

PREVALENCE OF MUTANT *Plasmodium falciparum* chloroquine resistance transport gene (Pfcr) IN HUMAN IMMUNE DEFICIENCY VIRUS PATIENTS ATTENDING UNIVERSITY OF UYO TEACHING HOSPITAL, UYO-NIGERIA

Running Title:-Prevalence of mutant Pfcr gene in HIV Patients attending University of Uyo Teaching Hospital Uyo-Nigeria

ABSTRACT: In this study, the prevalence of mutant *Plasmodium falciparum* chloroquine resistant transport gene in HIV positive patients attending University of Uyo Teaching Hospital, Uyo was evaluated. The risk factors associated with HIV was evaluated by testing for the significant difference between Genders, Ages, CD4 count level in association with malaria parasite. Blood samples were collected from 67 participants attending the HIV adult Clinic, University of Uyo Teaching Hospital, Uyo. Of the 67 cases, 35 were HIV+ve adult patients while 32 were HIV -ve (Controls). Twenty-one percent of subjects admitted that they took Anti-malaria drugs every three months. Upon microscopic examination, 2(5.7%) of *falciparum* malaria was detected, while Nested PCR Assay detected 3(8.7%) *falciparum* malaria in the DNA sample extracts of HIV patients. All 32 HIV negative subjects had no *falciparum* malaria. Five HIV positive patients were infected with *falciparum* malaria; the prevalence rate of Pfcr in HIV patients was 14.2%. There was no significant difference of malaria parasite infection by gender (P = 0.88), age group (P = 0.17), and CD4+ count (O.R:1.0, P = 0.81). There is an urgent need to administer effective anti-malaria drug to HIV patients infected with *falciparum* malaria.

Keywords: Prevalence, Mutant *Plasmodium falciparum* chloroquine resistant transport gene, Human Immune Deficiency Virus Patients, University of Uyo Teaching Hospital

1. INTRODUCTION

Malaria and HIV are two major public health challenges to patients in developing African countries (1). Sub-Saharan Africa has the most case of HIV and AIDS epidemic in the world (2). In 2013, an estimated 24.7 million people were living with HIV, accounting for 71% of global total (2), In the same year, there was an estimated 1.5 million new HIV infections and 1.1 million AIDS related deaths (3) while in 2015, there were about 214 million malaria cases that led to 438,000 deaths while in 2015; there were about 214 million

38 malaria cases that led to 438,000 deaths (4). The geographical overlap in sub-Saharan Africa
39 and South America has led to an equivalence in co-infection with *Plasmodium* and HIV, this
40 has resulted to the quick progression and severity of both infectious diseases notably among
41 the poor, and contributes to the financial conditions of sub-Saharan African nations by taking
42 a toll on young people who contribute greatly to the workforce of the economy (5). These two
43 infectious diseases have been documented to account for an enormous morbidity and
44 mortality rates in sub-Saharan Africa (6).

45 Malaria and Human Immunodeficiency Virus (HIV) frequently coexist in patients in
46 many parts of the world due to geographical overlaps of these two diseases. Presently, most
47 epidemiologic data on HIV infection with malaria are derived from *P. falciparum* endemic
48 regions of sub-Saharan Africa, Nigeria inclusive. These two infectious disease interact bi-
49 directionally and synergistically with each another (6). Thus, HIV infection can increase the
50 risk and severity of malaria infection and parasite burden which may facilitate higher rate of
51 malaria transmission. People living in malaria endemic areas, such as Uyo, Nigeria,
52 particularly, are considered semi-immune to malaria and can develop clinical malaria if they
53 are untreated. This has become a significant public health threat as a result of increase risk
54 factors associated with high malaria burden in HIV infected persons including low CD4⁺ cell
55 counts, low immune status, and gender among others, and the use of standard diagnostic
56 methods. HIV –1 RNA concentration and CD4 cells count are moderately but inconsistently
57 associated with *parasitaemia*. High parasite count with fever is associated with HIV –1 sero-
58 positivity and low CD4 cell count (7). Studies have shown that vulnerability and frequency of
59 malaria infection appear to be increased in HIV-positive adults particularly those with low
60 CD4 cell counts (8 9 10). Another challenge, is the chemotherapy failure in HIV patients,
61 when there is inability of a drug to produce the desired therapeutic effect. Treatment failure is
62 caused by various factors which include drug resistance (11). Chloroquine resistant *P.*

63 *falciparum* parasites arose in the late 1960s and have since spread to malaria endemic area
64 rendering chloroquine ineffective in many patients, including HIV patients (12). Currently,
65 the utilization of molecular diagnostic procedures in detecting and quantifying the mutant
66 resistant gene of *Plasmodium* species, particularly *P. falciparum* in HIV positive patients
67 have provided a new set of tools that facilitates both epidemiologic investigation and patients
68 treatments. However, there is paucity of information on molecular detection of *P. falciparum*
69 in HIV-infected patients and thus efficient treatment options

70 The sensitivity and accuracy of Polymerase Chain Reaction in detection and
71 quantifying of *P. falciparum* mutant resistant gene in the human blood is not
72 widely known. Detailed molecular information of *P. falciparum* is neglected in
73 most parts of Nigeria. This study will provide a baseline of a molecularly
74 assessment, detection and quantification of mutant *Plasmodium falciparum* chloroquine
75 resistant transport gene in HIV infected patients in Akwa Ibom, Nigeria

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77 **2. MATERIALS AND METHODS**

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79 **2.1 Study Design**

80 This study was a case control hospital based study consisting of HIV infected adult
81 patients, and non HIV infected adults attending the University of Uyo Teaching Hospital for
82 treatment.

83 **2.2 Study Area**

84 This study was carried out in the University of Uyo Teaching Hospital for
85 a period of 6 months. The hospital is located in Uyo, the capital of Akwa Ibom State,
86 Nigeria. It offers general medical treatment and surgical operation to HIV infected and
87 non infected patients in Uyo and other local government Areas in Akwa Ibom State.

88 The Latitude of Uyo, Nigeria is 5.038963, and longitude is 7.909470 with GPS

89 coordinates of 5° 2' 20.2668'' N and 7° 54' 34.0920'' E and elevation of 65m, 213 feet
90 above sea level.

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92 **2.3 Study Population**

93 This involved a total of 67 consisting of 35 HIV infected adult patients, and 32 non
94 HIV infected adults as controls with *falciparum* malaria attending the HIV Clinic University
95 of Uyo Teaching Hospital in Uyo.

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100 **3. Ethical Consideration**

101 Approval from the ethical Review Board of the University of Uyo Teaching Hospital,
102 Uyo was obtained prior to the inception of this research study. Permission was granted
103 by participants through their consent before the commencement of the study.

104

105 **3.1 Collection of Sample**

106 A sterile multi sample needle was used to draw 5ml of intravenous blood sample
107 into an EDTA Vacutainer glass tubes.

108

109 **3.2 HIV Test**

110 A plastic pipette was used to add 1 drop of whole blood to the sample well of Uni
111 gold Cassette kit. Three drops of diluents was added into the sample well. In 20 minutes,
112 result was read (13).

113

114 **3.3 CD4⁺ Count Test**

Twenty (20µl) of CD4 PE antibody was added into a Partec test tube, followed by an addition of 20 µl well mixed whole blood (EDTA) to the Partec test tube. This was incubated in the dark for 15 minutes at room temperature. The mixed CD4 PE antibody and blood solution was mixed every 5minutes with a 230 volt vortex mixer. Eight hundred (800µl) CD4 buffer solution was added and mixed gently by the same vortex machine for 5 seconds. The mixed sample was plugged in to a counter machine for CD4⁺ count reading (14).

3.4 Microscopic Analysis

A Plastic pipette was used to add 3 drops of blood sample on a labelled clean glass slide. Another clean glass slide was used to make a thick and thin blood film sample at a spot on the slide glass. After 10 minutes, the dried thin films were stained with 1-2 drops of methanol to affix the blood samples and then 10% of Giemsa stain, leaving the filmed glass slide to dry for 10 minutes. Distilled water was then poured to wash, drain and air dry for microscopy examination (15).

3.5 DNA Molecule Extraction

Four hundred (400µl) microlitre pipette was used to add Genomic Lysis Buffer to 100µl of blood sample, mixed completely by a vortex mixer for 4-6 seconds, thereafter allowed to stand for 5-10 minutes at room temperature. The mixture was then transferred to a Zymo-SpinTM Column in a collection tube. Two hundred (200µl) microlitres of DNA Pre-Wash Buffer was added to the spin column. This was centrifuged at 10,000 x for one minute. The spin column was transferred to a clean microcentrifuge tube and less than five

hundred (<500µl). Elution Buffer was added to the spin column and incubated for 2-5 minutes at room temperature (16).

3.6 **Pfcr1** gene Amplification (Nested PCR)

Thirty five (35) PCR tubes and an additional 32 were labelled as controls (HIV Negative) and placed in an Ice rack. A calibrated pipette of 1.0 µl was used to draw up a master mix and transferred to a 1.5µl Eppendorf tube. This was followed by adding 0.4µl *Pfmdr* 1 primers- (Forward IMDR1/F and reverse R IMDRI/R) 0.5µl MgCl₂, 5mM DNA template and a high molecular grade water of 3.29µl to dilute the other reagent in their correct concentrations. The primary nested PCR final volume (20µl) was taken from PCR cocktail and then transferred into the labelled PCR tubes for amplification by a Bio system PCR machine.

In the secondary stage of Nested PCR, all PCR tubes were labelled like that of the primary stage of Nested PCR and placed in an Ice rack. A calibrated pipette of 12.5 was used to draw up a master mix and transferred to a 1.5µl Eppendorf tube. This was followed by adding 0.2µl **Pfcr1** primers (Forward IMDR1/F and reverse R IMDRI/R) 0.5µl MgCl₂, 0.5µl DNA template, and an 11.1µl high molecular grade water to dilute the other reagent in their correct concentrations. The primary and secondary nested PCR final volume (25µl) was taken from PCR cocktail and then transferred into the labelled PCR tubes for amplification by a Bio system PCR machine. The secondary PCR process was carried out by using the amplicons from the primary nested PCR to carry out the secondary nested PCR process to ascertain the desired DNA gene of interest **Pfcr1**. Agar gel Electrophoresis was then carried out to separate the DNA gene molecule (**Pfcr1**) of interest.

3.7 Agar Gel Electrophoresis

Casting moulding tray was used to mould the agarose gel. Combs were used to create wells into which the PCR products (amplicons) were loaded. The warm agarose was poured gently into the casting tray with the comb already fixed appropriately. The poured agarose was allowed to set for 30 - 60 minutes. After which it was stored in the fridge for use. DNA is negatively charged and so the wells of the set agarose is placed to face the negative pole of the electrophoresis tank (filled with 1 x Tris Boric buffer to the maximum level). Ten (10µl) amplicons were loaded as appropriate and allowed to run at 110 volt for 25 minutes. The gel was viewed with an Ultra Violent Trans-illuminator to observe the lanes of DNA molecules.

3.8 Statistical Analysis

The statistical parameters for the analysis of data were Pearson's Chi-Square Test. All charts were created using Microsoft Excel software application.

4. RESULTS

4.1 Prevalence of malaria parasite according to HIV status and gender

Of the 67 subjects recruited in the study, 38 were of males while 29 were females, only 5(7.0%) HIV positive patients had *parasitaemia*. High prevalence of *parasitaemia* was seen in male subjects who had a prevalence rate of 10.3%. (Table 1)

Table 1: Prevalence of malaria parasite according to HIV status and gender

(n= 67)

HIV Positive		Malaria Parasite		
Gender	No. of Samples	Positive (%)	χ^2	P-value
Male	13	2(5.38)	0.02	0.88
Female	22	3(4.7)		
Total	35	5(14.2)		
HIV Negative (Control)				
Male	25	0(0)		
Female	7	0(0)		
Total	32	0(0)		

No significant difference P=0.005

4.3 The Risk Factors Associated with HIV Positive Patients

Malaria positive and negative cases were higher in the female gender of the HIV patients. Age group of 30-39 had the highest number of malaria positive and negative cases. (Table 2)

Table 2. Risk factors to malaria parasite in HIV positive Patients

(n = 35)

Variables	No. of samples	No. of malaria positive cases	No. of malaria negative cases	χ^2	df	P-value	Odd Ratio	95% C.I.
	35	5 (%)	30 (%)					
CD4⁺ counts (cells/μl)								
<200	9	2(7.7)	7(29.6)	0.056	1	0.81	1	0.43-30
$\geq$ 200	26	3(4.0)	23(30.2)				0.46	
Gender								
Male	13	2(5.3)	11(29.6)	0.02	1	0.88	1	0.13-6.0
Female	22	3(4.7)	19(30.2)				0.87	
Age in Years								
20-29	9	0(0)	9(35.0)	6.13	3	0.17	1	0.35-
							3.11	27.5
30-39	17	3(6.1)	14(28.8)					
40-49	5	2(14.0)	3(21.0)					
50 and above	4	0(0)	4(35.0)					

No significant difference P=0.005

4.4 Molecular detection of Mutant Plasmodium falciparum chloroquine resistant transport gene 1 in HIV positive patients

Gel electrophoresis showing the Pfcrt gene of malaria parasites from HIV positive patients. Lane 11, 21, 23, 24, 30 had Pfcrt gene. CD4⁺ counts 81 and 105µl were of 2(5.7%) +ve cases of malaria respectively, while 424, 450, 488µl were of 3 (8.7%) +ve of malaria parasite

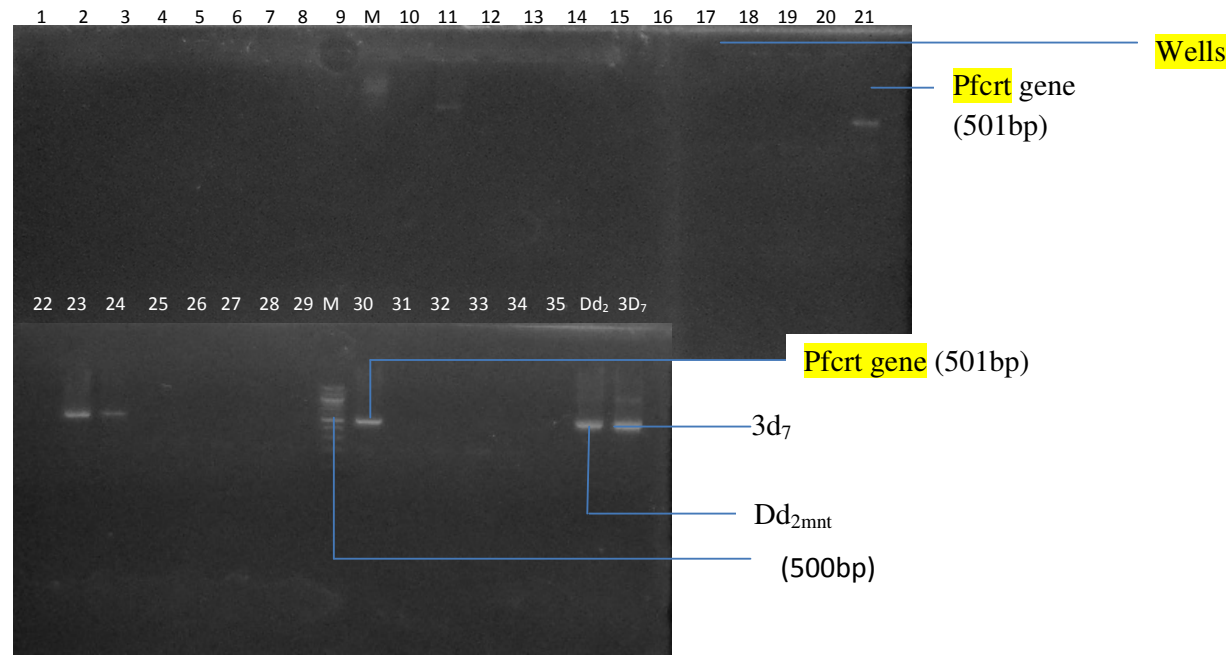


Fig 1.0: Molecular detection of Plasmodium falciparum chloroquine resistant transport gene in HIV positive patients

4.5 Molecular Detection of Mutant *Plasmodium falciparum* chloroquine resistant transport gene in HIV negative participants (Controls)

Lane M represents the Quick- Load 100bp molecular ladder while Dd₂ and 3d₇ are the mutant and Wild type controls respectively.

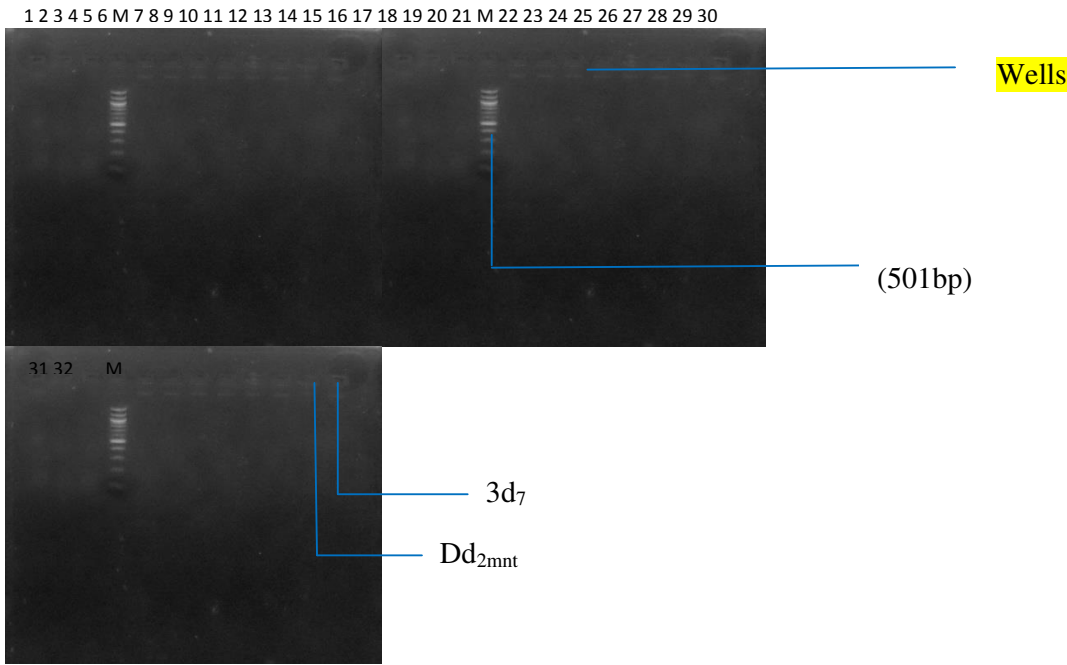
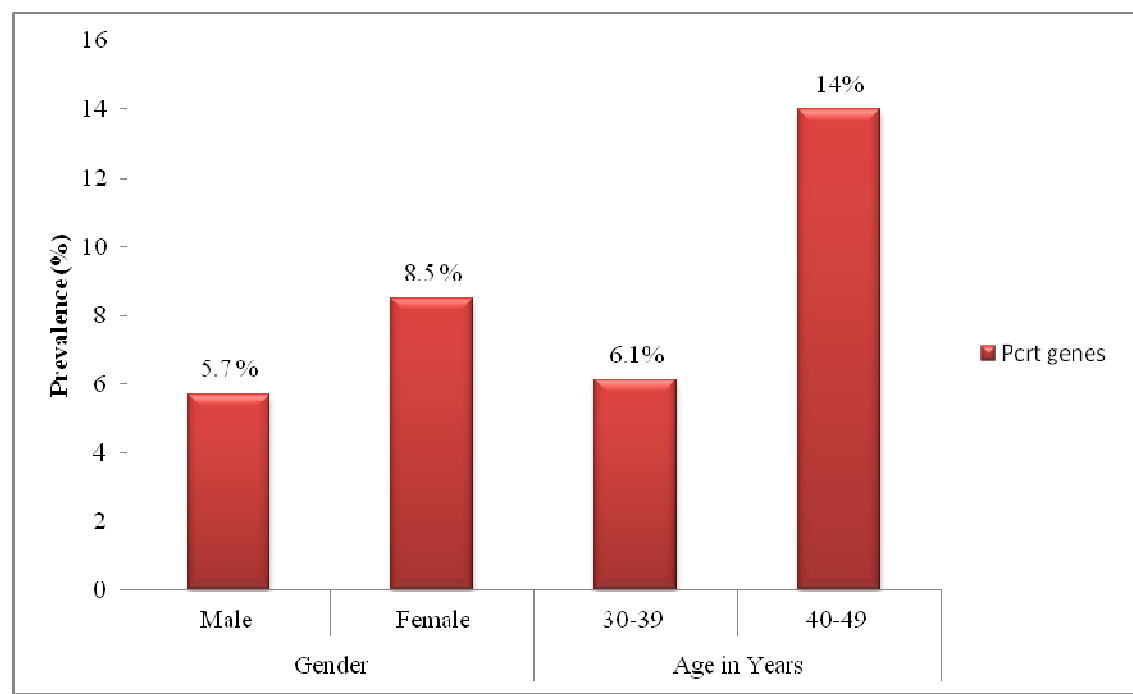


Fig 1.1: Molecular detection showing the absence of mutant *Plasmodium falciparum* chloroquine resistant transport gene of malaria parasites from HIV negative patients as a control.

4.6 Prevalence of Mutant *Plasmodium falciparum* chloroquine resistant transport gene according to gender and age in HIV positive patients

Females had 8.5% of Pfcrt gene , while 5.7% of Pfcrt was determined in males. Age group 30-39 years had 6.1% of Pfcrt gene while 40-49 years had a percentage of 14%,.



$\chi^2 = 4.1, 29.0, df=1, P = 0.005$

$\chi^2 = 0.17 df=1, P = 0.005$

Figure 1.2: Prevalence of mutant *Plasmodium falciparum* chloroquine resistant transport gene according to gender and age in HIV positive patients

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DISCUSSION

Malaria and HIV are two major Public Health challenges to patients in developing African countries (1). Recent studies have elucidated on the link between HIV and malaria and the potential public health implications of co-infection (17). Human Immunodeficiency Virus (HIV) infected adults in malaria endemic areas are at risk of contracting malaria, as HIV infection increases the incidence and severity of clinical malaria. HIV infection has been found to double the risk of malaria *parasitaemia* in clinical malaria (7). The prevalence of *falciparum* malaria and its resistant (Pfcrt) genes were evaluated during this study. In this study, Microscopic and Nested PCR assay of 67 HIV blood samples revealed that 5 (14.2%) of the samples were positive for *Plasmodium falciparum*. This indicated that microscopic method of screening of malaria parasite is very reliable. Method of diagnosis of malaria using microscopy continues to be the gold standard for laboratory confirmation (18); however a molecular diagnostic method (Nested Polymerase Chain reaction) was used to detect three positive cases compared to two cases earlier detected through microscopy. This confirms that Nested PCR was more sensitive and accurate than thick and thin film s of microscopy diagnostic method (19). The Nested PCR has been reported to be preferable to other PCR techniques because the target DNA sequence would be more abundant than the original solution before the first PCR is performed (20). Molecular analysis of the five positive *falciparum* parasites detected from DNA extract sample of the HIV positive patients, revealed the presence of Pfcrt. (Pfcrt) genes, the molecular weight of Pfcrt genes was 501bp. A prevalence of 14.2% Pfcrt was determined in this study.

During this study, HIV female patients were examined for *falciparum* malaria than males. Pfcrt genes were dominant in middle aged grouped female patients than the male patients, this was similar to that of Oke *et al.* (2017) (21) who reported the predominance of Pfcrt in

middle aged adult men who took chloroquine . However, in Oke *et al.* (2017) study, men were mostly examined than females. Statistically, there was significant difference between gender; $\chi^2 = 4.1, 29.0, df=1 P=0.005$, age $\chi^2 = 0.17 df=1, P=0.005$ with Pfcrt (Figure 1.2). The high prevalence of Pfcrt in females (8.5%) in this study could be as a result of malaria transmission determined in large part by social, economic and cultural factors, which intercross gender-specific vulnerabilities to impact women's ability to prevent malaria infection. Women's traditional household task, such as cooking the evening meal outdoors or waking up before sunrise to prepare the household for the day, may also put them at greater risk of malaria infection (22).

The presence of Pfcrt genes among the subjects agrees with the findings of Motta, 1995; Happi *et al.*, 2006; Okungbowa and Mordi (23, 24, 25). This indicates that Pfcrt gene is widely spread throughout Nigeria, the spread of this resistant gene is associated with the presence of the causative agent (*P. falciparum*) of the disease across Nigeria. However, the prevalence recorded in this study is dissimilar to that of Okungbowa and Mordi (2013) (25) who recorded a higher percentage of Pfcrt 24%. The prevalence of Pfcrt (14.2%) in our study area could be as a result of high indiscriminate use of drugs (drug abuse) for treatment of malaria in people living with HIV. Also, it could also be as a result of long time use of chloroquine drugs. Constant exposure of the parasite to drugs could lead to development of these resistance genes (26).

Investigation on risk factors of malaria parasite in HIV patients in this study revealed that there was no significant difference in relationship between the level of CD4⁺, gender, and age group and malaria parasite. Subjects in this study were aware of the outcome of malaria illness if exposed to mosquito bites indoors and outdoors..

In this study, two subjects with lesser CD4⁺ count of <200 cells / μ l had malaria parasite, while three other subjects with CD4⁺ counts ≥ 400 cells/ μ l also had malaria parasite. The patients in our study followed the High Active Antiretroviral Treatment

regimen (Lamivudine, Tenofovir and Efavirenze) which helped increase the CD4⁺ count of more than half of the subjects. This explained why the results showed no significant relationship between low CD4⁺ T-cell count and increased *parasitaemia* in these patients ($\chi^2 = 0.056$, df = 1, P = 0.81). Thus, there was no significant difference between low CD4⁺ T-cell counts and increased *parasitaemia*. This is because both subjects that had higher and lesser CD4⁺ of <200 cells / μ l counts had *parasitaemia* in the peripheral blood. This differs to that obtained in a study by Chavale *et al.* (2012) (27), which showed that there is a significant relationship between high amount of *parasitaemia* and decreased CD4⁺ T-cell count in HIV positive patients. A possible reason for this difference could be that patients in the former study were neither taking Antiretroviral nor Antimalarial drugs. This situation worsens if both diseases are left untreated. This explains the significance of lower CD4⁺ T-cell count and high amount of *parasitaemia* in the study by Chavale *et al.* (2012) (27).

HIV and malaria are diseases that have specific regimen of five to six drugs for treatment and it is important to study the possible interactions these drugs might have on each other. New information on HAART and Antimalaria drugs interactions will generate new guidelines for malaria in HIV-infected people on anti-retroviral (28). Therefore, there is an urgent need to develop efficient and effective Antimalarial treatment to contain this situation since millions of people are co-infected with *Plasmodium* and HIV worldwide.

CONCLUSION

In conclusion, this study revealed that prevalence rate of *Plasmodium falciparum* chloroquine resistant transporter gene in HIV patients at the University of Uyo Teaching Hospital, Uyo, Nigeria was high. Majority of the patients had healthy CD4⁺ count levels which helped to prevent them from illness such as *falciparum malaria*. Subjects with lower CD4⁺ of 200cells/ μ l were associated with an increased risk of having malaria parasite. Females had more positive cases of *falciparum malaria*.

Females in their middle age were likely to have more mutant Pfert in the peripheral blood.

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