Innate immune response in *Plasmodium* coinfection with HBV and HCV using complement C3, C4, and CD4+ T cells

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Study background
Complement, CD4+ T cells, and antibodies are immune materials for protection or are expressed for the detection of pathogens. Complements are activated by the classical or alternative pathway complement to cause cell death.

Aim and objective
This study was designed to evaluate the innate immune response in *Plasmodium* coinfection with hepatitis B virus (HBV) and hepatitis C virus (HCV) using complement C3, C4, and CD4+ T cells to provide information for useful directions in the management of malaria and hepatitis B and C.

Materials and methods
The participants recruited for this study include controls who were neither infected with *Plasmodium* nor hepatitis B and C (n = 50) and *Plasmodium*-infected participants (n = 50) and hepatitis C-infected participants (n = 30) and hepatitis B-infected participants (n = 30) and *Plasmodium* coinfected with hepatitis B participants (n = 30) and *Plasmodium* coinfected with hepatitis C participants (n = 30) aged 19–65 years. Complement C3, C4, CD4+ T cells HCV and HBV were determined in each of the participant by enzyme-linked immunosorbent assay method. Identification of *Plasmodium* was carried out using the thick and thin film technique using Giemsa and Leishman staining, while test and controls with elevated and normal total bile acids, respectively, were negative to HIV and acid fast bacilli tests were included.

Results
The results obtained in this study showed a significant decrease in plasma complement C3 in patients with monoinfection of *Plasmodium*, HCV, HBV, and *Plasmodium* patients coinfected with HCV and HBV compared with the control ($P < 0.005$). There was also a significant decrease in plasma complement C4 in *Plasmodium* patients coinfected with HCV and HBV compared with the control ($P < 0.005$). The results obtained in *Plasmodium* patients coinfected with HBV and HCV, and HBV patients coinfected with HCV showed a significant decrease in CD4+ T cells compared with the results obtained in the control ($P < 0.005$). There was significantly lower plasma complements C3, C4, and blood CD4+ T cells in *Plasmodium* monoinfected patients than in HBV or HCV monoinfected patients ($P < 0.005$). There was also a significantly lower plasma complement C4 and CD4+ T cells in *Plasmodium* patients coinfected with HBV or HCV than patients who were monoinfected with HCV and HBV ($P < 0.005$).

Conclusion
There was significant alterations in the blood complement C3, C4, and CD4+ T cells in response to *Plasmodium*, HBV, HCV mono- and coinfection which was more influenced by *Plasmodium* infection.

Keywords:
C4, CD4+ T cells, complement C3, hepatitis B virus, hepatitis C virus, *Plasmodium* coinfection

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Introduction

Complements are inborn immune materials that upgrade or supplement the capacity of antibodies and phagocytic cells to clear microbes and advances inflammation. Complements are present in the blood and are produced by the liver [1–3]. Complement is a complex system made up of more than 30 proteins that have the ability to eliminate infectious microbes in the body system. Specifically, complements cause lysis of foreign and infected cells, phagocytosis of foreign particles and cell debris, and also inflammation of the surrounding tissue. Complements can be stimulated by *Plasmodium*, hepatitis B virus (HBV), and hepatitis C virus (HCV) including their coinfections to cause the proteases in the system cleave specific proteins leading to release cytokines and initiate an amplifying cascade of further cleavages [1–5] that will stimulate phagocytes to remove foreign particles and debris, and also will stimulate inflammation to attract additional phagocytes, and activation of the cell-killing membrane attack complex [3–5]. Pathways

for the activation of the complement system include: the classical complement pathway, alternative complement pathway, and the lectin pathway [6–8].

HBV and HCV are hepatotropic viruses that cause inflammation of the liver known as viral hepatitis [6]. The life cycle plasmodia and the pathophysiology of malaria or *Plasmodium* infection involve liver which can trigger an inflammatory process [7–12]. These pathogens are potential triggers of cellular and humoral immune responses [6–12]. CD4 (cluster of differentiation 4) is an epitope of protein found on the surface of immune cells like T-helper cells, monocytes, macrophages, and dendritic cells. All cells that possess this glycoprotein on their surface are known as CD4-bearing cells ($CD4^+$ cells). This epitope of protein is for the attachment of virus such as HIV and decrease in number as the infection progresses [13–15].

This study was designed to evaluate innate immune response in *Plasmodium* coinfection with HBV and HCV using complement C3, C4, and $CD4^+$ T cells to provide information for useful directions in the management of malaria, and hepatitis B and C.

Materials and methods

Materials

Study area

The study was carried out in the Baptist Medical Centre, a faith-based hospital in Saki West Local Government Area Headquarters in Nigeria. The local government is located at the Northern part of Oyo State. The hospital is also a training institution for family physicians, midwives, nurses, and medical laboratory technicians.

Study population

In all, 220 participants classified into controls who were neither infected with *Plasmodium* nor hepatitis B and C (n = 50), *Plasmodium*-infected participants (n = 50), hepatitis C-infected participants (n = 30), hepatitis B-infected participants (n = 30), *Plasmodium* coinfected with hepatitis B participants (n = 30), and *Plasmodium* coinfected with hepatitis C participants (n = 30) aged 19–65 years were recruited for the study from Saki-West Local Government of Oyo State.

Inclusion criteria

Plasmodium, HCV, HBV mono-infected and co-infected participants and tested negative to HIV and acid fast bacilli with elevated total bile acids were included as test participants, while those individuals free of *Plasmodium*, HCV, HBV, acid fast bacilli, and HIV with normal total bile acids were studied as controls.

Exclusion criteria

Patients/participants who were positive to HIV and acid fast bacilli tests were excluded from the study.

Methods

C3 ELISA

This was carried out using Abcam's complement C3 human in-vitro enzyme-linked immunosorbent assay (ELISA) kit.

Principle: antibody specific to complement C3 coated onto micro-titer well plates is added. Standards, test samples and control and subsequently a complement C3 specific biotinylated detection antibody is added, incubated, and washed, which is then followed by the addition of streptavidin–peroxidase conjugate incubated and washed with buffer. The activities of streptavidin–peroxidase enzyme is made observable by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) to produce a blue-colored product that changes into yellow after adding acidic stop solution. The intensity of the yellow color is directly proportional to the concentration of complement C3.

C4 ELISA

This was analyzed using Abcam's complement C4 human in-vitro competitive ELISA kit.

Principle: this involves the addition of test sample/standard/control to micro-titer well plates coated with antibody specific to complement C4, incubated and washed followed by the addition of streptavidin–peroxidase incubated and washed again. The activities of the enzyme is visualized by the addition of 3,3',5,5'-TMB to form a blue color which changes to yellow on addition of stop reagent. The intensity of the yellow color is directly proportional to the concentration of complement C4 in sample/standard/control.

$CD4^+$ T-cell

This was enumerated by cytoflowmetry using Partec machine and reagent.(Partec Equipment, 1 Ubi View, Singapore 408555 Phone: +65 6295 5331) IBM SPSS 18.0'New York.

Laboratory identification of *Plasmodium*

This was carried out using Giemsa thick and Leishman thin blood film staining techniques as described by CDC [10,11].

Anti-HCV ELISA assay

This was assayed using antihepatitis C virus core antigen antibody (ab50288) Abcam kit.

It involves the immobilization of antigen to HCV on a micro-titer plate to trace anti-HCV in the sample/control/standard involving addition of enzyme, substrate/HRP conjugate (horseradish peroxidase)) and chromogen (TMB) to visualize the reaction leading to the formation of color indicating the presence or concentration of anti-HCV in the sample/control/standard.

HBsAg ELISA test

This was assayed using diagnostic automation/Cortez Diagnostics, INC kit by ELISA method

It involves the immobilization of antibody to HBsAg on a micro titer plate to trace HBsAg in the sample/control/standard involving addition of enzyme, substrate/HRP conjugate (horseradish peroxidase) and chromogen (TMB) to visualize the reaction leading to the formation of color indicating the presence or concentration of HBsAg in the sample/control/standard.

Acid fast bacilli test by Ziehl Neelsen staining technique

This was carried out by Ziehl Neelsen staining technique as described by Cheesbrough [16].

HIV test

HIV test was carried out using Genscreen Ultra HIV1/2 antibody and HIV1 p24 antigen ELISA kit of BIO-RAD (Laboratories, Hercules, California, United States). The kit detects both HIV antigen and HIV antibody.

Estimation of total bile acid by enzymatic colorimetric method using reagent kit of Randox principle

Bile acids are converted to 3-ketosteroids and thio-NADH in the presence of thio-nicotinamide-adenine dinucleotide (NAD) and under the catalysis of the enzyme 3-α-hydroxysteroid dehydrogenase (3-α HSD). This is a reversible reaction as 3-α HSD can convert 3-ketosteroids and thio-NADH to bile acids and thio-NAD. In the presence of excess NADH, enzyme cycling occurs efficiently, and the rate of formation of thio-NADH is determined by measuring change of absorbance at 405 nm on a spectrophotometer.

Ethical consideration

The proposal of this work was presented, reviewed, and approved by the Ethics and Research Committee of Baptist Medical Center Saki, Oyo State, Nigeria before the start of this research work. Oral and written informed consent was also obtained from patients and controls.

Method of statistical analysis

The results obtained were subjected to statistical analysis using IBM SPSS 18.0 to determine mean, SD, probability, Student's '*t*' test, and level of significant at 0.05.

Results

The results obtained in this study show a significant decrease in plasma complement C3 in patients with monoinfection of *Plasmodium*, HCV, HBV, and *Plasmodium* patients coinfected with HCV and HBV compared with the control ($P < 0.005$; Tables 1–3 and Figs. 1, 2). There was also a significant decrease in plasma complement C4 in *Plasmodium* patients coinfected with HCV and HBV compared with the control ($P < 0.005$; Tables 1–3 and Figs 1, 2). The results obtained in *Plasmodium* patients coinfected with HBV and HCV and HBV patients coinfected with HCV showed a significant decrease in $CD4^+$ T cells compared with the results obtained in the control ($P < 0.005$; Tables 1–3 and Figs. 1, 2).

There was a significantly lower plasma complements C3, C4, and blood $CD4^+$ T cells in *Plasmodium*-monoinfected patients than in HBV or HCV-monoinfected patients ($P < 0.005$; Tables 1–3 and Figs 1, 2). There was also a significantly lower plasma complement C4 and $CD4^+$ T cells in *Plasmodium* patients coinfected with HBV or HCV than patients who were monoinfected with HCV and HBV ($P < 0.005$; Tables 1–3 and Figs 1, 2).

Discussion

The results obtained in this study showed a significant decrease in plasma complement C3 in patients with monoinfection of *Plasmodium*, HCV and HBV, and *Plasmodium* patients coinfected with HCV and HBV compared with the control. This outcome is in line with the findings of a significant decrease in C3 in the sera of patients with chronic hepatitis C as reported [17]. This is because HCV proteins can inhibit C3 complement synthesis [17]. Complement C3 is the most important central molecule in the complement system as it is being activated by both classic and alternative pathways which mediate opsonization and anaphylactic bioactivity. Further studies have shown the role of complement C3 in human immune responses,

Table 1 Mean and SD of C3, C4, and CD4⁺ T cells in patients and controls

	C3 (mg/dl)	C4 (mg/dl)	CD4⁺T cells (×10⁹/l)	HIV infection	Acid fast bacilli	TBA (μmol/l)
HBV (*n*=30)	100±4.0	9.8±1.5	0.55±0.01	Negative	Negative	11±0.5
HCV (*n*=30)	95±5.0	9.1±1.0	0.6±0.01	Negative	Negative	11±0.3
Plasmodium (*n*=50)	98±5.0	11±1.0	0.5±0.02	Negative	Negative	10±0.4
Plasmodium+HBV (*n*=30)	87±5.0	8.1±2.0	0.46±0.01	Negative	Negative	11±0.7
Plasmodium+HCV (*n*=30)	80±5.0	7.2±2.0	0.4±0.02	Negative	Negative	12±1.0
Control (*n*=50)	198±3.0	14±2.0	0.6±0.02	Negative	Negative	4.0±0.3

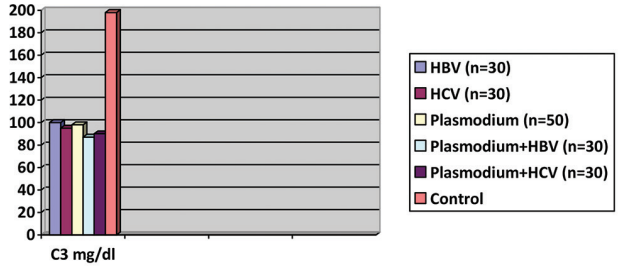
HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 2 Variations of complement C3, C4, and CD4+T cells in patients and controls

Parameters	HBV vs control	HCV vs control	*Plasmodium* vs control	*Plasmodium*+HBV vs control	*Plasmodium*+HCV vs control	HBV vs HCV	HBV vs *Plasmodium*
C3 (mg/dl)							
t	19.6	17.66	17.15	19.04	20.23	0.78	0.31
P	0.001**	0.002**	0.002**	0.001**	0.001**	0.26	0.39
C4 (mg/dl)							
t	1.68	2.19	1.34	2.05	2.4	0.85	–1.34
P	0.12	0.07	0.16	0.04*	0.04*	0.24	0.1
CD4⁺T cells (×10⁹/l)							
t	2.24	1	3.54	6.55	7.07	–3.53	2.23
P	0.08	0.05	0.04*	0.01*	0.009**	0.04*	0.08

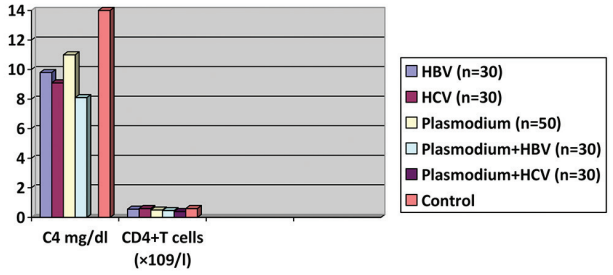
HBV, hepatitis B virus; HCV, hepatitis C virus. *Significant; **Highly Significant

Figure 1



Comparative description of complement C3 obtained in patients and control groups.

Figure 2



Comparative description of complement C4 and CD4⁺ T cells in patients and control groups.

lysis of pathogens, and that the C3 level correlates with infectious diseases [18].

There was also a significant decrease in plasma complement C4 in *Plasmodium* patients coinfected with HCV and HBV compared with the control. These results support the research report that there was a significant decrease in complement C4 in chronic viral hepatitis [19].

The results obtained in *Plasmodium* patients coinfected with HBV and HCV, and HBV patients coinfected with HCV showed a significant decrease in CD4⁺ T cells compared with the results obtained in the control. This could be as a result of immune response to the invasion of pathogens like *Plasmodium*, HCV, and HBV leading to the activation of CD4⁺ T cells. Induced intracellular expression of HCV has been linked with the shift in T-cell phenotype [20].

There was a significantly lower plasma complements C3, C4, and blood CD4⁺ T cells in *Plasmodium* monoinfected patients than in HBV or HCV monoinfected patients which agrees with the previous findings [21].

There was also a significantly lower plasma complement C4 and CD4⁺ T cells in *Plasmodium* patients coinfected with HBV or HCV than patients who were monoinfected with HCV and HBV. It has been reported that the bioactivities of complements include enhancement of chemotaxis, anaphylaxis, opsonization, and phagocytosis of microorganisms [22].

Generally, complement activity varies throughout the body like in patients with rheumatoid arthritis; complement activity may be normal or higher-than-normal in the blood, but much lower-than-normal in the joint fluid [23].

Table 3 Variations of complement C3, C4, and CD4+ T cells in patients and controls

Parameters	HBV vs *Plasmodium*+HBV	HBV vs *Plasmodium*+HCV	HCV vs *Plasmodium*	HCV vs *Plasmodium*+HBV	HCV vs *Plasmodium*+HCV	*Plasmodium* vs *Plasmodium*+HBV	*Plasmodium* vs *Plasmodium*+HCV	*Plasmodium*+HBV vs *Plasmodium*+HCV
C3 (mg/dl)								
t	2.03	3.12	-0.42	1.13	2.12	1.56	2.54	0.99
P	0.09	0.04*	0.36	0.19	0.08	0.13	16	0.35
C4 (mg/dl)								
t	0.44	0.85	-1.34	0.44	0.85	1.29	1.7	0.32
P	0.24	0.16	0.02*	0.003**	0.006**	0.12	0.12	0.39
$CD4^+$T cells ($\times 10^9$/l)								
t	7.6	6.7	4.47	12.07	8.94	1.69	3.53	3.15
P	0.008**	0.01*	0.02*	0.003**	0.006**	0.12	0.12	0.39

HBV, hepatitis B virus; HCV, hepatitis C virus. Note: *Significant, **Highly Significant

Participants were recruited based on their level of total bile acids. Those with elevated total bile acids were recruited as test participants who might be in active hepatocellular damage, while those with normal level of total bile acids were studied as controls. A significantly higher value of this parameter was found in test participants than the control and in those with viral hepatitis than the *Plasmodium* monoinfected participants. Viral coinfection with *Plasmodium* also showed a significant increase in the value of TBA. It has been reported that elevated serum bile acids is found in liver disease with the greatest increase in acute viral hepatitis but moderate or slight increase in chronic active hepatitis, liver cirrhosis, and hepatoma as what causes liver cirrhosis and hepatoma include accumulation of fibrotic tissues as a result of hepatocellular damage [24]. Fasting bile acids is a sensitive index of hepatocellular dysfunction but deficient in its use to differentiate liver diseases which is a limitation of this study [24]. This is because liver is constantly exposed to large varieties of antigens such as dietary antigens through meal, pathogens, and toxins. Liver is a major immune organ. Liver forms a major innate immune system with many cellular components such as monocytes, macrophages, granulocytes, natural killer cells, and dendritic cells, which coordinate to exert a tolerogenic environment and at the same time detect, respond, and eliminate invading pathogens, infected or transformed self to mount immunity [25,26].

Complements are produced in the liver and available in the blood. Significant alterations in the level of complements in this study can be linked with the explanation that complements destroy antibody-coated targets, apoptotic cells, and infectious agents which include bacteria, viruses, and fungi [27].

CD4 is known as a cluster of differentiation 4 or $CD4^+$ T-helper cells or CD4 cells. T-helper cells or T4 cells are essential white blood cells in the human immune system because they send signals to other types of immune cells like CD8 killer cells to release cytotoxin to destroy infectious particles such as HBV, HCV, *Plasmodium* and some other immune cells including the production of antibodies. Continual or excessive use of this cell in these processes can deplete its value especially in immune suppression [13–15].

It has been reported that innate immunity is an indispensable factor in early virus infection that facilitates virus clearance [28] and hence immunochemical alterations in the values of the complements and $CD4^+$ cells obtained in this study.

Furthermore, innate immune response to viral infection is in three phases [28], which involves various sensors

in the cytoplasm that recognize pathogen-associated molecular patterns, such as foreign DNA or RNA, sending a warning message to initiate downstream signals; the proteins of the downstream signaling pathways transmitting the danger message to the nucleus, activating effector elements, and the consequently upregulated effectors that degrade exogenous viral elements [28].

In addition, HCV is a leading cause of chronic viral hepatitis that can lead to cirrhosis and hepatocellular carcinoma as a result of liver damage [29]. During acute infection very few patients can clear the virus. Elimination of HCV during acute infection has been associated with a rapid induction of innate, especially interferon-induced genes, and delayed induction of adaptive immune responses [29] which can also be linked with the results obtained in this study.

Considering innate immune response to malaria as evidenced by the significant alterations in the levels of complements and $CD4^+$ cells in this study, it has clearly shown that innate immune response is an essential pathophysiological protective process to the infection as it has been reported [30] that innate immune responses have great contribution to the control of *Plasmodium* infection which involves natural killer T cells as significant to the immunity of the liver-stage of the infection [30]. Innate immune responses can also influence the nature and magnitude of the adaptive immune response to *Plasmodium* infection [30].

The results obtained in the evaluation of innate immune response in *Plasmodium* coinfection with HBV and HCV considering a significant immunochemical alterations in the values of complement C3, C4, and $CD4^+$ T cells in this study is in support of the above explanations and reports, hence affirming the evidence of innate immune response in *Plasmodium* coinfection with HBV and HCV.

Conclusion

This study showed the possible innate immune response as shown by a significant decrease in plasma levels of C3, C4, and blood $CD4^+$ T cells in *Plasmodium*, hepatitis B and C virus mono- and coinfections, including a significant influence of *Plasmodium* infection on the values of the innate immune materials.

Limitation and strength

This study investigated patients who were mono- and coinfected with *Plasmodium*, HBV, and HCV with active liver damage as signified by elevated total bile acids without a clear demarcation between acute and chronic phases. However, the study has been used to provide information for useful directions on the possible immune responses through the evaluation of blood complement C3, C4, and $CD4^+$ T cells in patients who were mono- or coinfected with *Plasmodium*, HBV, and HCV for effective management of malaria, and hepatitis B and C infections.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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