

## Co-Endemicity of *Plasmodium falciparum* and HIV-Infections in Treated Patients is Uncorrelated in Benin City, Nigeria

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### Abstract

The Human immunodeficiency virus (HIV) and malaria are two of the world's most formidable pathogens. Co-infection has been shown to amplify the effects of both diseases with HIV infection enhancing the severity of malaria. Previous work in our laboratory has shown that individuals infected with malaria and HIV who are taking anti-retrovirals have the *Plasmodium* parasite in their bloodstream suggesting that the lack of anti-malarials in their drug regimen resulted in *Plasmodium* infection. In this study, we set out to determine the status of *Plasmodium* infection in a cohort of patients taking both anti-malarial and anti-retroviral drugs. Blood samples were collected from patients of the Edo district of Nigeria in Benin City co-infected with *Plasmodium* and HIV. We have found that 31 out of the 317 (9.78%) HIV patients on HAART and ACT had *Plasmodium* in their blood based on microscopic counts. Surprisingly, using the polymerase chain reaction (PCR), the prevalence was at 25.6% for *Plasmodium*. In addition, we have identified by PCR that *Plasmodium falciparum* is the only species infecting these patients. Furthermore, no significant relationship was found to exist between CD4<sup>+</sup> T-cell counts and malarial infections (CD4 count < 200 cells/μL (7.20%)) nor was the malaria parasite density significantly associated with CD4 count < 200 cells/μL ( $P=0.595$ ) in this study population in Benin City, Nigeria. These results suggest that other factors are involved in this complex interaction.

**Keywords:** Co-infection; Human immunodeficiency virus (HIV); Malaria; *Plasmodium falciparum*; Prevalence; Highly Active Antiretroviral Therapy (HAART); Artemisinin combinatorial therapy (ACT); CD4<sup>+</sup> T-cells; Polymerase chain reaction (PCR)

### Introduction

The human immunodeficiency virus (HIV) and *Plasmodium* species that infect human are responsible for two of the most prevalent and important infectious diseases on the planet. According to the World Health Organization (WHO), 219 million cases of malaria developed in the year 2010 alone and the 90% of these cases occur in sub-Saharan Africa where 67% of the world's HIV infections take place [1-3]. This has led to significant geographical intersection of the two diseases with a negative impact on both malaria and AIDS-related illnesses.

The immunosuppression caused by HIV infection has a negative effect on the immune response against the parasite leading to distinct disease outcomes between HIV-infected and HIV-uninfected individuals. At late stages of HIV infection or AIDS, the reduction of CD4<sup>+</sup> T-cells results in decreased CD8<sup>+</sup> T-cell counts and function, leading to severe alterations of the immune response against other pathogens, including *Plasmodium* [4,5]. HIV infection increases the risks of severe malaria and death from *Plasmodium* infection while malaria leads to increased illness in HIV-infected individuals under treatment [5]. Indeed, co-infection has been shown to increase HIV viral load 10-fold in patients with malaria [6]. HIV infections have been associated with increased prevalence and severity of malaria causing severe morbidity in malaria patients in Ivory Coast [7,8]. This also occurs during pregnancy. Several studies have documented a higher *Plasmodium* parasitemia in HIV-infected pregnant women [9-11]. Likewise, *Plasmodium* infections were shown to cause a doubling of the HIV viral load in pregnant women [12,13].

In previous surveys of HIV and malaria co-infections in Benin City, Nigeria, patients prescribed with HAART and ACT were excluded and a higher *Plasmodium* infection rate was observed in HIV-infected individuals [14]. Recently, Akinbo and Omoregie [15] have

found a 2.11% prevalence of malaria, using microscopy techniques among 285 HIV-infected individuals taking HAART (zidovudine, stavudine, and nevirapine) and ACT (sulfadoxine, pyrimethamine, and dihydroartemisinin). Their study also reported a significant correlation between CD4<sup>+</sup> T-cell counts and malaria. In this study, we surveyed HIV and *falciparum* malaria co-infections, in Benin City, Nigeria, among patients prescribed with HAART (zidovudine, stavudine, and nevirapine) and ACT (artesunate-lumefantrine combination) medications. We also took this study a step further to determine if *Plasmodium falciparum* was the only species present in these co-infected individuals.

### Materials and Methods

#### Study area

The study was carried out at the University of Benin Teaching Hospital and Center for the United States President's Emergency Plan for AIDS Relief (PEPFAR) and according to Institute of Human Virology of Nigeria (IHVN) policy (PE, Benin City, Edo State, Nigeria). The teaching hospital is a tertiary health institution with a referral status serving 5 States of the Nigerian Federation: Edo, Delta, Ondo,

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Anambra and Kogi. The study area is located in the Midwestern part of Nigeria and has an estimated population of 3.2 million people [16]. This study was conducted and samples were collected between February and late May of 2012.

### Study population

The study population consisted of a total of 317 HIV-infected outpatients treated with HAART (zidovudine, stavudine, and nevirapine). One hundred and forty five males and 172 females, ranging from 20 to 68 years of age were selected. Patients with AIDS-defining conditions and those not on antiretroviral therapies were excluded from this study [17]. All HIV-infected patients were on a prophylactic ACT (artesunate-lumefantrine combination) regimen and had no signs or symptoms of malaria.

### Ethical aspects

Informed consent was obtained from all participants prior to specimen collection. The Ethical Committee of the University of Benin Teaching Hospital approved the protocol for this study.

### Specimen collection and processing

Five milliliters of blood was collected from each participant, dispensed into an ethylene diamine tetra-acetic acid (EDTA) container, and mixed. A small sample of each blood sample was applied to Whatman® filter paper and allowed to dry.

### Parasite density

Malaria was diagnosed using a method previously described in Akinbo et al. [15]. Briefly, thick blood films were made from each blood specimen and allowed to air-dry. The blood films were stained in a 3% Giemsa solution for 30 minutes, rinsed in tap-water, and allowed to air-dry. A total of 200 fields per stained thick film were examined for malaria parasites by microscopy [18]. The parasite density was calculated from Giemsa-stained thick films by multiplying the ratio of the number of malaria parasite per 500 white blood cells by the assumed total white blood cell count of 4000 cells/ $\mu$ L to give the malaria density in cells/ $\mu$ L.

### Quantitation of CD4<sup>+</sup> T-lymphocytes

The CD4<sup>+</sup> T-lymphocyte count was evaluated using flow cytometry (Partec, GmbH, Germany). Briefly, 20  $\mu$ l of CD4 PE antibody and 20  $\mu$ l of mixed whole EDTA blood were placed in Partec test tubes. The tube contents were mixed gently and incubated in the dark for 15 minutes at room temperature. This mixture was agitated during incubation every 5 minutes. To this mixture, 800  $\mu$ l of CD4 buffer was added and mixed gently. The CD4<sup>+</sup> T-cells were then counted.

### DNA isolation

Blood samples dried on Whatman® filter paper were punched (3.0 mm in size) in triplicate from Whatman® filter paper blood smears. DNA was isolated from these punches at room temperature by incubating them in a high pH solution (35  $\mu$ l 0.1 N NaOH, 0.3 mM EDTA, pH 13.0) for 5 minutes, followed by a neutral solution (65  $\mu$ l 0.1 M Tris-HCl, pH 7.0) for 10 minutes [19].

### Polymerase chain reaction

Polymerase chain reactions (PCR) were carried out in half reactions containing 2  $\mu$ l of extracted gDNA to which was added to 1 illustra™puReTaq Ready-To-Go PCR bead containing 2.5 units of puReTaq DNA polymerase, 400  $\mu$ M each of dATP, dGTP, dCTP, dTTP, 10

mMTrisHCl (pH 9.0), 50 mM KCl, and 1.5 mM MgCl<sub>2</sub> (GE Healthcare, Buckinghamshire, UK), 2  $\mu$ M forward primer and 2  $\mu$ M reverse primer in nuclease-free water. The DNA was amplified using a forward primer specific to four of the five different species of *Plasmodium* known to infect humans (*falciparum*: 5'-AAC AGA CGG GTA GTC ATG ATT GAG; *vivax*: 5'-CGG CTT GGA AGT CCT TGT; *ovale*: 5'-CTG TTC TTT GCA TTC CTT ATGC; and *malariae*: 5'-CGT TAA GAA TAA ACG CCA AGCG) and a reverse primer conserved in all four of these *Plasmodium* species (5'-GTA TCT GAT CGT CTT CAC TCCC). The DNA was incubated at 95°C for 2 minutes, followed by 43 cycles each consisting of 45 seconds at 95°C, 90 seconds at 60°C and 60 seconds at 72°C, and a final step at 72°C for 5 minutes [20]. PCR products were analyzed by agarose gel electrophoresis. PCR products were visualized by ethidium bromide staining.

### Statistical analysis

The parametric data were analyzed with student t-tests, while the non-parametric data were analyzed using a Chi-squared ( $\chi^2$ ) test and an odds ratio (OR) analysis, in the statistical software INSTAT® (GraphPad Software Inc, La Jolla, CA, USA) or an independent samples t-test in SPSS (IBM Corp, Armonk, NY).

### Results

Previously, we assessed the prevalence of *Plasmodium* infections in HIV-infected patients prescribed HAART and a specific type of ACT formulation containing sulfadoxinepyrimethamine, and dihydroartemisinin (SPD), and those without HAART and antimalarials. We found a low prevalence of asymptomatic malaria among HIV patients on HAART and SPD compared to HIV-infected patients not receiving these therapies [14,15]. To verify the prevalence of malaria in HIV-infected patients prescribed HAART and a ACT treatment different from our previous study, artesunate-lumefantrine combination therapy (ACT), we recruited 317 HIV-infected patients representative of the population in Benin City, Nigeria. These patients were grouped according to gender, age, and symptoms of AIDS (based on AIDS defining conditions) [15]. Among the recruited HIV-infected patients there were 145 males and 172 females ranging in age from 20 to 68 years. To ascertain malaria infections in our study population, thick blood smears were prepared and counted by light microscopy. The malaria parasite density ranged from 40-240 parasites/ $\mu$ L of blood (Table 1). The results of these counts showed that 31 out of the 317 (9.8%) HIV patients prescribed HAART and ACT had malaria parasites in their blood (Table 1). To determine if the HIV infection status increased the prevalence of malaria in these patients, we determined the CD4<sup>+</sup> T-cell count of the patients by flow cytometry. The results showed that 40 out of 317 patients had CD4<sup>+</sup> T-cell counts <200 cells/ $\mu$ L (12.6%) and 277 out of 317 patients had CD4<sup>+</sup> T-cell of  $\geq$  200 cells/ $\mu$ L (87.4%) (87.4%)

Characteristic	CD4 count (cells/ $\mu$ L)		OR+	95% CI*	p value
	$\leq$ 200	>200			
<b>Malaria parasite detection</b>					
Positive	3 (7.5%)	28 (10%)	0.721	0.209, 2.492	0.815
Negative	37 (92.5%)	249 (89.9%)			
Total	40 (12.6%)	277 (87.4%)			
<b>Malaria parasitemias</b>					
Density (parasite/ $\mu$ L)	146.67	115.71			0.595
Standard Deviation	$\pm$ 48.07	$\pm$ 11.14			

\*CI Confidence Interval  
+Odds ratio

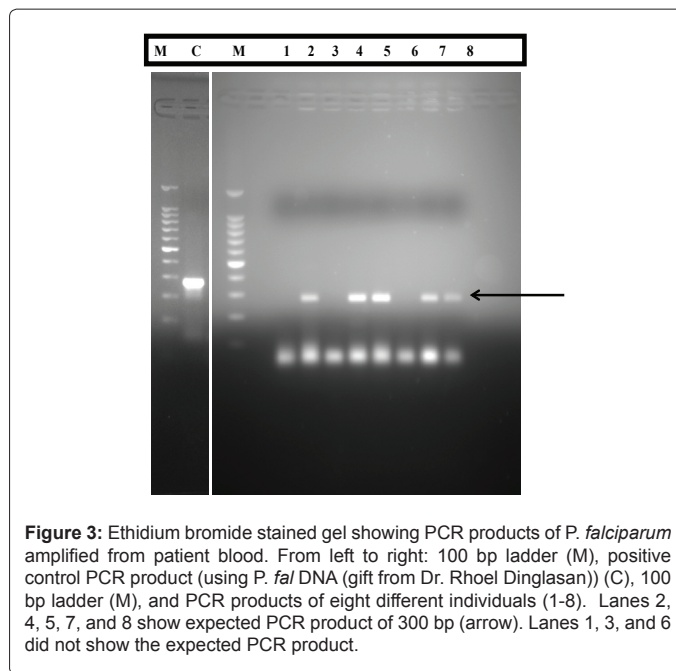
**Table 1:** Prevalence of malaria in HIV-infected and non-infected individuals.

(Table 1). These results suggest that a CD4<sup>+</sup> T-cell count of <200 cells/ $\mu$ L was not significantly associated with asymptomatic malaria among HIV-infected patients prescribed HAART and ACT (7.2%) when compared to those with CD4<sup>+</sup> T-cell count  $\geq$  200 cells/ $\mu$ L (10.1%) (OR 0.72, 95% CI: 0.21-2.49,  $p=0.82$ ) (Figure 1). The parasite density, though higher in HIV patients with CD4 count <200 cells/ $\mu$ L (M=146.67, SE=48.1), again was not significantly associated with asymptomatic malaria ( $p=0.60$ ) (Figure 2). This observation is in accordance with our previously published report [15].

To identify the species of *Plasmodium* in our study cohort, PCR was conducted on 207 samples with primers specific for four of the five *Plasmodium* species known to infect humans, namely, *falciparum*, *vivax*, *malariae*, and *ovale*. We found a prevalence of 25.6% of *P. falciparum* (expected PCR product 300 bp) infection, while *P. vivax*, *P. malariae*, and *P. ovale* were not detected (Figure 3 and Table 2). This identifies the parasites in the blood of these patients prescribed ACT as *P. falciparum* and suggests that only *P. falciparum* is predominant in Benin City, Nigeria. In addition, data collected from the molecular study showed that the number of individuals in this study cohort infected with *P. falciparum* was much higher than that detected by microscopic counts. We did not test for *P. knowlesi* in this study.

## Discussion

The brunt of the HIV pandemic has been borne disproportionately by resource-poor regions of the world, where tropical infectious diseases predominate [21]. *P. falciparum*, helminthes, and the HIV virus are endemic in sub-Saharan Africa making co-infection a reality. Akinbo et al have previously reported co-infections of *P. falciparum* and *Ascaris lumbricoides* in HIV-infected patients in Benin City, Nigeria [22]. In this study involving 2,000 HIV-infected patients that were examined from August 2007 to August 2009, we found that 25.2% of the enrolled

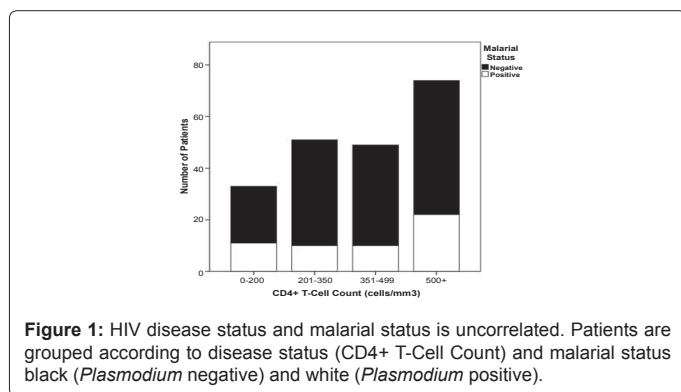


**Figure 3:** Ethidium bromide stained gel showing PCR products of *P. falciparum* amplified from patient blood. From left to right: 100 bp ladder (M), positive control PCR product (using *P. fal* DNA (gift from Dr. Rhoel Dinglasan)) (C), 100 bp ladder (M), and PCR products of eight different individuals (1-8). Lanes 2, 4, 5, 7, and 8 show expected PCR product of 300 bp (arrow). Lanes 1, 3, and 6 did not show the expected PCR product.

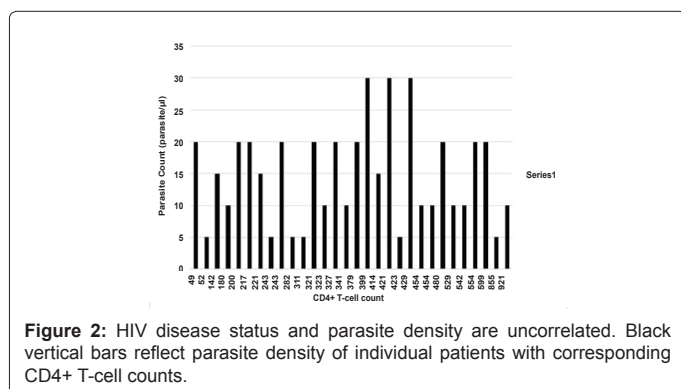
individuals were positive for *P. falciparum* [15]. Among them, 34.4% had a CD4<sup>+</sup> T cell count of <200 and 10.1% had CD4<sup>+</sup> T-cell counts greater than 200, suggesting the *P. falciparum* infection was related to the CD4<sup>+</sup> T-cell counts. It is duly noted that patients who were on highly active antiretroviral therapy (HAART), antiparasitic agents, and those with AIDS-defining conditions were excluded from the study [15]. Another interesting finding was that none of the subjects tested showed co-infections with *P. falciparum* and intestinal parasitic infections other than in HIV-positive subjects. Thus, subjects with cell <200 cells/mm<sup>3</sup> showed an increased risk of opportunistic infections [15].

Furthermore, a low prevalence of *P. falciparum* infection among HIV-positive individuals undergoing treatment with HAART and sulfadoxine, pyrimethamine, and dihydroartemisinin was previously reported [15]. Patients on HAART with signs and symptoms of malaria and AIDS (by conditions) and those not on HAART were excluded from that study. In the same study, small subsets of these patients were observed to have asymptomatic malaria. The prevalence of anemia observed was 45.26% and is an important cause of anemia among HIV-infected patients on HAART [15]. Both HIV and *Plasmodium* infections can lead to anemia independently [21-23].

In this study, we examined the prevalence of *Plasmodium* infection in a cohort of HIV-infected individuals undergoing both HAART and ACT (artesunate-lumefantrine combination) chemotherapies. By microscopic counts 9.8% HIV patients prescribed HAART and ACT had malaria parasites in their bloodstream (Table 1). By molecular studies the number of infections was much higher at a 25.6% prevalence of *P. falciparum* infection despite treatment with ACT (Table 2). The CD4<sup>+</sup> T-cell counts <200 cells/ $\mu$ L was 12.6% (Table 1). However, there was no correlation between co-infection of HIV and *Plasmodium*. We asked whether there was a correlation between CD4<sup>+</sup> T-cell count and parasite density in the samples that tested positive for *P. falciparum*. In our analysis, we found no correlation between CD4<sup>+</sup> T-cells and the amount of parasites in these patients. A previous study done by Chavale et al. [24] showed that patients co-infected with HIV-1 and *Plasmodium* have a high amount of parasitemia and decreased CD4<sup>+</sup> T-cell count. Their results showed significantly lower CD4<sup>+</sup> T-cells values observed



**Figure 1:** HIV disease status and malarial status is uncorrelated. Patients are grouped according to disease status (CD4<sup>+</sup> T-Cell Count) and malarial status black (*Plasmodium* negative) and white (*Plasmodium* positive).



**Figure 2:** HIV disease status and parasite density are uncorrelated. Black vertical bars reflect parasite density of individual patients with corresponding CD4<sup>+</sup> T-cell counts.

Malaria status	Frequency	Percent	Valid Percent	Cumulative Percent
Negative	154	74.4	74.4	74.4
Positive	53	25.6	25.6	100.0
Total	207	100.0	100.0	

Table 2: Malaria status using PCR.

in the *P. falciparum*/HIV (Pf/HIV) co-infected patients, which indicates a correlation between CD4<sup>+</sup> T-cell count and parasitemia. This finding does not agree with our study. A possible reason for this discrepancy is that the patients in Chavale's study were not taking either anti-retroviral or anti-malarial drugs. If both diseases are left untreated, symptoms worsen which explains the significant lower CD4<sup>+</sup> T-cell count and high amount of parasitemia found in their studies.

Of the species of *Plasmodium* that were tested, only *P. falciparum* was found to be present in this cohort (Table 2 and Figure 3). This high prevalence of *P. falciparum* infection among this cohort of HIV-infected individuals subjected to HAART and ACT treatment may be due to either interference of the antiretrovirals on the antimalarial drugs or the action of the HIV virus, or that the parasite may be developing resistance to the ACT drugs. At the time of this study, resistance to ACT has not been reported in Nigeria. The lack of correlation with the CD4<sup>+</sup> T-cell count suggests that the virus is not implicated. CD4<sup>+</sup> T cell counts in these patients suggest that the antiretroviral drugs are controlling the viral replication. The interference of antiretrovirals drugs with antimalarials has been suggested to occur [5]. Moreover, the treatment of HIV and malaria in co-infected patients create the potential for drug interactions with the potential for either increased drug toxicity or decreased concentration, which might lead to drug resistance. In addition to increasing malaria morbidity and mortality, HIV co-infection has been linked to increased antimalarial drug resistance [25]. We are actively investigating the mechanisms by which this interference occurs and the possibility of resistance that may have already developed in Nigeria. This is particularly important as *P. falciparum* isolates from Nigeria were shown to display increased susceptibility to artemether *in vitro* [26]. Another possibility is the stems from the observation that individuals living in malaria endemic regions have been known to have circulating parasites that the immune system controls. It is possible that the patients have circulating antibodies and possibly CD8<sup>+</sup> T-cells that are maintaining some control of the infection. This is another indication that would again point toward resistance to ACT in the Nigerian population. Our results have implications for the future treatment and prevention of malaria in HIV-infected individuals.

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