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Genotypic Speciation of Four Plasmodium among Human Immunodeficiency Virus Positive Individuals Attending HIV Clinics in Abakaliki, South-Eastern Nigeria

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Abstract

A total of 150 blood samples 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). Of the 150 positive individuals, 75(50%) blood samples were positive for malaria (*P. falciparum*). The comparison of blood films microscopy and PCR results were evaluated thus, 88 malaria positive cases recorded a prevalence of 58.68% for malaria parasites by PCR analysis while the overall prevalence of malaria infections by microscopy gave 50% prevalence. However, there were a number of disagreements in the identification of Plasmodium species by these two methods. Ten (6.67%) subjects were identified by PCR to be infected by P. malariae while blood film microscopy yielded 4(2.67%). Microscopy gave 70(46.67%) malaria positive cases of P. falciparum while PCR analysis yielded 75(50%). Two percent of the subjects screened were determined to be a mixed infection of P.falciparum and P. malariae by PCR while microscopy result revealed 0.67% prevalence. Therefore, PCR examination proves more sensitive than the parasitological technique used in malaria parasite studies.

Keywords: Genotypic; Plasmodium Species; HIV; PCR; Microscopy

Introduction

Malaria is caused by a protozoan parasite of the genus Plasmodium (phylum: Apicomplexa). In humans, malaria is caused by mosquito born *Plasmodium falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. However, *Plasmodium falciparum* is the most important cause of the disease and is responsible for about 80% of the reported cases and 90% of deaths in Africa especially Nigeria [1]. Malaria causes about 400-900 million cases of fever and approximately one to three million deaths annually. A vast majority of cases occur in children under the age of 5 years [2] and more so, pregnant women are particularly vulnerable.

Malaria and Human immunodeficiency virus (HIV) often co-exist in patients in most parts of the world due to overlap of these two diseases [3]. An estimated 28 million individuals are infected with HIV in sub-Saharan Africa with almost 3 million deaths annually [4]. 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.

Early diagnosis and prompt treatment is the key to minimizing the morbidity and mortality due to malaria, but the diagnostic capabilities for malaria remain a challenge for the laboratories and a hindrance for effective malaria control [5,6].

The 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. Inaccurate microscopic evaluations of blood smear resulting in misdiagnoses and misclassification of malaria severity [7]. It is against these back-drops that this study was designed to evaluate the efficacy of blood film microscopy and detection of Plasmodium species DNA with PCR-based rapid diagnostic tests (RDTs) among HIV positive individuals.

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, Ebonyi State University, Abakaliki while the polymerase chain reaction (PCR) was done at Veterinary Medicine Molecular Laboratory Unit in University of Ibadan, Oyo State.

Study Population

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.

Ethical Clearance

Ethical approval was obtained from the above mentioned health Institution. 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.

Blood Smear Preparation for the Identification of *Plasmodium Species*

Thin Blood Film Preparation

A drop of fresh blood was gently made to touch one end of a clean grease-free slide. A spreader was used to spread the blood along the glass slide. The spreader was held at a suitable angle of 450 and pushed along the slide, drawing the blood behind it until the whole of the drop has been smeared, forming a thin film. The thin film was allowed to air dry; then fixed with three drops of methanol or by dipping it into a container of methanol for a few seconds. With prolonged fixation it may be difficult to detect Schiffner's dots and Maurer's cleffs. Forceps was used to place the slide on a staining trough. Three percent (3%) Giemsa solution was prepared in buffer or distilled water and added in sufficient quantity to fill the number of staining trough. The stain was poured gently into the staining trough, until all the slides are completely covered. The staining was allowed to stain for 30-45 minutes out of sunlight. Clean water was poured gently on the surface to remove staining solution deposit. The remaining water on the surface of the glass slide was air dried gently drained. The forceps was used to remove the slide and placed it on the slide rack to drain off and allow to air dry at room temperature. The back of the slide was cleaned and examine microscopically for the presence of plasmodium species [8].

Thick Blood Film Preparation

A fresh drop of blood was gently touched onto the center of a clean grease free slide. A beveled piece of glass (spreader), a little narrower than the slide was used to spread a drop of the blood in a circular form. The thick film was allowed to air dry thoroughly; and was not fixed so as to permit dehaemoglobinization. Forceps was used to place the slide on a staining trough. Three percent (3%) Giemsa solution was prepared in buffer or distilled water in sufficient quantity to fill the number of staining trough. The stain was poured gently into the staining trough, until all the slides are completely covered and staining was allowed to stain for 30-45 minutes out of sunlight. Clean water was poured gently on the surface to remove staining solution deposit. The remaining water on the surface of the glass slide was gently poured off. Forceps was used to remove the slide and placed it on the slide rack to drain off and allowed to air dry at room temperature. The back of the slide was cleaned and examine microscopically for the presence of plasmodium species.

Plasmodium Species DNA Extraction

Whole Blood DNA Extractions

Exactly 150 µl of whole blood was pipette into micro-centrifuge tube. 95 µl of 2 x digestion buffer and 5 µl of proteinase were added to the microcentrifuge simultaneously. The content in the tubes were mixed well and then incubated at 55°C for 20 minutes. Seven hundred (700 µl) 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 minutes. Two hundred (200 µl) of DNA pre-wash buffer were added to the spin column in a new collection tube and allowed to centrifuge at 13,900rpm for 2 minutes. Four hundred (400 µl) of g-DNA wash buffer was measured and introduced in the spin column. The content was centrifuged at 13,900 rpm for 2 minutes. The spin column was carefully transferred into a clean micro-centrifuge tube. Forty (40 µl) of DNA elution buffer was added to the spin column and incubated at 2-5 minutes at room temperature. The final mixture was centrifuged at 8,000 rpm for 2 minutes. The eluted DNA was immediately used for molecular based applications or stored at -200C 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 [9].

Genomic DNA Electrophoresis

ethidium bromide

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 [rPLU5:5^1 CTGTTGTTGCCTTAAACTTC-3^1 and rPLU6: 5^1-TTAAAATTGTTGCAGTTAAAACG-3^1] for first step PCR. Thirty-five cycles (940 C for 1 min, annealing at 60°C for 2 min and extension at 720 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*

[rFAL1:5^1-TTAAACTGGTTTGGGAAAACCAAATATATT-3^1 and rFAL2:5^1-ACACAATGAACTCAATCATGACTACCCGTC-3^1; P. vivax, rVIV1:5^1-CGCTTCTAGCTTAATCCACATAACTGATAC-3^1 and rVIV2:5^1 ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3^1; P. malariae, rMAL1:5^1-ATAACATAGTTGTACGTTAAGAATAACCGC-3^1 and rMAL2:5^1-AAAATTCCCATGCATAAAAAATTATACAAA-3^1] while 45 thermal cycling was used for P. ovale with species-specific primer (rOVA1: 5^1- ATCTCTTTTGCTATTTTTTAGTATTGGAGA-31 and rOVA2: 5^1-GGAAAAGGACACATTAATTGTATCCTAGTG-3^1) of denaturation temperature at 94° C for 30s, annealing temperature at 45°C for 30s and extension temperature at 72°C for 1 min 30s. The above method is according to Johnston et al., with little modifications.

Electrophoresis of the PCR Products

The PCR products were separated in 1.5% agarose gels 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 hrs 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 bp and 144 bp, respectively.

Result

A total of 150 blood samples were collected from Human immunodeficiency virus (HIV) positive patients who visited some selected hospitals in Ebonyi State. The samples were screened for the presence of four human malaria parasites using parasitological examination of blood stained films and Polymerase Chain Reaction (PCR).

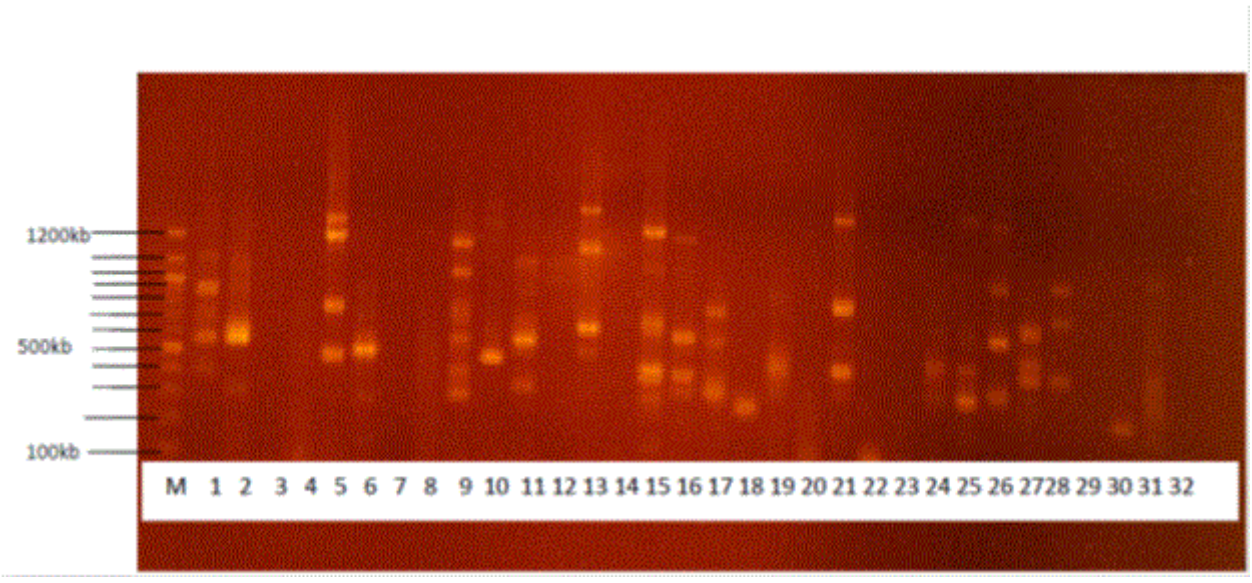
The comparison of blood films microscopy and polymerase chain reaction (PCR) results were evaluated thus: Exactly half of the 150 blood samples collected from patients were identified to contain Plasmodium species but more specifically for *Plasmodium falciparum* and these correlated between microscopy and PCR. A total of 150 blood samples from HIV positive patients were screened using 18s rRNA based nested PCR. Of the 150 samples, 88(58.67%) were positive for malaria parasites by PCR analysis while the overall prevalence of malaria by microscopy gave 75(50%). The majority of the malaria infections were due to *Plasmodium falciparum* 75(50%). *Plasmodium malariae* positive cases were seen in only 10(6.67%) of the blood samples. The dual infections with *Plasmodium falciparum* and *Plasmodium malariae* was observed in 3 (2%) blood samples (Table 1).

However, there were a number of disagreements in the identification of Plasmodium species including *Plasmodium falciparum*. Prevalence of 6.67% *Plasmodium malariae* infections were identified by PCR while microscopy recorded 4(2.67%). Microscopy gave 70(46.67%) while PCR analysis yielded 75(50%) for *Plasmodium falciparum*. Three (2%) samples were determined to be a mixed infection of *Plasmodium falciparum* and *Plasmodium malariae* by PCR while microscopy result revealed 0.67% prevalence (Table 1).

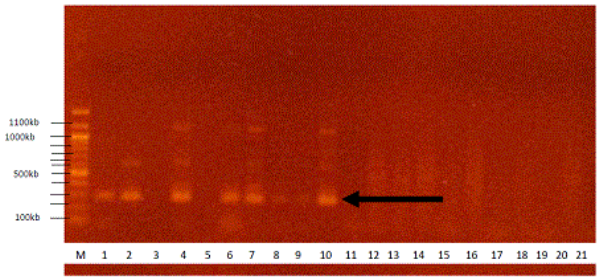
PCR products with primer rPLU5 and rPLU6 for Plasmodium species was shown in gel 1 which demonstrated different DNA band patterns of Plasmodium species while gel 2 and 3 with specific primers revealed definite DNA band patterns that correspond to base pairs of P. falciparum and P. malariae respectively. Gel pictures on band 4 and 5 showed that no DNA band was detected.

Plasmodium species isolated	PCR positive results (%)	Microscopy positive results (%)
Number of negative samples	62(41.33)	75(50)
Plasmodium falciparum (Pf)	75(50)	70(46.67)
Plasmodium malariae (Pm)	10(6.67)	4(2.67)
Pf + Pm	3(2)	1(0.67)
Total	150(58.67)	150(50)

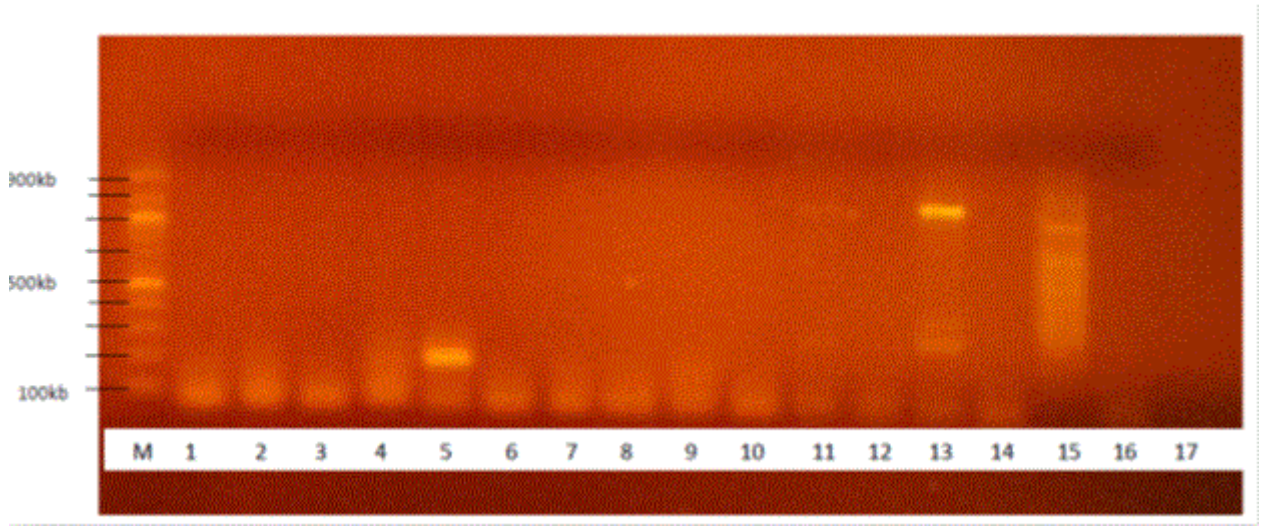
Table 1: Comparison of Blood Films Microscopy and PCR Results for malaria prevalence



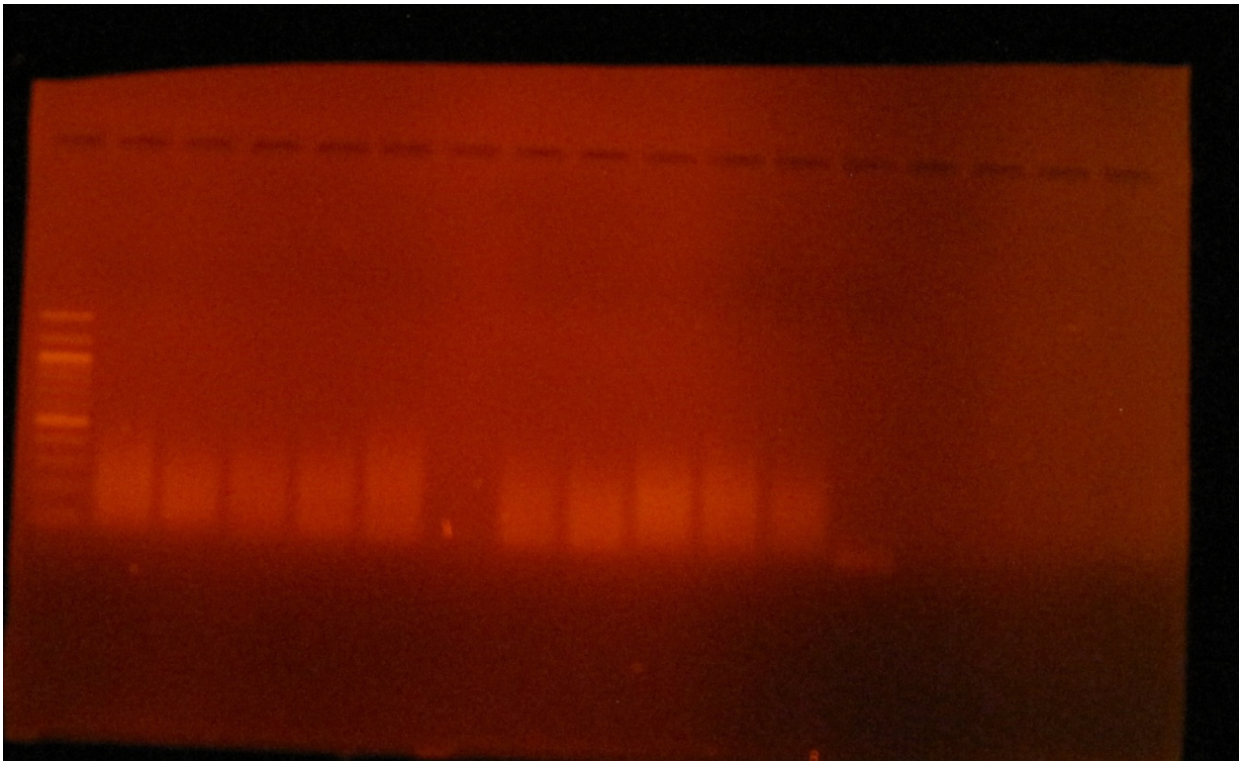
Gel 1: PCR products with primer rPLU5 and rPLU6 for Plasmodium species observed



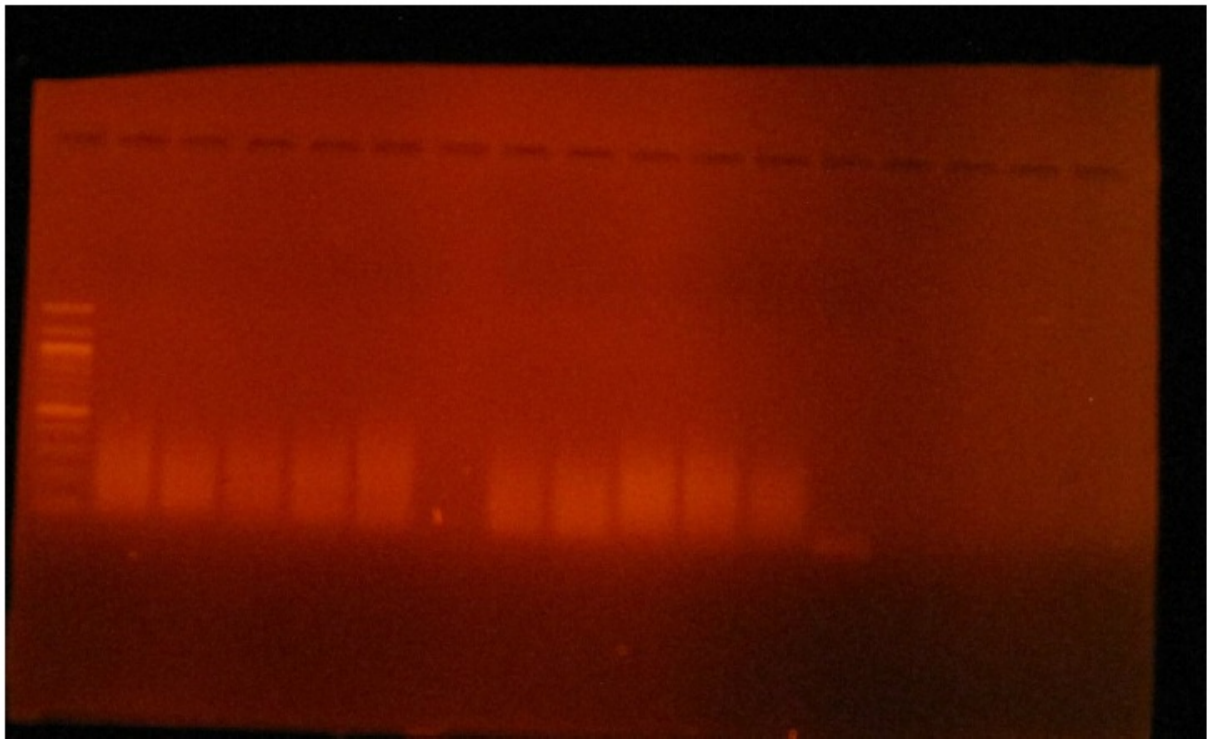
Gel 2: PCR products with primer rFAL1 and rFAL2 for *Plasmodium falciparum*



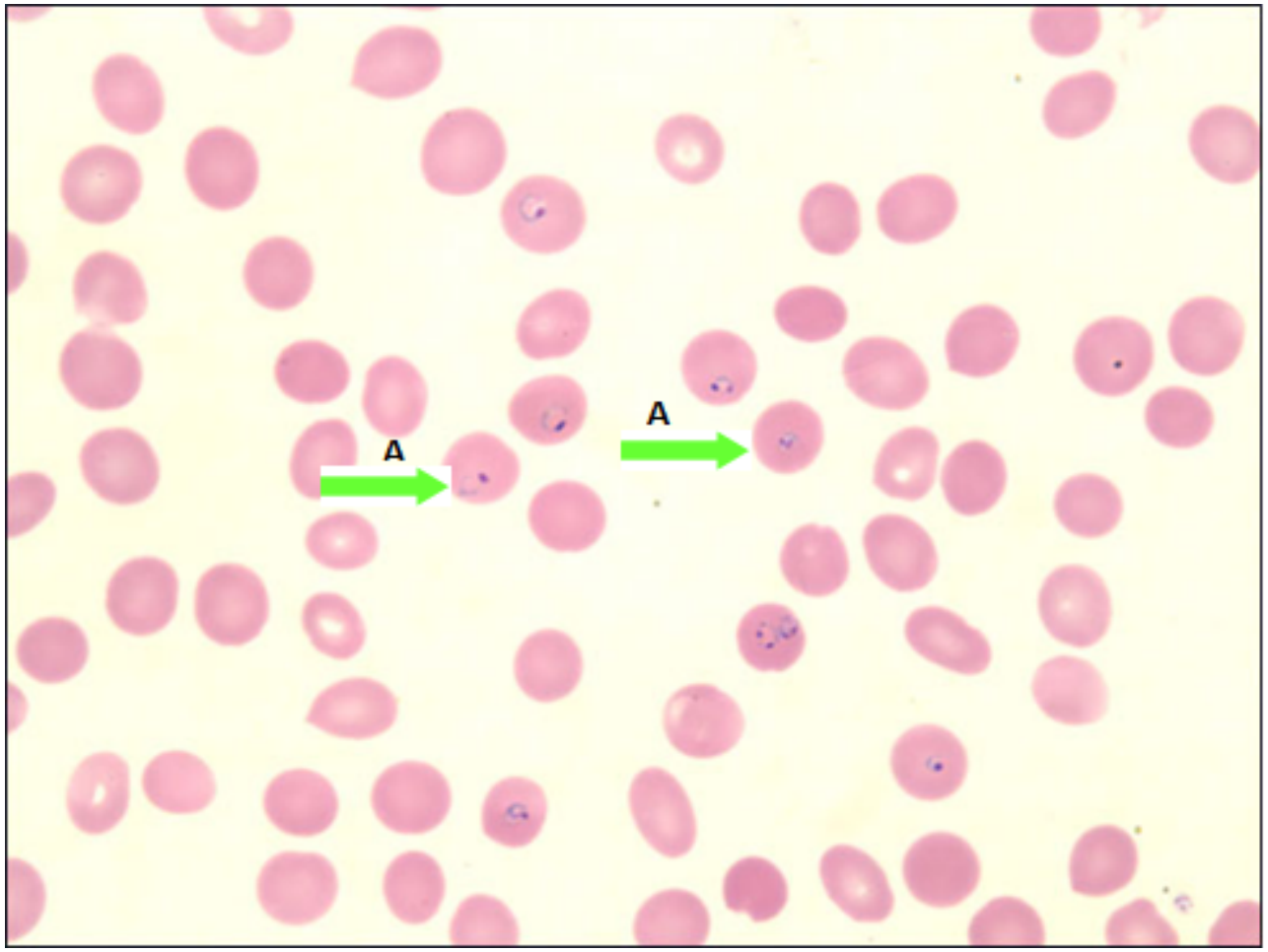
Gel 3: PCR products with primer rMAL1 and rMAL2 for Plasmodium malariae



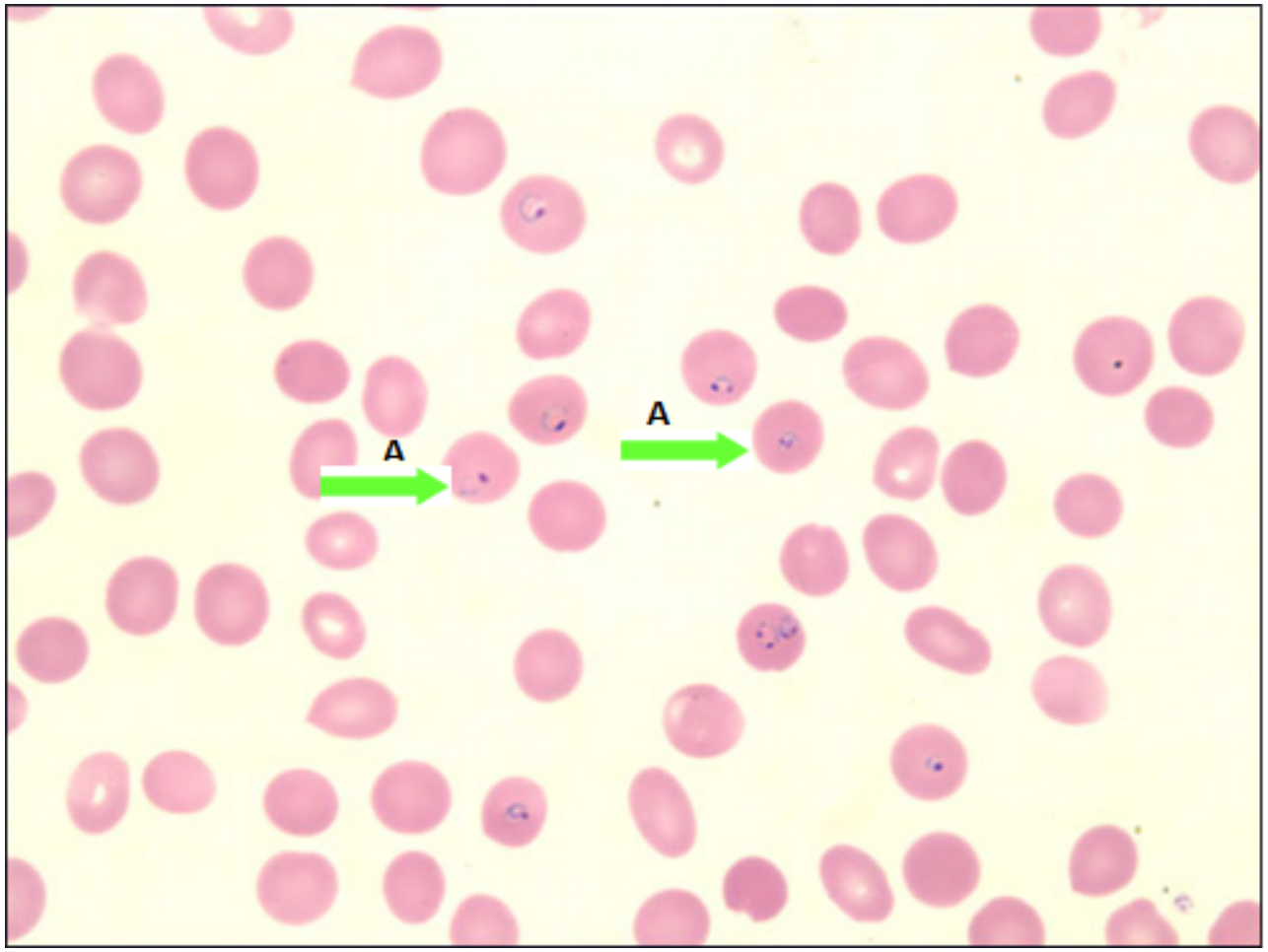
Gel 4: PCR products with primer rV1V1 and rVIV2 for Plasmodium vivax



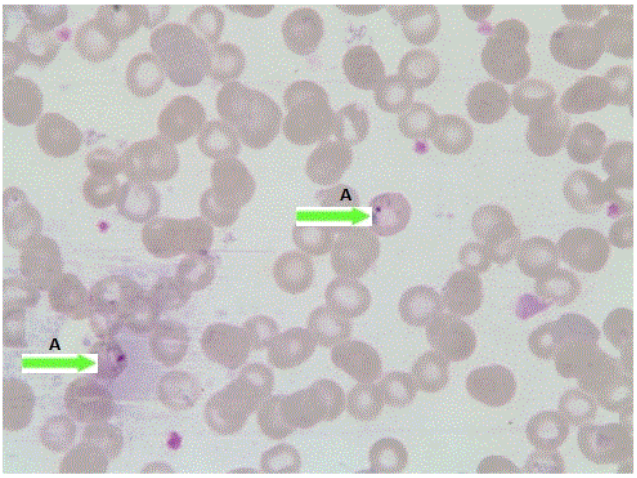
Gel 5: PCR products with primer rOVA1 and rOVA2 for Plasmodium ovale



Slide 1: Thin Film *Plasmodium falciparum* Trophozoites Stage of Ideal Stain Showing Pink RBCs, Parasites with Red Nuclei Delicate Blue Rings of Cytoplasm, and Vacuoles Cells Appear Red, and the Cytoplasm in the Parasites Appears Blue.



Slide 2: A= Elongated Appearance of the Parasite is Called a "Band Form," which is commonly Associated with P. malariae Trophozoites. C=*Plasmodium falciparum* Trophozoites and this is a Good Representation of P. falciparum and P. malariae Dual Infection.



Slide 3: Thin Film Showing *Plasmodium Malariae* Trophozoites Stage. These are Two Ring Trophozoites that are not Stippled, Distorted or Enlarged in RBCs. P. malariae does not Distort the Morphology of the Host Cell.

Discussion

Our investigation revealed the prevalence of 100% and 50% for HIV and malaria infections respectively. It also showed prevalence of 50%, 6.67% and 2% for *Plasmodium falciparum*, *Plasmodium malariae* and dual infection between *Plasmodium falciparum* and *Plasmodium malariae* infections by PCR analysis of blood samples respectively. Our finding on HIV and malaria prevalence is in consonance with WHO [10]. In that WHO reported that both HIV and malaria were among the most prevalent infectious diseases in sub-Saharan Africa and leading cause of morbidity and mortality. Our findings were also supported by Federal Ministry of Health, [11] and Mendis et al., [1] where they reported prevalence of P. falciparum to be 80.2% and 80% respectively.

Our results further demonstrated percentage prevalence of 46.67%, 2.67%, and 0.67% against P. falciparum, P. malariae and dual infection of P. falciparum and P. malariae respectively. Comparing the PCR and blood film microscopy results, microscopy gave low prevalence suggesting that PCR analysis is more highly sensitive even picking up an infection that is a day old. The above finding was in agreement with NACA, [11] though, with higher percentage prevalence of 80.2% for P. falciparum, P. malariae (13.6%), P. ovale yielded 4.4% while prevalence rate of 1.6% of dual infection was recorded against P. falciparum and P. malariae. Though, in this study we did not found the presence of P. ovale and P. vivax but NACA, [11] reported percentage prevalence of 4.4 against P. ovale. In another work by Johnston et al., [11] is also in consonance with our findings though, presented different mixed infection reported of P. falciparum and P. ovale by microscopy while five mixed infections were identified by using nested PCR analysis.

The gel picture 1 showed DNA band patterns of the presence of Plasmodium species isolated from blood samples collected from HIV positive individuals in this study. The results on gel 2 and 3 revealed that the gene fragments were approximately 205 bp that depicts the presence of P. falciparum. When the results of microscopy were compared with the gel band patterns, it was observed that those blood films that were identified to be positive for P. falciparum gave the same band pattern (Slide 1). The gel band pattern observed in the gel picture 2 corresponds to the DNA fragment approximately 144 bp and this relates to the P. malariae (Slide 3). The result here correlates with the results of blood film microscopy. The results also coincide with the results of blood film microscopy. The legends 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.

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). The reason of inaccurate microscopic evaluations of blood smears, resulting in misdiagnoses and misclassification of malaria severity Makler et al., [12]. From our analysis we found out that 50% of the total blood screened for malaria parasites was positive by microscopy while 50.67% was positive by PCR. 70(46.67%) yielded *Plasmodium falciparum* by microscopy while 75(50%) was recorded against *Plasmodium falciparum* by PCR. Ten blood samples gave 6.67% of *Plasmodium malariae* by PCR while microscopy recorded 4(2.67%) and 3(2%); (0.67%) mixed infection of Plasmodium falciparum and *Plasmodium malariae* were recorded by PCR and microscopy respectively. The above findings is in consonance with the reports of Mayxey et al., [13]; Snounou and White, [14] and Putapomtip et al., [15] who deduced that although, the microscopy method is feasible, economical and practical but a quite number of asymptomatic cases have been left undiagnosed when parasitaemia did not reach the microscopic detection threshold. Due to the high degree of PCR sensitivity it was able to capture and resolve one day old malaria infection than microscopy.

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