Original Research Article

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

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

ABSTRACT: In this study, the prevalence of mutant *Plasmodium falciparum* chloroquine mutant 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 Pfcrt 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 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 which there is inability of a drug to produce the desired therapeutic effect. Treatment failure
62 is caused by various factors which include drug resistance (11). Chloroquine resistant *P.*

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

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

2. MATERIALS AND METHODS

2.1 Study Design

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

2.2 Study Area

This study was carried out in the University of Uyo Teaching Hospital for a period of 6 months. The hospital is located in Uyo, the capital of Akwa Ibom State, Nigeria. It offers general medical treatment and surgical operation to HIV infected and non infected patients in Uyo and other local government Areas in Akwa Ibom State. The Latitude of Uyo, Nigeria is 5.038963, and longitude is 7.909470 with GPS

coordinates of 5º 2’ 20.2668” N and 7º 54’ 34.0920’’ E and elevation of 65m, 213 feet above sea level.

2.3 Study Population

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

3. Ethical Consideration

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

3.1 Collection of Sample

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

3.2 HIV Test

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

3.3 $CD4^+$ Count Test

115 Twenty (20µl) of CD4 PE antibody was added into a Partec test tube, followed by
116 an addition of 20 µl well mixed whole blood (EDTA) to the Partec test tube. This was
117 incubated in the dark for 15 minutes at room temperature. The mixed CD4 PE antibody and
118 blood solution was mixed every 5minutes with a 230 volt vortex machine. Eight hundred
119 (800µl) CD4 buffer solution was added and mixed gently by the same vortex machine for 5
120 seconds. The mixed sample was plugged in to a counter machine for $CD4^+$ count reading
121 (14).
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125 **3.4 Microscopic Analysis**
126 A Plastic pipette was used to add 3 drops of blood a sample on a labelled clean
127 glass slide. Another clean glass slide was used to make a thick and thin blood film sample at
128 a spot on the slide glass. After 10 minutes, the dried thin films were stained with 1-2 drops
129 of methanol to affix the blood samples and then 10% of Giemsa stain, leaving the filmed
130 glass slide to dry for 10 minutes. Distilled water was then poured to wash, drain and air dry
131 for microscopy examination (15).
132
133 **3.5 DNA Molecule Extraction**
134 Four hundred (400µl) microlitre pipette was used to add Genomic Lysis Buffer to
135 100µl of blood sample, mixed completely by a vortex machine for 4-6 seconds, thereafter
136 allowed to stand for 5-10 minutes at room temperature. The mixture was then transferred to
137 a Zymo-Spin™ Column in a collection tube. Two hundred (200µl) microlitres of DNA Pre-
138 Wash Buffer was added to the spin column. This was centrifuged at 10,000 x for one
139 minute. The spin column was transferred to a clean microcentrifuge tube and less than five

140 hundred (<500µl). Elution Buffer was added to the spin column and incubated for 2-5
141 minutes at room temperature (16).
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143 **3.6 Pfcrt gene Amplification (Nested PCR)**
144 Thirty five (35) PCR tubes and an additional 32 were labelled as controls (HIV
145 Negative) and placed in an Ice rack. A calibrated pipette of 1.0 µl was used to draw up a
146 master mix and transferred to a 1.5µl Eppendorf tube. This was followed by adding 0.4µl
147 *Pfmdr* 1 primers- (Forward IMDR1/F and reverse R IMDRI/R) 0.5µl $MgCl_2$, 5mM DNA
148 template and a high molecular grade water of 3.29µl to dilute the other reagent in their
149 correct concentrations. The primary nested PCR final volume (20µl) was taken from PCR
150 cocktail and then transferred into the labelled PCR tubes for amplification by a Bio system
151 PCR machine**.**
152 In the secondary stage of Nested PCR**,** all PCR tubes were labelled like that of the primary
153 stage of Nested PCR and placed in an Ice rack. A calibrated pipette of 12.5 was used to
154 draw up a master mix and transferred to a 1.5µl Eppendorf tube. This was followed by
155 adding 0.2µl Pfcrt primers (Forward IMDR1/F and reverse R IMDRI/R) 0.5µl $MgCl_{2,}$ 0.5µl
156 DNA template, and an 11.1µl high molecular grade water to dilute the other reagent in their
157 correct concentrations. The primary and secondary nested PCR final volume (25µl) was
158 taken from PCR cocktail and then transferred into the labelled PCR tubes for amplification
159 by a Bio system PCR machine. The secondary PCR process was carried out by using the
160 amplicons from the primary nested PCR to carry out the secondary nested PCR process to
161 ascertain the desired DNA gene of interest Pfcrt. Agar gel Electrophoresis was then carried
162 out to separate the DNA gene molecule (Pfcrt) of interest.
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164 **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 chats 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 Gender	No. of Samples	Malaria Parasite 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)

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

214 **(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
≥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 inYears								
20-29	9	0(0)	9(35.0)	6.13	3	0.17	1	0.35-27.5
30-39	17	3(6.1)	14(28.8)				3.11	
40-49	5	2(14.0)	3(21.0)					
50 and above	4	0(0)	4(35.0)					

215 No significant difference P=0.005

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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. Lanes without band represent the absence of Pfcrt gene while those with bands indicate presence of the gene.

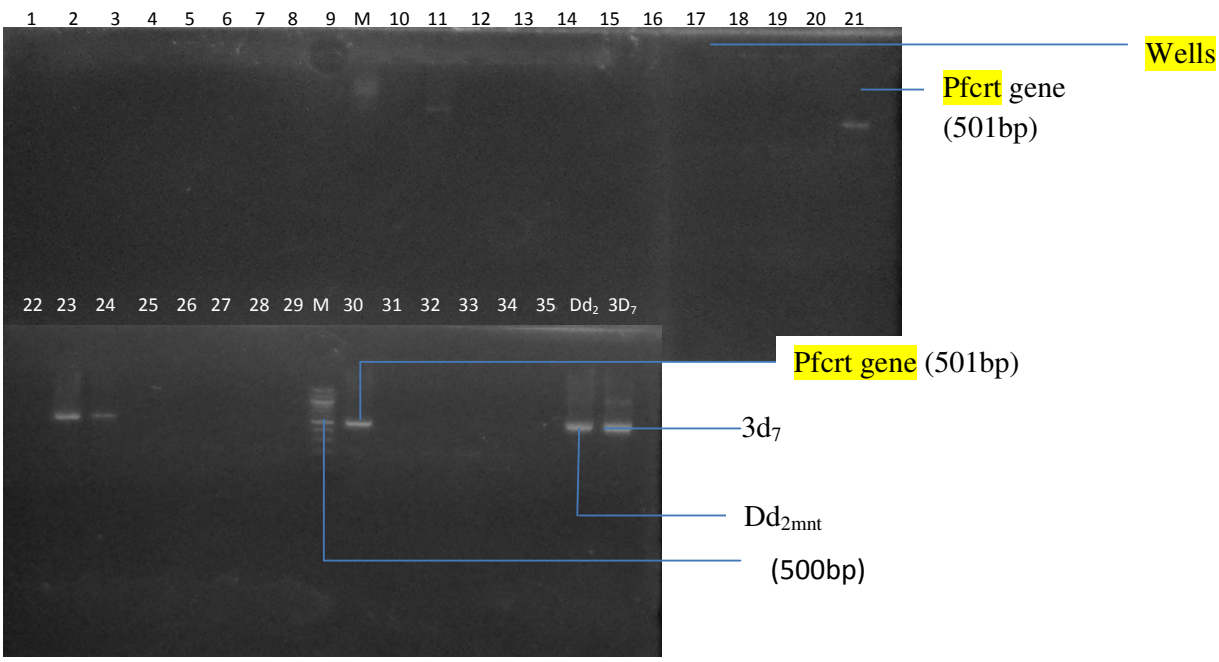


Fig 1.0: Molecular detection of *Plasmodium falciparum* chloroquine resistant transport gene in HIV positive patients

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 Dd_2 and $3d_7$ are the mutant and Wild type controls respectively.

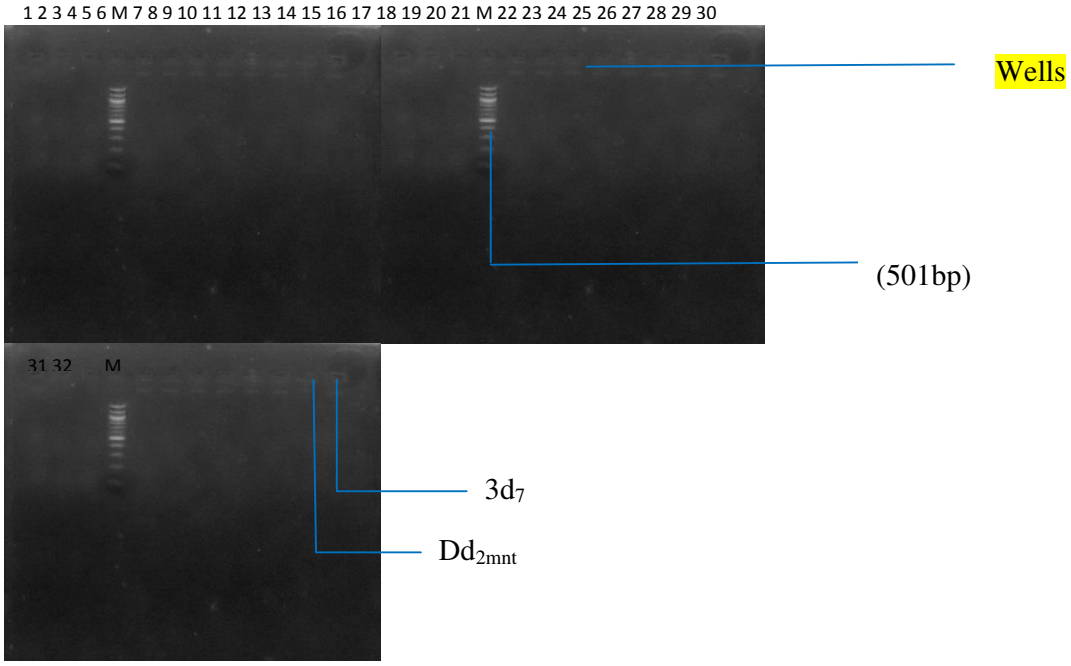
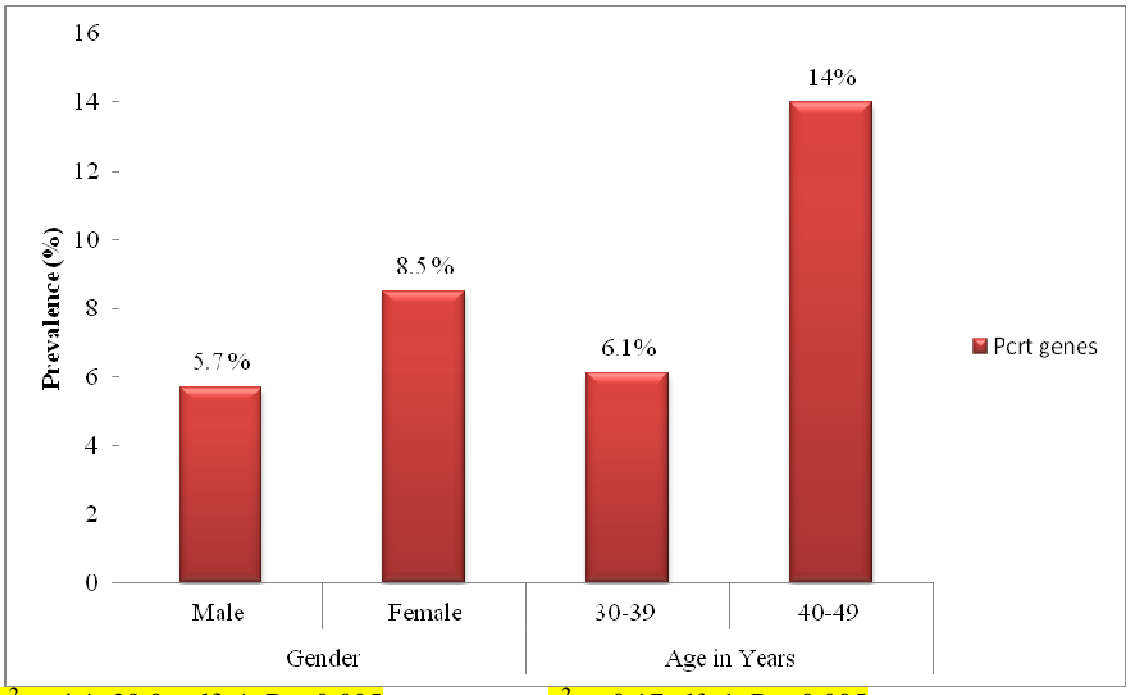


Fig 1.1: Molecular detection showing the absence of mutant *Plasmodium falciparum* chloroquine resistant transport gene of malaria parasites from HIV negative patients as a control.

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%,.



χ^2 = 4.1, 29.0, df=1, P = 0.005 χ^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 where dominant in middle aged grouped females patients than the male patients, this was similar to that of Oke *et al.* (2017) (21) who reported the predominance of Pfcrt in

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

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

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

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

331 regimen (Lamivudine, Tenofovir and Efavirenze) which helped increase the $CD4^+$ count of
332 more than half of the subjects. This explained why the results showed no significant
333 relationship between low $CD4^+$ T-cell count and increased *parasitaemia* in these patients (χ^2
334 = 0.056, df = 1, P = 0.81). Thus, there was no significant difference between low $CD4^+$ T-
335 cell counts and increased *parasitaemia*. This is because both subjects that had higher and
336 lesser $CD4^+$ of <200 cells /µl counts had *parasitaemia* in the peripheral blood. This differs
337 to that obtained in a study by Chavale *et al*. (2012) (27), which showed that there is a
338 significant relationship between high amount of *parasitaemia* and decreased $CD4^+$ T-cell
339 count in HIV positive patients. A possible reason for this difference could be that patients in
340 the former study were neither taking Antiretroviral nor Antimalarial drugs. This situation
341 worsens if both diseases are left untreated. This explains the significance of lower $CD4^+$ T-
342 cell count and high amount of *parasitaemia* in the study by Chavale *et al*. (2012) (27).
343 HIV and malaria are diseases that have specific regimen of five to six drugs for
344 treatment and it is important to study the possible interactions these drugs might have on
345 each other. New information on HAART and Antimalaria drugs interactions will generate
346 new guidelines for malaria in HIV-infected people on anti-retroviral (28). Therefore, there
347 is an urgent need to develop efficient and effective Antimalarial treatment to contain this
348 situation since millions of people are co-infected with *Plasmodium* and HIV worldwide.
349

350 **CONCLUSION**

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

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

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