(0.57 - 0.84)**	**66.67(9.4 - 99.2)**	**86.67(73.2 - 94.9)**	**25.0**	**97.5**
		HBV Recovery				
IL-12p70	>8	0.51(0.38 - 0.65)	91.67(61.5 - 99.8)	40.00(25.7 - 55.7)	28.9	94.7
TNF-α	≤102	0.56(0.42- 0.69)	91.67(61.5 - 99.8)	35.56(21.9 - 51.2)	24.3	85.0
IFN-γ	≤59.4	0.63(0.49 - 0.75)	75.00(42.8 - 94.5)	46.67(31.7 - 62.1)	27.2	87.5
IL-4	**>37.5**	**0.72 (0.58 - 0.83)**	**66.67(34.9 - 90.1)**	**84.44(70.5 - 93.5)**	**53.3**	**90.5**
IL-6	>99	0.67(0.53 - 0.79)	50.00(21.1 - 78.9)	84.44(70.5 - 93.5)	46.2	86.4
IL-10	≤3.6	0.51(0.37 - 0.64)	50.00(21.1 - 78.9)	60.0 (45.4 - 72.9)	25.0	81.8

AUC = Area under the curve, CI = confidence interval, PPV = positive predictive value, NPV = Negative predictive value.

Table 4 summarizes the diagnostic performance of the various Th1 and Th2 cytokines tested in HBeAg positive and HBeAg negative CHB infections. In the HBeAg positive CHB, IL-6 had an AUC of 0.75 which at 95% confidence interval was significantly different from that of IFN-γ. At a threshold value of ≤30pg/mL, IL-6 had a sensitivity of 80% and the highest specificity of 71.1% and was statistically different from that of INF-γ but similar to all the other cytokines.

In HBeAg negative CHB,IL-10 had the highest AUC of 0.66, its sensitivity of 67.0%, specificity of 60.0%, PPV of 78.8%, NPV of 45.0% at a threshold of >4.8 pg/ml. Taken together, IL-10 was the best marker for HBeAg negative CHB although IL-6 had similar AUC, sensitivity and specificity of 0.53, 43% and 73.3% respectively.

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Table 4. Diagnostic performance of cytokines in HBeAg positive and HBeAg negative CHB infections

Parameters (pg/ml)	Threshold value (pg/mL)	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95%)	PPV (%)	NPV (%)
		HBeAg positive CHB				
IL-12p70	>45	0.78(0.64 - 0.88)	100.00(47.8 - 100.0)	62.22(46.50 - 76.20)	22.7	100
TNF-α	>90	0.56(0.42 - 0.70)	80.00(28.4 - 99.5)	62.22(46.50 - 76.20)	19.1	95.4
IFN-γ	≤126	0.51(0.37 - 0.66)	60.00(14.7 - 94.7)	17.78(8.00- 32.10)	7.5	80
IL-4	>10	0.66(0.52 - 0.79)	100.00(47.8 - 100.0)	40.00(25.70 - 55.70)	15.6	100
IL-6	**≤30**	**0.75(0.61-0.86)**	**80.00(28.4 - 99.5)**	**71.11(55.70-83.60)**	**23.5**	**96.9**
IL-10	>6.6	0.53(0.39-0.68)	60.00(14.7 - 94.7)	68.89(53.4 - 81.80)	17.6	93.9
		HBeAg negative CHB				
IL-12p70	>15	0.60(0.51 - 0.68)	67.0(56.9 - 76.1)	53.33(37.9 - 68.3)	76.1	42.1
TNF-α	>12	0.57(0.49 - 0.65)	81.0(71.9 - 88.2)	35.56(21.9 - 51.2)	73.6	45.7
IFN-γ	>75	0.60(0.52 - 0.68)	51.0(40.8 - 61.1)	66.67(51.0 - 80.0)	77.3	37.9
IL-4	>13.8	0.64(0.56 - 0.72)	84.0(75.3 - 90.6)	46.67(31.7 - 62.1)	77.8	56.8
IL-6	>66	0.53(0.44-0.61)	43.0(33.1 - 53.3)	73.33(58.1 - 85.4)	78.2	36.7
IL-10	**>4.8**	**0.66(0.58-0.74)**	**67.0(56.9 - 76.1)**	**60.00(44.3 - 74.3)**	**78.8**	**45**

AUC = Area under the curve, CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value.

Discussion

This study explored the disease pattern of immunosuppressive, Th1 and Th2 cytokines and their diagnostic accuracy in identifying the various stage of HBV infections among a Ghanaian population. IL-4 was significantly elevated in both HBV recovery and HBeAg negative CHB whilst IL-10 was significantly elevated in HBeAg negative CHB compared to controls. For the first time, we identified IL-4 as the most specific and sensitive marker for identifying HBV recovery infection, IL-6 as the most specific and accurate diagnostic marker for identifying HBeAg positive CHB and IL-10 was identified as the most accurate, sensitive and specific for identifying both acute HBV infections and HBeAg negative CHB. Levels of IL-12p70, and TNF-α were elevated in HBeAg positive chronic infection and IFN-γ in HBeAg negative CHB but showed no significant difference compared to controls.

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This study found a significantly increased concentration of IL-4 among participants with HBV recovery and HBeAg negative CHB(Table 3).The significant increase in IL-4 among HBV recovery group is consistent with a case-control study by Benhamou (2004)among Italian population. He *et al.* (2013) and Falasca *et al.* (2006) also observed a significant elevation in IL-4 among CHB infections which is in agreement with the present study. Increased concentration of IL-4 have been shown to suppress HBsAg mRNA, the pregenomic RNA and the precore mRNA which secrets the HBeAg(Lin *et al.*, 2003). This may lead to sero-clearance of HBsAg and HBeAg and subsequently sero-conversion to HBsAb and HBeAb respectively.

This study also observed a reduced IL-10 levels among participant who had recovered from HBV infection but elevated in acute infections, HBeAg positive CHB and significantly in HBeAg negative CHB compared to controls. The significant elevation in IL-10 among participants with HBeAg negative CHB is consistent with findings by Khan *et al.* (2011) and Falasca *et al.* (2006). IL-10 has immune-regulatory properties able to inhibit the secretion of Th1 cytokines and suppressing cellular immune response to HBV thus shifting the balance towards a Th2 type profile which favours persistent or chronic infection(Asadullah *et al.*, 2003; Das *et al.*, 2012).

Although IL-12p70 levels were elevated in acute infection, HBeAg positive and HBeAg negative CHB infections but reduced in HBV recovery compared to control there was no statistical significant difference(Table 3). Similarly, no statistical significant difference was observed for the increase in TNF-α concentration among participants with acute infection, HBeAg positive and HBeAg negative CHB infections as well as a reduction in concentration among those with recovery HBV infection compared to controls (Table 3). Our study also observed an increase in IFN-γ concentration among participants with HBeAg positive and HBeAg negative CHB though not statistical significantly different from the control group(Table 3). Furthermore, a reduced concentration of IL-6 was observed among participants with acute HBV infection though elevated levels were observed in HBV recovery, HBeAg positive and HBeAg negative CHB compared to controls with no statistically significant difference (Table 3).

At a threshold point of >37.5pg/ml with specificity of 84.4%, sensitivity of 66.67%, PPV of 53.3%, NPV of 90.5%, and AUC of 0.72, IL-4 was identified as the most specific and sensitive marker for HBV recovery(Table 4). Th2 cytokine like IL-4 are able to stimulate B cell culminating in an increase production of hepatitis B antibodies. This buttresses its role in the pathogenesis of recovery infection. Increase level of IL-6 cytokine in chronic HBV infection have been shown to inhibit Hepatitis B viral replication and also probable in predictor of hepatocellular carcinoma (Porta *et al.*, 2008; Kuo *et al.*, 2009). In this study IL-6 was identified as better specific and accurate marker with a sensitivity of 80.0%, specificity of 71.1%, PPV of 23.5%, NPV of 96.9% and a threshold point of ≤30pg/ml for HBeAg positive CHB infection(Table 5).The most specific, sensitive and accurate cytokine for acute infections was IL-10 as depicted by a sensitivity of 66.7%, specificity of 86.7%, PPV of 25.0%, NPV of 97.5%, AUC of 0.72 and a threshold of >15pg/ml(Table 4). At a threshold of >4.8 pg/ml and a sensitivity of 67.0%, specificity of 60.0%, PPV of 78.8%, NPV of 45.0% and AUC of 0.66, IL-10 was identified as a better marker for HBeAg negative CHB infection(Table 5).

The limitation of the current study is our inability to get equal number of participants in various classification of HBV infection. We therefore recommend increasing the number of study participants proportionally in these various groups of HBV in future studies.

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Conclusion

IL-10 was identified as the best diagnostic marker for identifying acute HBV and HBeAg negative CHB, IL-4 the best diagnostic marker for identifying HBV recovery and IL-6 the best marker for identifying HBeAg positive CHB. The correlation of these cytokines to the various stage of HBV infections highlights the critical role of these molecules in the pathogenesis of HBV from infection. This may ultimately lead to improvements in the clinical management of HBV infection patients based on the diagnostic value of defined cytokine profiles.

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