

Full Length Research Paper

A study of *Plasmodium* species DNA in urine sample of HIV positive individuals using PCR amplification method

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A total of 150 blood and urine samples each were collected from human immunodeficiency virus (HIV) positive patients who visited selected hospitals in Ebonyi State. The subjects were made up of 57 males and 93 female patients. The blood samples were screened for the presence of four human malaria parasites using parasitological examination of blood stained films and polymerase chain reaction (PCR). Out of the 150 urine samples from the HIV positive individuals, 88 urine specimens were identified to harbor *Plasmodium* species. 75 (50%) urine specimens were identified to harbor *Plasmodium falciparum* while 10 (6.67%) and 3 (2%) were recorded against *Plasmodium malariae* and dual infection of *P. falciparum* and *P. malariae*, respectively. The result of the comparison of the specimens used showed the same result. None of the isolates that were negative by PCR test using DNA primers (template) from blood gave positive results by urine samples. Furthermore, when the primers (rOVA₁ and rOVA₂; rV₁V₁ and rV₁V₂) specific for *Plasmodium ovale* and *Plasmodium vivax*, respectively were used, none of the two species mentioned were detected in both urine and blood samples, signifying that these species may be absent in our environment. Our study demonstrated highly, the presence of *P. falciparum* and *P. malariae*, especially when specific *Plasmodium* species DNA markers were used for the analysis.

Key words: Urine, *Plasmodium* species, human immunodeficiency virus (HIV), polymerase chain reaction (PCR), microscopy.

INTRODUCTION

Malaria is caused by protozoan parasites of the genus *Plasmodium* (phylum Apicomplexa). In humans, malaria is caused by mosquito born *Plasmodium falciparum*,

Plasmodium malariae, *Plasmodium ovale* and *Plasmodium vivax*. However, *P. falciparum* is the most important cause of the disease and is responsible for about

80% of the reported cases and 90% of deaths (Mendis et al., 2001). Malaria causes about 400 to 900 million cases of fever and approximately one to three million deaths annually (Bremner, 2001). A vast majority of cases occur in children under the age of 5 years (Greenwood et al., 2005) and more so, pregnant women are particularly vulnerable.

Malaria is a major public health problem in Nigeria where it accounts for more cases and death than any other country in the World. Malaria is a risk for 97% of Nigerian population. The remaining 3% of the population live in the malaria free highlands. There are estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria. This compares with 215, 000 deaths per year in Nigeria from human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). Malaria contributes an estimated 11% of maternal mortality.

Malaria accounts for 60% outpatient visit and 30% of hospitalization among children under five years of age in Nigeria. Malaria has the greatest prevalence, close to 50% in children age 6 to 59 months in the South-West, North-Central and North-West region. Malaria has the least prevalence 27.67% in children age 6 to 59 months in the South-East region.

Malaria prevalence and mosquito vector abundance in Uli town in Ihiala Local Government Area of Anambra State, Nigeria was studied between April and July, 2010. Oral interviews were used to get personal data of participants. 178 participants made up of 111 (62.45%) males and 67 (37.6%) females from the ten villages of the town were involved in the study.

Participants were aged between 0 to 70 years and subdivided into 7 age group of ten years intervals each; in occupational and education groups of the 178 participants examined, 126 (70.8%) were positive with malaria parasite. Species detected include *P. falciparum* (80.2%), *P. malariae* (13.6%), *P. ovale* (4.4%) and mixed infection of *P. falciparum* and *P. malariae* (1.6%). The age group 31 to 40 years had the highest malaria prevalence 42 (85.71%), while 0 to 10 year has the least 6 (42.86%). The malaria prevalence in relation to age was significant (15.100 df = 5; $p < 0.05$). The males 82 (73.9%) were slightly more affected than the females 45 (65.7%) but not statistically significant (0.683 m df – 5; $p > 0.05$). Malaria prevalence was significantly higher among those without formal education 7 (77.7%) and least among those with tertiary education 5 (35.7%, $p < 0.05$). Malaria prevalence among different through farmers were slightly more infected than others ($p > 0.5$) (Umeanaeto, 2011).

HIV/AIDS and malaria are among the most prevalent infectious diseases in sub-Saharan Africa and the leading cause of morbidity and mortality in the region (World Health Organization (WHO), 2004). HIV/AIDS and malaria together cause more than 4 million deaths annually, significantly affecting those in poverty and impeding sustainable development. The dual infections of malaria and HIV increase the risk of morbidity and mortality for all individuals infected. The complexity of co-infection has an impact on public health approaches and effective management of this interaction will require integration and strengthening of current public health care delivery system (WHO, 2004). HIV is a lentivirus (classified into retroviridae family) and is the major etiological agent of AIDS, a condition in humans in which the immune system begins to deplete, leading to life-threatening opportunistic infections (Weiss, 1993).

HIV is transmitted through direct contact of a mucous membrane or the blood stream with a contaminated body fluid such as blood, semen, vaginal fluid, seminal fluid and breast milk (Centre for Disease Control (CDC), 2003). The transmission can be through anal, vaginal or oral sex with infected individual, blood transfusion, infected contaminated blood, use of contaminated hypodermic needles, vertical route from mother to her baby during pregnancy or childbirth among other routes.

Malaria and HIV often co-exist in patients in most parts of the world due to overlap of these two diseases (Hewitt et al., 2006). An estimated 28 million individuals are infected with HIV in sub-Saharan Africa with almost 3 million deaths annually (Idemiyor, 2007). These diseases demonstrate a great deal of an overlapping distribution. Co-infection with HIV and malaria is very common in sub-Saharan Africa, and an understanding of how the two infections interact is important for the control of both diseases. The mainstay of malaria diagnosis has been the microscopic examination of blood, utilizing blood films (Krafts et al., 2011). Although blood is the sample most frequently used to make a diagnosis, both saliva and urine have been investigated as alternative, less invasive specimens (Sutherland and Hallett, 2009). More recently, modern techniques utilizing antigen tests or polymerase chain reaction have been discovered, though these are not widely implemented in malaria endemic regions (Ling et al., 1986; Mens et al., 2006). Areas that cannot afford laboratory diagnostic tests often use only a history of subjective fever as the indication to treat malaria.

The most economic, preferred and reliable diagnosis of malaria is microscopic examination of blood films because each of the four major parasite species has distinguishing characteristics.

Two sorts of blood film are traditionally used. Thin films are similar to usual blood films and allow species identification because the parasite's appearance is best preserved in this preparation. Thick films allow the microscopist to screen a larger volume of blood and are about eleven times more sensitive than the thin film, so picking up low levels of infection is easier on the thick film, but the appearance of the parasite is much more distorted and therefore distinguishing between the different species can be much more difficult. With the pros and cons of both thick and thin smears taken into consideration, it is imperative to utilize both smears while attempting to make a definitive diagnosis (Warhurst and Williams, 1996).

From the thick film, an experienced microscopist can detect parasite levels (or parasitemia) as few as 5 parasites/ μ l blood (Richard et al., 2006). Diagnosis of species can be difficult because the early trophozoites ("ring form") of all four species look identical and it is never possible to diagnose species on the basis of a single ring form; species identification is always based on several trophozoites.

Plasmodium malariae and *P. knowlesi* (which is the most common cause of malaria in South-east Asia look very similar under the microscope. However, *P. knowlesi* parasitemia increases very fast and causes more severe disease than *P. malariae*, so it is important to identify and treat infections quickly. Therefore modern methods such as PCR (see "Molecular methods") or monoclonal antibody panels that can distinguish between the two should be used in this part of the world (McCutchan et al., 2008). Malaria transmission and mortality rates remain unchanged in endemic countries lacking adequate health care and malaria control programme despite the use of preventive measures and treatments against the disease (Bell et al., 2006). A major obstacle for effective malaria control is the lack of affordable and accurate malaria diagnostics and prompt treatment. This has led to misuse and abuse of anti-malarial drugs of which the consequence is the development of drug resistance in strain.

Microscopic examination of blood smears as the conventional method for *Plasmodium* species detection, is currently being replaced gradually with polymerase chain reaction (PCR) based rapid diagnostic tests (RDTs) for blood, because of inaccurate microscopic evaluations of blood smears resulting in misdiagnoses and misclassification of malaria severity (Makler and Hinrich, 1993). Failure to detect cryptic *P. falciparum* infections could lead to the risk of developing severe or fatal outcomes while missed *P. vivax* malaria may result in recurrent debilitating infections and economic loss (Mayxay et al., 2004).

Nevertheless, repeated examination of blood samples from malaria patients during post-treatment follow-up may at times result in poor compliance, especially among

infants and young children. Therefore, an alternative means for a noninvasive malaria diagnosis is required hence this study was designed to detect *Plasmodium* species DNA in urine sample of HIV positive individuals using PCR amplification method.

MATERIALS AND METHODS

The study area includes, General Hospital Onuigboji Ikwo LGA, Federal Teaching Hospital, Abakaliki and Onueke General Hospital, Ezza South LGA, Abakaliki, Ebonyi State. The investigation was carried out in Applied Microbiology Laboratory Complex, Eboyin State University (EBSU) while the PCR was done at Veterinary Medicine Molecular Laboratory, University of Ibadan, Oyo State. The study population includes 57 males and 93 females that were HIV positive. One hundred and fifty (150) blood and urine samples each were collected from one hundred and fifty out-patients for this study. An easy-to-read and friendly questionnaire was provided for the collection of demographic and clinical data. A physical examination with a clinical note of any reported sign or symptom was done by a physician. Ethical approval was obtained from the mentioned health Institution.

Parasitological identification of *Plasmodium* Species

Each blood sample was subjected to parasitological examination using thick and thin blood film microscopy according to microbiological standards (Cheesbrough, 2002).

Plasmodium Species DNA extraction of using whole urine samples

Exactly 150 μ l of whole urine was pipette into micro-centrifuge tube. 95 μ l of 2 \times digestion buffer and 5 μ l of proteinase k were added to the microcentrifuge simultaneously. The content in the tubes were mixed well and then incubated at 55°C for 20 min. Seven hundred (700 μ l) of genomic lysis buffer were added to each tube and thoroughly mixed by vortexing. The mixture were carefully transferred to a Zymo-SpinTM IIC column in a collection tube and centrifuged at 13,900 rpm for 2 min. Two hundred (200 μ l) of DNA pre-wash buffer were added to the spin column in a new collection tube and allowed to centrifuge at 13,900 rpm for 2 min. Four hundred (400 μ l) of g-DNA wash buffer was measured and introduced in the spin column. The content was centrifuged at 13,900 rpm for 2 min. The spin column was carefully transferred into a clean micro-centrifuge tube. Forty (40 μ l) of DNA elution buffer was added to the spin column and incubated at 2 to 5 min at room temperature. The final mixture was centrifuged at 8,000 rpm for 2 min. The eluted DNA was immediately used for molecular based applications or stored at -20°C for future use. The resultant supernatant, containing DNA, was carefully transferred into a pre-labeled 1.5 ml microcentrifuge tube, excluding chelex, for immediate PCR analysis or stored at -20°C (Johnston et al., 2006).

Whole blood DNA extractions

The aforementioned procedure for whole urine DNA extraction was adopted for whole blood DNA extraction.

Table 1. Sex distribution of HIV Co-infected with malaria parasite (*P. falciparum*) among the population studied.

Sex	Number of sample examined	Number positive for HIV (%)	Percentage positive for malaria (%)
Male	57	57 (38.00)	47 (82.46)
Female	93	93 (62.00)	28 (30.12)
Total	150	-	75 (50.00)

Genomic DNA electrophoresis

The extracted genomic DNA were run on a 1.5% agarose gel in ethidium bromide (TBE) buffer (pH 8.0) at 100 mV and 500 mA for 3 h after which the gel was viewed under UV light for DNA bands. This confirms the presence and quality of the extracted genomic DNA.

PCR based detection

Plasmodium species detection was carried out using nested PCR in the thermal cycler. Two (2 µl) of the genomic DNA was added to a total volume of 23 µl amplification reaction mixture with plasmodium genus-specific outer primers [rPLU5:5' - CCTGTTGTTGCCTTAAACTTC-3' and rPLU6:

5'-TTAAAATTGTTGCAGTTAAAACG-3'] for first step PCR. Thirty-five cycles (94°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 2 min) were carried out. Two (2 µl) of the PCR product from the first step PCR reaction was measured and used as a DNA template for the nested PCR reaction in which amplification for *P. falciparum*, *P. vivax* and *P. malariae* was done in separate reaction tubes. The amplification reaction and thermal cycling of the first step PCR reaction was the same with the second nested PCR reaction except that the annealing temperature was reduced to 55°C for 2 min and species-specific primers for *P. falciparum* [rFAL1: 5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3' and rFAL2:5'-ACACAATGAACTCAATCATGACTACCCGTC-3'; *P. vivax*, rVIV1:5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3' and rVIV2:5'-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3'; *P. malariae*, rMAL1:5'-ATAACATAGTTGTACGTTAAGAATAACCGC-3' and rMAL2:5'-AAAATCCCATGCATAAAAAATTATACAAA-3'] while 45 thermal cycling was used for *P. ovale* with species-specific primer (rOVA1: 5'-ATCTCTTTTGTATTTTTAGTATTGGAGA-3' and rOVA2: 5'-GGAAAAGGACACATTAATTGTATCCTAGTG-3') at denaturation temperature at 94°C for 30 s, annealing temperature at 45°C for 30 s and extension temperature at 72°C for 1 min 30 s. The aforementioned method is according to Johnston et al. (2006), with little modifications.

Electrophoresis of the PCR products

The PCR products were separated in 1.5% agarose gel for both first and second nested PCR analyses. It was thereafter stained with 1 µl ethidium bromide (TBE) and allowed to run at 100 mV and 500 mA for 3 h after which the gel was visualized under a UV light for DNA bands. The PCR amplified fragments of *P. falciparum* and *P. malariae* genes were 205 and 144 bp, respectively.

RESULT

A total of 300 samples comprising 150 each of both blood

and urine were collected from HIV positive patients who visited some selected hospitals in Ebonyi State. The subjects were made up of 57 males and 93 female patients. The samples were screened for the presence of HIV and four human malaria parasites using enzyme linked immunosorbent assay (ELISA) and parasitological examination of blood stained films. The HIV positive samples were further subjected to confirmatory test using two rapid tests. Both blood and urine samples were further analyzed using PCR.

The sex distribution of HIV patients co-infected with malaria parasite revealed that female patients had the highest occurrence of 93 (62%) and 28 (30.12%) positive cases, respectively. The least prevalence was found among male patients presenting 57 (38%) positive cases for HIV and 47 (82.46) positive results for malaria infection (Table 1). The distribution of *Plasmodium* species in blood and urine specimens using PCR identification is shown in Table 2. Out of the 150 urine samples from the HIV positive individuals 88 urine specimens were identified to harbor *Plasmodium* species. 75 (50%) urine specimens were identified to harbor *P. falciparum* while 10 (6.67%) and 3 (2%) were recorded against *P. malariae* and dual infection of *P. falciparum* and *P. malariae*, respectively. The result of the comparison of the specimens used showed the same result. None of the isolates that were negative by PCR test using DNA primers (Template) from blood gave positive results by urine samples.

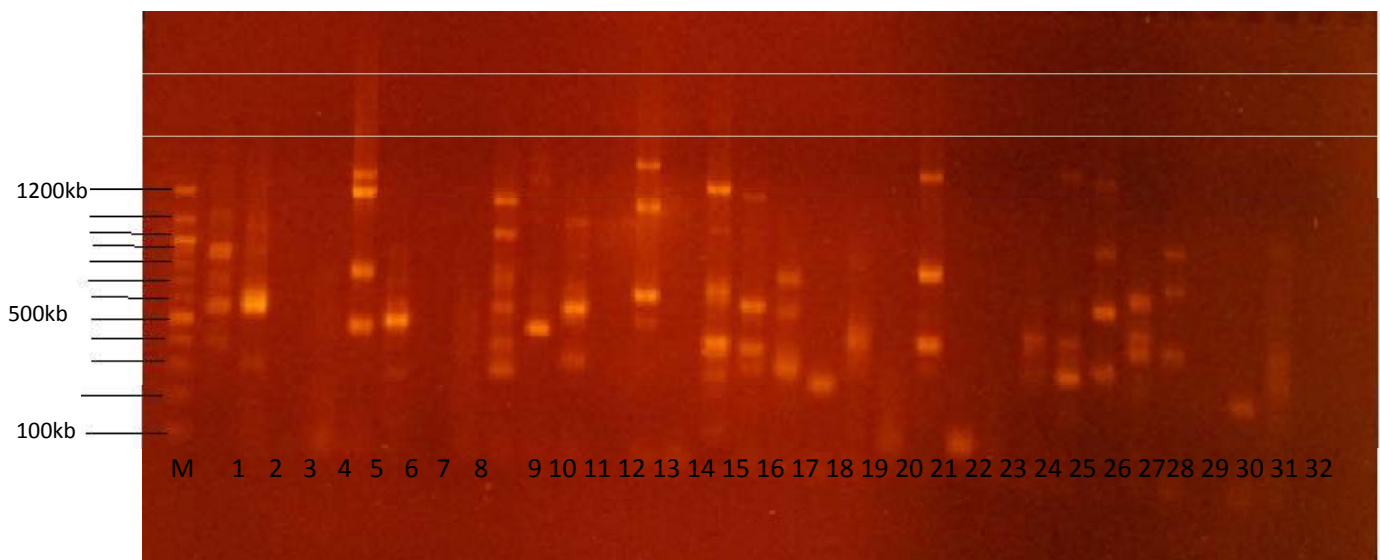
Furthermore, when the primers (rOVA₁ and rOVA₂; rV₁V₁ and rV₁V₂) specific for *P. ovale* and *P. vivax*, respectively were used none of the two species mentioned were detected in both urine and blood samples, signifying that these species may be absent in our environment (Figures 4 and 5). Figures 2 and 3 highly demonstrated the presence of *P. falciparum* and *P. malariae*, respectively especially when specific *Plasmodium* species DNA markers were used for the analysis while Figure 1 revealed the presence of *Plasmodium* species with different binding patterns.

DISCUSSION

Our investigation revealed the prevalence of 100 and 50% for HIV and malaria infections, respectively. The sex distribution of HIV patients co-infected with malaria

Table 2. Distribution of *Plasmodium* species in blood and urine specimens collected from HIV positive individuals using PCR identification method.

Type of specimen	Number of sample examined	Plasmodium species isolated	Number of isolates (%)
Blood	150	<i>Plasmodium falciparum</i> (Pf)	75(50.00)
		<i>Plasmodium malariae</i> (Pm)	10 (6.67)
		Pf + Pm	3(2.00)
Urine	150	<i>Plasmodium falciparum</i> (Pf)	75(50.00)
		<i>Plasmodium malariae</i> (Pm)	10 (6.67)
		Pf + Pm	3(2.00)
Total	300		176 (58.67)

**Figure 1.** PCR products with primer rPLU5 and rPLU6 for *Plasmodium* species.

infection revealed that female patients gave high HIV occurrence of 62% and low malaria prevalence 30.12% while low HIV prevalence of 57(38%) was observed among the males with high malaria prevalence of 47(82.46%). Our findings here as it affect prevalence among HIV positive individuals is in agreement with UNAIDS and WHO (2008) and WHO (2004) where they reported high percentage prevalence among female subjects than male subjects. These were also supported by National Agency for the Control of AIDS (NACA) (2012) where they recorded 4.0% prevalence among HIV female patients and males 3.2%. They further observed a higher prevalence of 4.5% among female police than their male colleagues at 2.0% (Federal Ministry of Health (FMH), 2008).

In another development, population based survey observed that gender inequality is an important factor for

epidemic prevalence higher in females than males (FMH, 2011). Generally, women are more vulnerable to HIV infection than men because during sexual intercourse the receptive partner (the person who is penetrated) is more at risk than the incentive partners. Unprotected sexual intercourses among other factors were believed to be contributory factors especially among sexually active persons (UNAIDS and WHO, 2008).

High malaria profile recorded in this study against the male patients and low prevalence among female patients is in line with the report by NACA (2012) in which male patients recorded 73.9% and the female patients had 65.7%. The high percentage prevalence of malaria infection recorded in this study against the male patients could be attributed to high- risk exposure to the vector that transmits malaria. Malaria and HIV/AIDS co-exist in patients in most parts of the world due to overlap of the

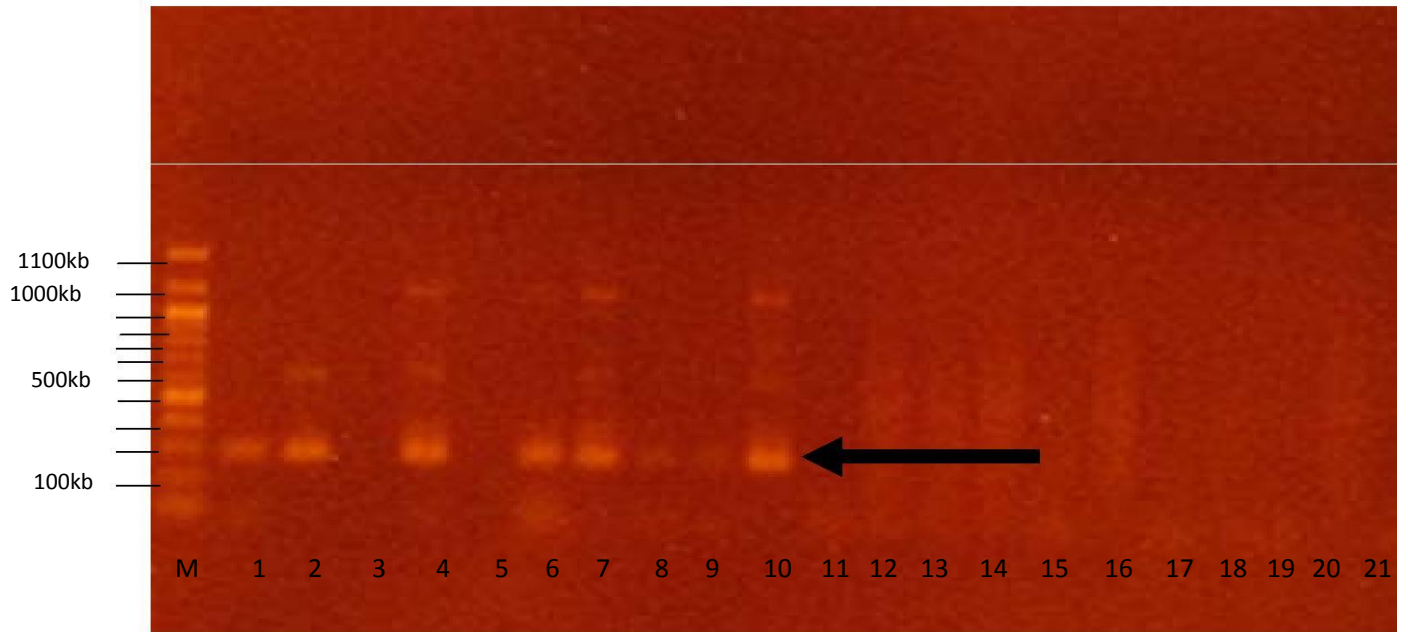


Figure 2. PCR products with primer rFAL1 and rFAL2 for *Plasmodium falciparum*.

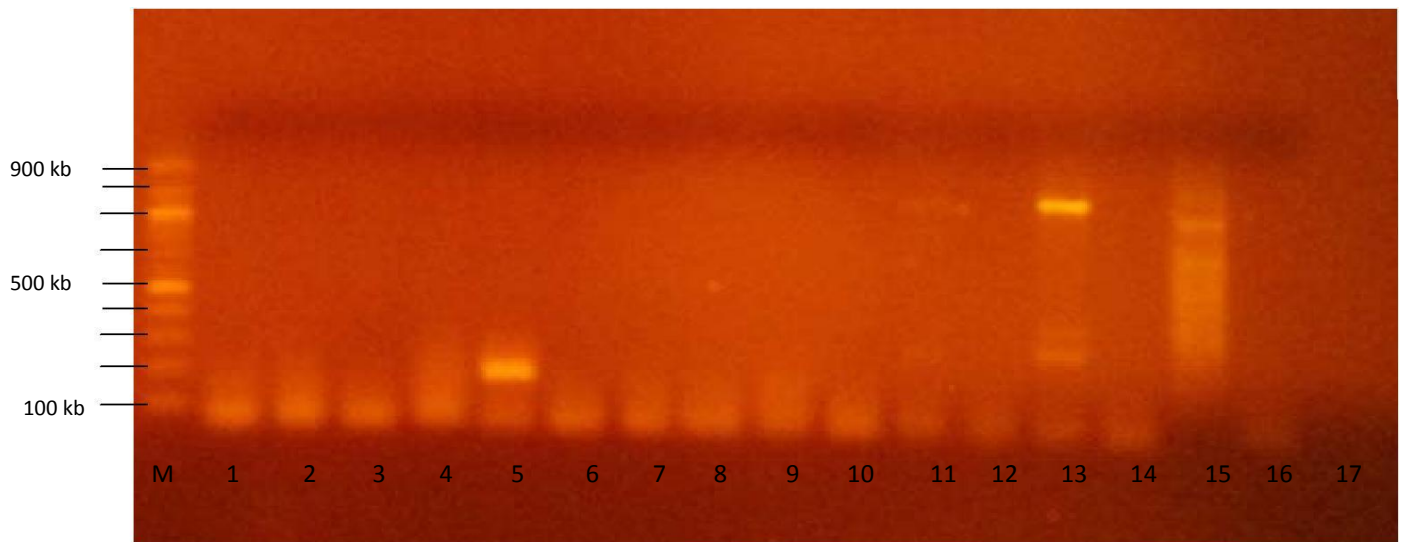


Figure 3. PCR products with primer rMAL1 and rMAL2 for *Plasmodium malariae*.

two (Hewitt et al., 2006). The Figure 2 band patterns of the presence of *Plasmodium* species isolated from the malaria and HIV individuals in this study revealed that the gene fragments are approximately 205 bp that depicts the presence of *P. falciparum*. When the results of microscopy were compared with the gel band pattern, it was observed that those blood films that were identified to be positive for *P. falciparum* gave the same band pattern

(Figure 2). The gel band pattern observed in the Figure 3 corresponds to the DNA fragment approximately 144 bp and this relates to the *P. malariae*. The result here correlates with the results of blood film microscopy. In Figure 1, the isolates in the group 2 lanes 5, 10, 13, 16, 17, 22, 28 and 29 contain multiple band patterns and they are suspected to harbor more than one *Plasmodium* species. The results also coincide with the results of blood film microscopy.

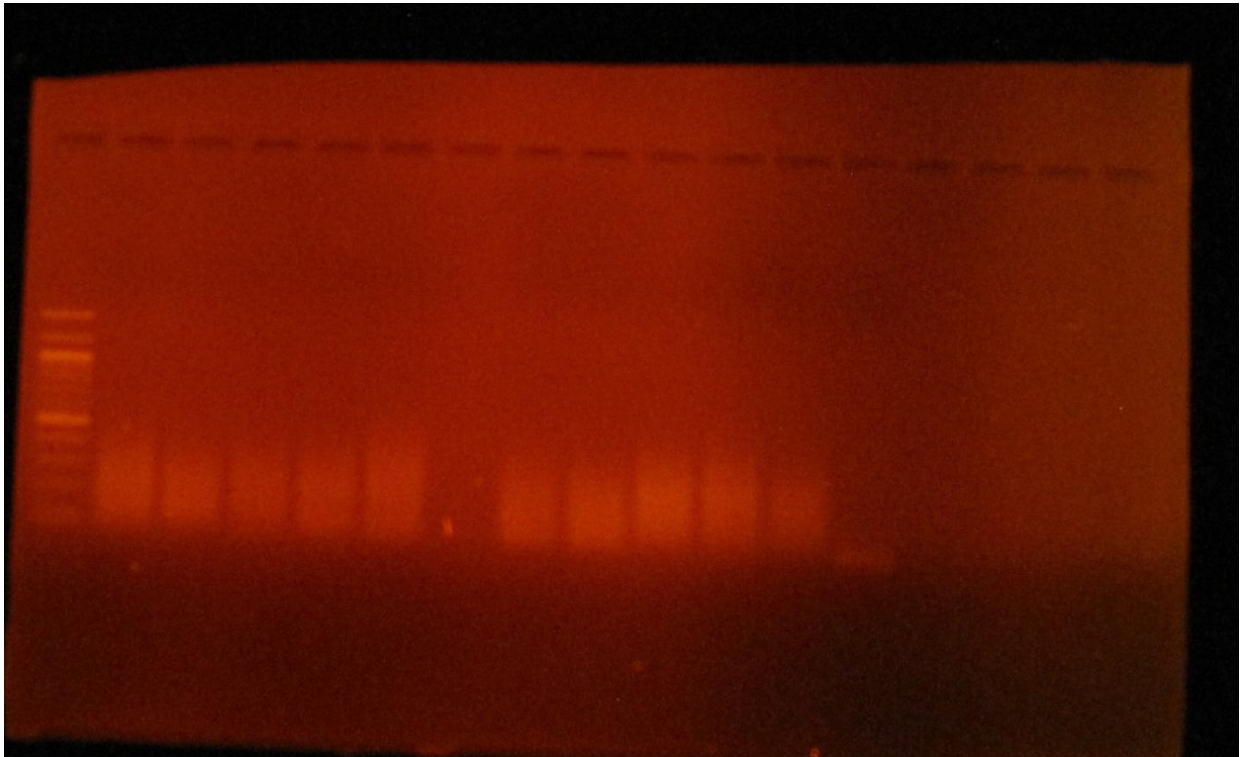


Figure 4. PCR products with primer rV1V1 and rVIV2 for *Plasmodium vivax*.

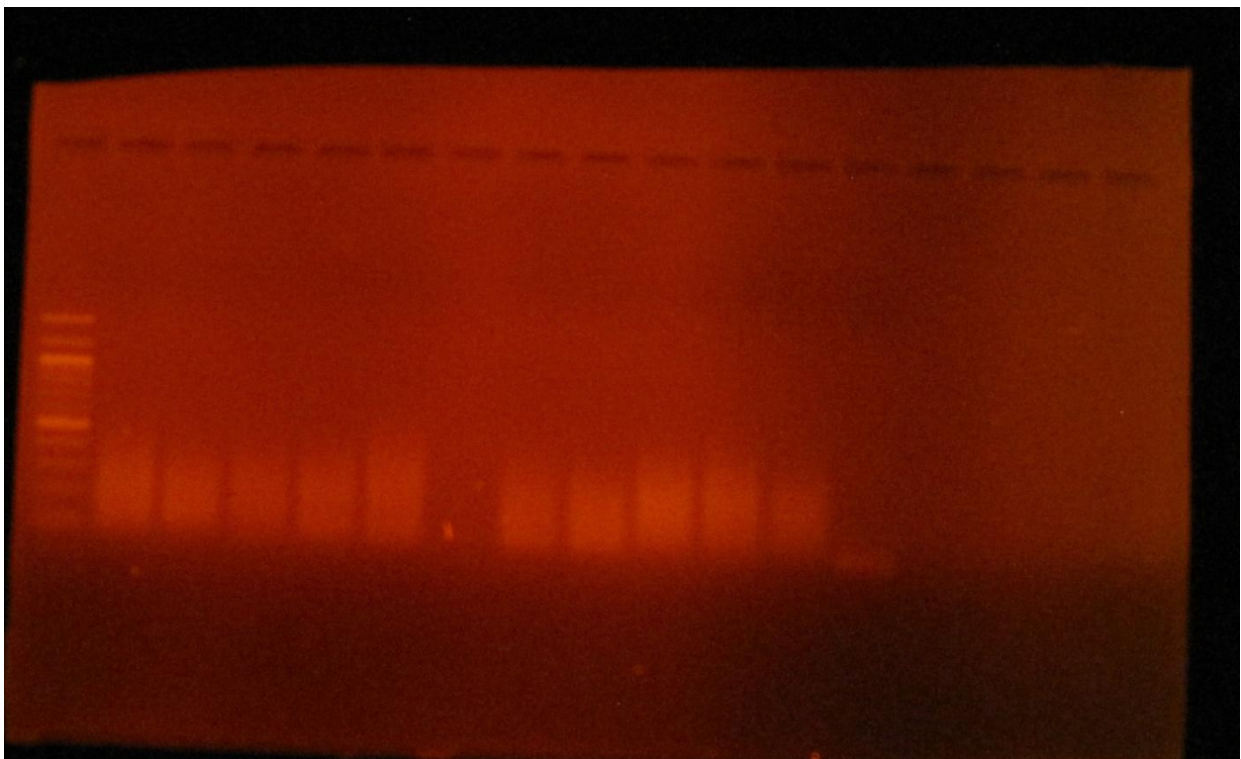


Figure 5. PCR products with primer rOVA1 and rOVA2 for *Plasmodium ovale*.

The lanes where no bands are found were suspected to be devoid of *Plasmodium* species and of course the results correspond to blood films microscopy that was negative to *Plasmodium* parasites.

The verification on the authenticity of using urine as a clinical specimen for the diagnosis of malaria in our environment was also investigated using polymerase chain reaction (PCR). Out of the 150 urine samples collected from the HIV positive individuals, 88 urine specimens were identified to harbor *Plasmodium* species. 75 (50%) was identified to be *P. falciparum* while 10 (6.67%) and 3 (2%) were recorded against *P. malariae* and dual infection of *P. falciparum* and *P. malariae*, respectively. A subsequent study in Gambia has shown that detection of the small subunit ribosomal RNA gene (SSU rRNA) of *P. falciparum* in urine has high specificity comparable to that obtained from blood samples (Nwakanma et al., 2009).

Despite a lower sensitivity of PCR detection for *P. falciparum* from urine sample than that obtained from blood-derived DNA template, repeated noninvasive sample collection during drug trials or monitoring vaccine efficacy may be warranted (Sutherland and Hallett, 2009). The findings correlate with the results gotten from blood sample PCR analysis collected from same patients. A study conducted by Buppan et al. (2010) reported 100% specificity of urine specimen for the identification of *P. falciparum* and *P. vivax* when compared with nested PCR results from blood. Results from our study also validates that *Plasmodium* species DNA could be identified in urine samples of an infected individual thereby paving way for an alternative source of non-invasive clinical specimens for potential diagnosis of malaria in our environment.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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