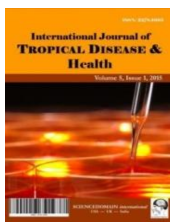

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Prevalence of Mutant *Plasmodium falciparum* Chloroquine Resistance Transport Gene (PFCRT) in Human Immune Deficiency Virus Patients Attending University of Uyo Teaching Hospital, Uyo-Nigeria

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Authors' contributions

This work was carried out in collaboration between both authors. Author ACP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors IUC and ACP managed the analyses of the study. Author IUC managed the literature searches. Both authors read and approved the final manuscript.

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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

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

Malaria and Human Immunodeficiency Virus (HIV) frequently coexist in patients in many parts of the world due to geographical overlaps of these two diseases. Presently, most epidemiologic data on HIV infection with malaria are derived from *P. falciparum* endemic regions of sub-Saharan Africa, Nigeria inclusive. These two infectious diseases interact bi-directionally and synergistically with each another [6]. Thus, HIV infection can increase the risk and severity of malaria infection and parasite burden which may facilitate higher rate of malaria transmission. People living in malaria endemic areas, such as Uyo, Nigeria, particularly, are considered semi-immune to malaria and can develop clinical malaria if they are untreated. This has become a significant public health threat as a result of increase risk factors associated with high malaria burden in HIV infected persons including low $CD4^+$ cell counts, low immune status, gender and among others. HIV –1 RNA concentration and CD4 cells count are moderately but inconsistently associated with *parasitaemia*. High parasite count with fever is associated with HIV – 1 sero-positivity and low CD4 cell count [7]. Studies have shown that vulnerability and frequency of malaria infection appear to be increased in HIV-positive adults particularly those with low CD4 cell counts [8,9,10]. Another challenge is the chemotherapy failure of Antimalarial drugs in HIV patients, when there is inability of Antimalarial drugs to produce the desired therapeutic effect. Treatment failure 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 areas 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 patient treatment. However, there is paucity of information on molecular detection of *P. falciparum* in HIV-infected patients' thus efficient treatment options.

The sensitivity and accuracy of Polymerase Chain Reaction in detection and quantifying of *P. falciparum* mutant resistant gene in 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 for molecular 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 in 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 65 m, 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.

2.4 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.

2.4.1 Collection of sample

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

2.4.2 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].

2.4.3 $CD4^+$ count test

Twenty (20 µl) 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 (800 µl) 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].

2.4.4 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].

2.4.5 DNA molecule extraction

Four hundred (400 µl) microlitre pipette was used to add Genomic Lysis Buffer to 100 µl 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-Spin™ Column in a collection tube. Two hundred (200 µl) 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 (<500 µl). Elution Buffer was added to the spin column and incubated for 2-5 minutes at room temperature [16].

2.4.6 PFCRT 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 1.5 µl Eppendorf tube. This was followed by adding 0.4 µl Pfcrt primers- (Forward CRTP1 5′ and Reverse CRTP2 5′) 0.5 µl $MgCl_2$, 5 mM DNA template and a high molecular grade

water of 3.29 µl to dilute the other reagent in their correct concentrations. The primary nested PCR final volume (20 µl) 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 1.5 µl Eppendorf tube. This was followed by adding 0.2 µl Pfcrt primers (Forward CRTP1 5′ and Reverse CRTP2 5′) 0.5 µl $MgCl_2$, 0.5 µl DNA template, and an 11.1 µl high molecular grade water to dilute the other reagent in their correct concentrations. The primary and secondary nested PCR final volume (25 µl) 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 Pfcrt. Agar gel Electrophoresis was then carried out to separate the DNA gene molecule (Pfcrt) of interest.

2.4.7 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 (10 µl) 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.

2.4.8 Statistical analysis

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

3. RESULTS

3.1 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).

3.2 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).

3.3 Molecular Detection of Mutant *Plasmodium falciparum* Chloroquine Resistant Transport Genes in HIV Positive Patients

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

Table 1. Prevalence of malaria parasite according to HIV status and gender (n= 67)

HIV positive	No. of samples	Malaria parasite		P-value
Gender		Positive (%)	χ^2	
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 = 0.05

Table 2. Risk factors to malaria parasite in HIV positive patients (n = 35)

Variables	No. of samples 35	No. of malaria positive cases 5 (%)	No. of malaria negative cases 30 (%)	χ^2	df	P-value	Odd ratio	95% C.I.
$CD4^+$ 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 in Years								
20-29	9	0(0)	9(35.0)	6.13	3	0.17	1 3.11	0.35-27.5
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 = 0.05

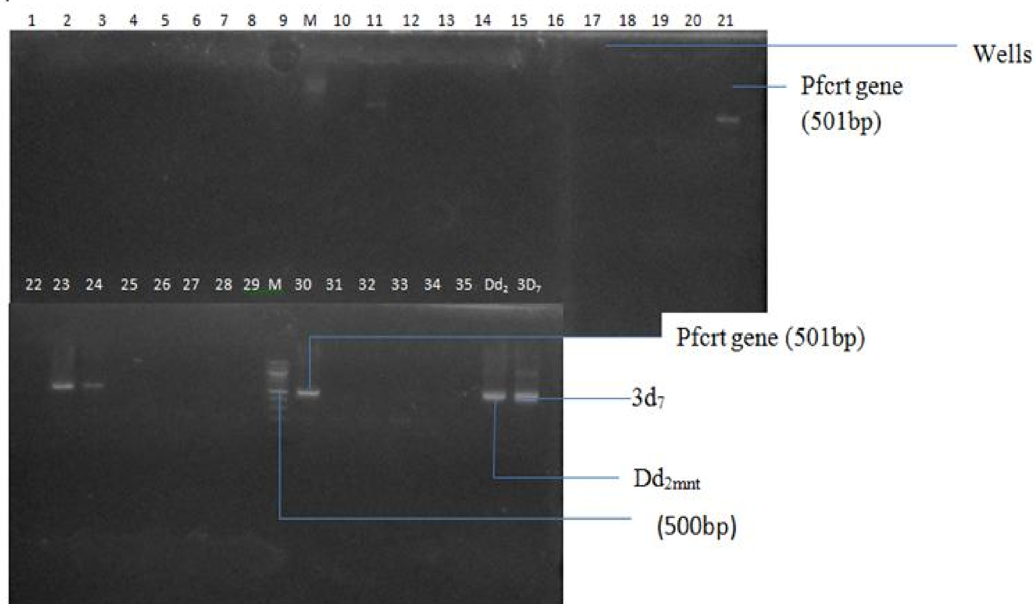

Fig. 1. Molecular detection of mutant *Plasmodium falciparum* chloroquine resistant transport genes in HIV positive patients

3.4 Molecular Detection of Mutant *Plasmodium falciparum* Chloroquine Resistant Transport Genes in HIV Negative Participants (Controls)

Lane M represents the Quick- Load 100 bp molecular ladder while Dd_2 and $3d_7$ are the mutant and Wild type controls respectively.

3.5 Prevalence of Mutant *Plasmodium falciparum* Chloroquine Resistant Transport Gene According to Gender and Age in HIV Positive Patients

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

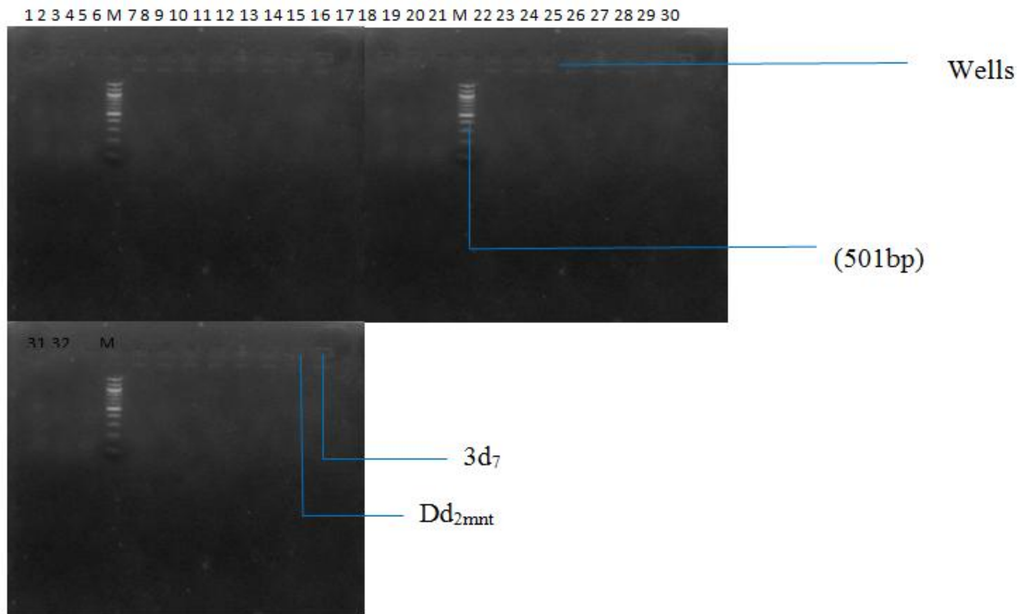

Fig. 2. Molecular detection showing the absence of mutant *Plasmodium falciparum* chloroquine resistant transport genes in HIV negative patients as a control

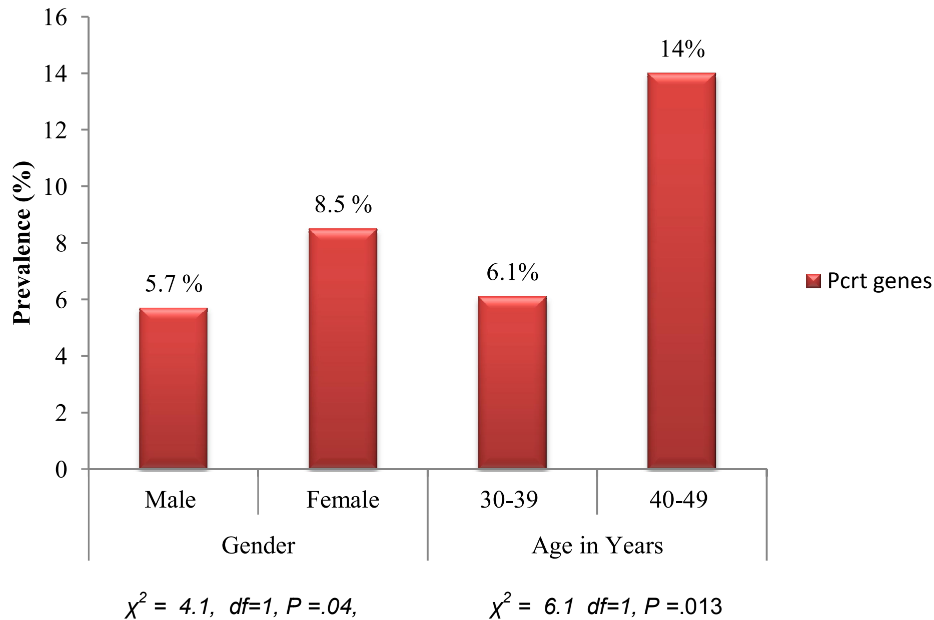

Fig. 3. Prevalence of mutant *Plasmodium falciparum* chloroquine resistant transport gene according to gender and age of HIV positive patients

4. 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 mutant 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 for *falciparum* malaria is very reliable. Method of diagnosis of *falciparum* 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 blood film 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 mutant Pfcrt genes, the molecular weight of mutant Pfcrt genes was 501bp. A prevalence of 14.2% mutant Pfcrt was determined in this study.

During this study, HIV female patients were examined for *falciparum* malaria than males. Mutant Pfcrt genes were dominant in middle aged grouped female patients than the male patients, this was similar to that of Oke et al. [21] who reported the predominance of mutant Pfcrt in middle aged adult men who took chloroquine. However, in Oke et al. [21] study, men were mostly examined than females. Statistically, there was significant difference between gender; χ^2 = 4.1,df=1 P =.04, age χ^2 = 6.1 df=1, P < .001 with Pfcrt (Fig. 3). The high prevalence of mutant 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 to malaria parasite infection [22].

The presence of mutant Pfcrt genes among the subjects agrees with the findings of Molta, [23]; Happi et al. [24]; Okungbowa and Mordi, [25]. This indicates that mutant Pfcrt gene is wildely 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 [25] who recorded a higher percentage of mutant Pfcrt genes 24%. The prevalence of mutant Pfcrt genes (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 chlororoquine 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 /µl had malaria parasite, while three other subjects with $CD4^+$ counts ≥ 400 cells/µl 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 (χ^2 = 0.056,df = 1, P =.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 /µl counts had *parasitaemia* in the peripheral blood. This differs to that obtained in a study by Chavale et al. [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. [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 Antimalarial 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.

5. CONCLUSION

In conclusion, this study revealed that the prevalent rate of mutant *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/µl 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 Pfcrt genes in the peripheral blood.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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