Detection of Artemisinin Target Gene (*PFATP6*) in Plasmodium Falciparum Infections among Asymptomatic Patients Attending NDUTH, FMC and General Hospital in Bayelsa State

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Abstract:*Malaria infections have proven to be a burden to Africa and the world in general. This study was carried out between January and October 2018 to detect Artemisinin target gene (PFATP6) in Plasmodium falciparum isolates from blood samples using Polymerase Chain Reaction technique. Two hundred and fifty (250) blood samples were collected from Federal Medical Centre, Niger Delta University Teaching Hospital and General Hospital in Yenagoa, Okolobiri and Amassoma community respectively in Bayelsa State and were immediately taken to the laboratory for analysis for the presence of malaria parasites using Rapid Diagnostic Test (RDT) kits, Conventional Microscopy and molecular analysis using Polymerase Chain Reaction technique. The findings showed that out of 250 subjects screened, 23 (9.2%) subjects were positive for malaria parasites using both Rapid Diagnostic Test Kit and Conventional Microscopy. Observed showed that of the 23 positive samples, three (3) others were positive by RDT only giving 26 (10.4%) positive samples for RDT and four (4) other samples were positive to Conventional Microscopy only giving 27 (10.8%). Twenty (20) out of the 23 positive samples were subjected to Nested-PCR amplification using specific primers after the parasite DNA extraction from the blood samples. Thirteen 13 (65%) out of 20 were positive for the Plasmodium falciparum Artemisinin target gene (PFATP6). In conclusion, this study revealed the presence of the Artemisinin target gene (PFATP6) in Plasmodium falciparum in Bayelsa state and elucidated a possible reason for the failure of Artemisinin-based chemotherapy in Clinical practice.*

Keywords: Malaria infections, Antimalaria agents, Artemisinin gene, *Plasmodium falciparum*

1. Introduction

Malaria is a zoonotic disease that is caused by the parasites belonging to the family Plasmodiidae. This disease is severe and has caused a high level of morbidity and mortality, particularly in underdeveloped and developing countries. Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes, it is preventable and curable (Caraballo, 2014). In 2016, there was an estimated 216 million cases of malaria in 91 countries, an increase of 5 million cases over 2015. The number of deaths however, was pegged at 445, 000 in 2016, a similar number (446, 000) to 2015. A heavy share of the global malaria burden is carried by the African nations with 90% of malaria cases and 91% of malaria deaths. In areas of high transmission of malaria, children under five are particularly susceptible to infection, illness and death. It is interesting to know that over 70% of all malaria deaths occur in this age group (World Health Organization, 2017).

Falciparum infections are more severe and when untreated can result in a death rate of 25% in adults. The presentation may include headache, fever, shivering, joint pain, vomiting, haemolytic anaemia, jaundice, haemoglobin in urine, retinal damage, and convulsions, paroxysm-a cyclical occurrence of sudden coldness followed by shivering and then fever and sweating, occurring every two days (tertian fever) in *P. vivax* and *P. ovale* infections, and every three days (quartan fever) for *P. malariae.P. falciparum* infection can cause recurrent fever every 36-48 hours, or a less pronounced and almost continuous fever (Eric *et al*., 2012).Complications include respiratory distress, pulmonary oedema, renal failure, encephalopathy, hepatosplenomegaly, hypoglycaemia, hemoglobinuria, coagulopathy, shock, as well as infant morbidity and mortality (Caraballo, 2014). The result of what has been called the pathology cascade includes renal insufficiency, renal failure, pulmonary oedema, neurologic symptoms and severe haemolytic anaemia. In the pregnant female falciparum malaria may result in stillborn, lower than normal birth weight, or abortion. Non-immunes and children may develop cerebral malaria, a consequence of the mechanical blockage of micro-vessels in the brain, or organ infarcts, due to sequestration of infected red cells via protuberances called knobs (Rosauro *et al*., 2018). If relapse occurs in falciparum malaria it is due to the increase in numbers of pre-existing erythrocytic forms, which were too low to be detected microscopically; this type of relapse is termed recrudescence. Falciparum malaria accounts for 50% of all the clinical malaria cases and is responsible for 95% of the deaths. Falciparum malaria occurs in tropical and subtropical Africa and Southeast Asia (Caraballo, 2014).

It has been reported by Caraballo (2014) that malaria is responsible for more than one million deaths per year; and most of the victims are young children in Africa. The prevalence of Malaria in tropical and sub-tropical regions is due to rainfall, consistent high temperatures and high humidity, along with stagnant waters in which mosquito larva readily mature, providing them with the environment they need for continuous breeding. Malaria in Africa is present in both rural and urban areas, though the risk is lower in larger cities.

The agents used to treat malaria infections are collectively known as Antimalarial drugs. They are of different groups and they have different modes of action. They are classified based on their therapeutic usage and their chemical structure. They are therapeutically Classified as Schizonticides which are pre-erythrocytic schizonticides and erythrocytic schizonticides are used for prophylaxis and treatment. Sporozoiticides prevent development of trophozoites and liver schizonts. Gametocides are those who destroy gametocytes and prevent transmission. Primaquine, Hypnozoiticides are also Primaquine. Tissue Schizonticides are Proguanil, Pyrimethamine, Primaquine, Tetracycline and Fansidar. Blood Schizonticides are Chloroquine, Quinine, Fansidar, Mefloquine, Halofantrine, Tetracycline, Artemisin, Sporozoitocides are Primaquine, Pyrimethamine. The adverse effect known by Artemisinins is that, they are generally well tolerated at the doses used to treat malaria. The side effects from the artemisinin class of medications are similar to the symptoms of malaria: nausea, vomiting, anorexia, and dizziness. Mild blood abnormalities have also been noted. A rare but serious adverse effect is allergic reaction. One case of significant liver inflammation has been reported in association with prolonged use of a relatively high-dose of artemisinin for an unclear reason (the patient did not have malaria). The drugs used in combination therapies can contribute to the adverse effects experienced by those undergoing treatment. Adverse effects in patients with acute *P. falciparum* malaria treated with artemisinin derivatives tend to be higher (Tilley *et al.,* 2016). The plasmodium parasites that cause the disease are *P. falciparum*, *P. ovale*, *P. malariae* and the recently identified *P. knowlesi*. Infection occurs with the bite of the female anopheles mosquito which usually have the infective forms, "Sporozoites" deposited in their saliva they inject via the proboscis into the individual during a blood meal. The primary mechanism of the pathogenesis is the lysis of erythrocytes during the blood stage of the infection. Symptoms subside with therapeutic intervention with any of the antimalarial drugs.

Artemisinin-based Combination Therapy has been the WHO recommended first-line drug for malaria infections for a number of years now. This has been due to the parasite's increased resistance to chloroquine, sulfadoxine, pyrimethamine, quinine, and mefloquine in recent years. However, the tides have turned again as resistance to the Artemisinins is rapidly emerging (Feng *et al.*, 2017). The medical community have sought out ways to identify genetic markers of resistance in order to effectively combat the infection Lalremuruta *et al.*, 2113.

Antimicrobial resistance is resistance of a microorganism to an antimicrobial agent to which it was previously sensitive. The artemisinins have been the drug of choice for the treatment of malaria. Decreased sensitivity to ARTs, manifesting clinically as slower rates of parasite is now documented in multiple Southeast Asian countries Leann *et al.*, 2016. Although true clinical resistance to artemisinin and its derivatives has not been confirmed in malaria parasites collected from patients, there have been sporadic reports of clinical failures of artemisinin treatment. A small number of cases with poor responses to artesunate or artemether have been reported in western Thailand, India

and Sierra Leone. Some clinical parasite isolates from Nigeria and Madagascar appear to exhibit reduced sensitivity to artemisinins. *In vitro* studies in Yunnan province of western China have detected reduced susceptibility to artemisinins, and drug sensitivity exhibits considerable geographic variation (Leann *et al.,* 2016).

The mode of action of ARTs in malaria parasites is still not completely understood, and the molecular basis of reduced ART susceptibility is unclear. So far, a number of genes have been proposed to be associated with reduced sensitivities to ARTs. It has been suggested that the Artemisinins have specific targets in the plasmodium parasites known as PfATP6. This is based on the structural similarity between artemisinins and thapsigarnin, an inhibitor of sarco/endoplasmic reticulum calcium-dependent ATPases (SERCAs).

This study aims to detect the ATPase6 gene which is a genetic marker of resistance to Artemisinins using specific primers. This is to compare Microscopy, RDT and PCR methods of detection of malaria.

2. Materials and Methods

Study Area
The study samples were collected from patients in the General Hospital, Amassoma and Federal Medical Centre, Yenagoa, Bayelsa state in the months of January and February 2018.

Approval
This was by individuals consent by the volunteers as they were coming from the hospital.

Sample Size
The sample size was determined using the Taro Yamen's formulae.

$$n = \frac{N}{1 + N\,(\alpha^2)}$$

Where n = required sample size
N = Population size
α = Level of significance at 0.05 or 0.10

Assuming N = 250 and   α = 0.05;

$$n = \frac{250}{1 + 250\,(0.05^2)}$$

n = 244 (minimum sample size)

Sample Collection
Through venous blood sample collection, 3mls of venous blood was collected into EDTA bottles from the individuals. A total of 250 blood samples were collected.

Sample Analysis

Malaria parasite screening
Thick blood films were made on a clean glass slide and stained with 10% Giemsa stain for 15mins after which the stain was washed off using buffered water, air dried and examined under the microscope using X100 oil immersion objectives. The stained thick smear was used for detection of

the presence of malaria parasite according to Samina *et al*., 2017.

Rapid Diagnostic Test
CoreStart, a rapid diagnostic test kit that detects histidine-rich protein 2(HRP-2) specific for *P.falciparum* was used for screening alongside the microscopic technique according to Wilson, 2012.

Procedure: Five (5) microlitres of whole blood were added into the sample well(small well) of the kit cassette using Pasteur pipette, 2 (60ul) of assay buffer was added vertically into the assay buffer well, the test result was read after 15mins but not exceeding 30 minutes, the presence of two colour bands indicated a positive result. The presence of only one band at the C line indicated a negative result, where the C line did not appear, the test was considered invalid and was repeated.

Molecular Analysis (Kesinee, Kamolrat and Nicholas 2007 methods)

DNA Extraction

DNA was extracted using the quick gDNA mini prep DNA extraction kit supplied by Inqaba Biotechnological, South Africa., One hundred microliter (100μl) of whole blood was pipetted into a microcentrifuge tube, and four hundred microliter (400μl) of Genomic lysis buffer was added. The samples were mixed by vortexing for five seconds and were allowed to stand at room temperature for ten minutes. The mixture was transferred to a zymo-spin column in a collection tube. It was centrifuged at twelve thousand revolutions per minutes (12, 000rpm) for one minute. The flow through and the collection tubes were discarded. The zymo-spin columns were transferred to a new collection tube and two hundred microlitre (200μl) of DNA pre-wash buffer was added and centrifuged at twelve thousand revolutions per minute for one minute. Five hundred microlitre (500μl) of g-DNA wash buffer was added to the spin column and centrifuged at 12, 000rpm for 1 min.The spin column was transferred into a 1.5 micro centrifuge tube and one hundred microlitres (100μl) of DNA elution buffer was added to the spin column and incubated at room temperature for five minutes and centrifuged at 14, 000rpm for 30secs to elute the DNA.

DNA Quantification
DNA quantification and purity testing was carried out on a Nano-drop 1000 spectrophotometer by loading 2ul of the extracted product on the lower pedestal. The concentration of the DNA was calculated by the nanodrop software installed on a desktop computer.

Amplification of PFATP6 Gene
Nested PCR was used to amplify *PFATP6* gene, amplification was done at final volume of 20ul. 1X master mix containing dNTPs, $MgCl_2$, buffer and Taq polymerase with 5ul and 0.5ul of DNA template and 0.5C of both forward and reverse primers were used respectively for the PCR reaction. Primers used for primary amplification were Atpase1 Forward: TTGGTAATAAAACTCCCGC Atpase1 Reverse: TATTCCTCTTAGCACCAC TCC and secondary

amplification were Atpase2 Forward: TCATCTACCGCTATTGTATG Atpase2 Reverse: TCCTCTTAGCACCACTCC. PCR conditions for primary amplification were as follows: initial denaturation temperature of 95°C for 5 minutes, denaturation at 92°C for 30seconds, annealing at 56°C for 40 seconds and initial extension at 65°C for 1:30 seconds followed by final extension at 65°C for 8minutes after the 35th cycle. The PCR condition of the secondary are the same with the primary, but differ from that of the annealing temperature (47°C for 40 seconds), initial extension (65°C for 1.30 seconds), final extension (65°C for 8 minutes) and the number of cycles was 30.

Agarose Gel Electrophoresis
Agarose gel prepared with ethidium bromide was used to run the amplified sample for visualization on UV trans-illuminator. Gel Electrophoresis was done with 1.5% agarose ran at 110v for 25mins and visualized under UV transilluminator. This was run alongside with the DNA molecular ladder and the control strains, different base pairs of the genes were compared with the DNA ladder.

3. Results

Table 1.0 shows the age distribution of patients and the number of positive subjects using Rapid Diagnostics Test (RDT) and Microscopy methods of Malaria parasite diagnosis. Positive samples to both diagnostic techniques were identified in all age groups.

Table 2.0 shows the gender distribution of the participants with malaria positive cases using RDT and microscopy methods. 147 (58.8%) subjects were males and 103 (41.2%) were females. 14 (9.5%) males were positive by RDT and 13 (8.8%) were positive by microscopy. 12 (11.7%) females were RDT positive and 14 (13.6%) were positive by microscopy.

Fig 1.0 is a Venn diagram showing the results of both RDT and Microscopy in the detection of malaria parasites in the study samples. 26 (10.4%) samples were malaria positive by RDT and 27 (10.8%) were positive by microscopy.

Fig 2.0 and Fig. 3.0 shows the Agarose Gel Electrophoresis separated bands on the PCR amplicons. 13 of 20 samples subjected to PCR amplification were positive for the PFATP6 gene.

Table 1: Age Distribution of subjects and diagnosis results

Age(yrs.)	Total Number of Samples (%)	RDT Positive (%)	Microscopy Positive (%)
1-10	93(37.2%)	13(14%)	10(10.7%)
11-20	54(21.6%)	4(7.4%)	7(13.0%)
21-30	32(12.8%)	1(3.1%)	2(6.25%)
31-40	35(14%)	2(5.7%)	2(5.7%)
41-50	20(8%)	4(20%)	4(20%)
>50	16(6.4%)	2(12.5)	2(12.5%)
TOTAL	250	26	27

Table 2: Gender Distribution of Subjects

Gender	Total Number of Samples (%)	RDT Positive (%)	Microscopy Positive (%)
Males	147	14(9.5%)	13(8.8%)
Females	103	12(11.7%)	14(13.6%)
Total	250	26(10.4%)	27(10.8%)
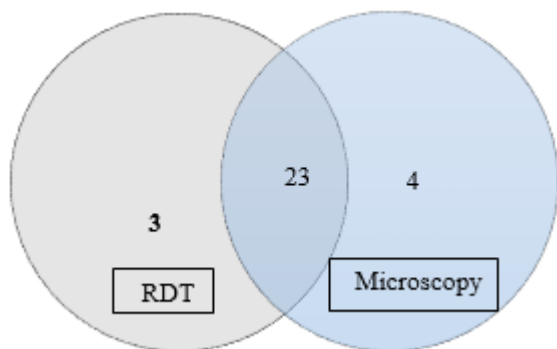
Figure 1: Venn diagram showing the positive results based on RDT and Microscopy methods of diagnosis
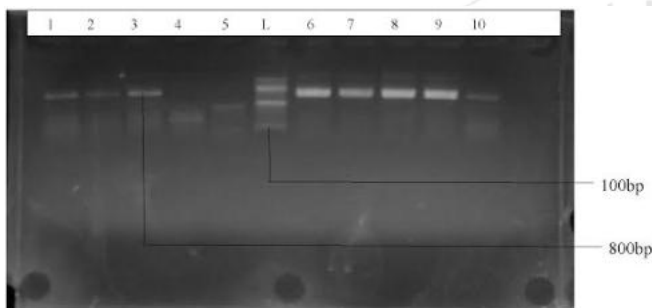
Figure 2: Agarose Gel Electrophoresis showing PFATP 6 genes (706bp). Lane 1-10 represents the samples while L represent 100bp molecular ladder.
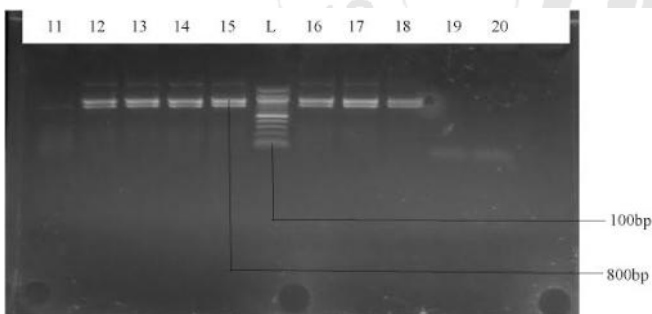
Figure 3: Agarose Gel Electrophoresis showing PFATP6 gene (800bp). Lane 11-20 represent the samples while L represent 100bp molecular ladder

4. Discussion and Conclusion

4.1 Discussion

A total of 250 blood samples screened for malaria parasites, 27 (10.8%) and 26 (10.4%) samples were found to be positive using conventional microscopy and RDT techniques respectively. Out of 147 participants within the ages of 1-20 screened, 17 (65.4%) were positive to malaria parasite using both RDT technique and conventional microscopy resulting in higher prevalence of malaria infection in children. These findings are in concordance with the work done by Tatfeng *et al.* (2011) which stated that it has long been observed that children within the ages of 1 and 16 are usually the most

affected. A key reason for this affinity to the young is their low immunity as compared to the adults who have gained relative immunity to the parasites over the years. The oldest participant that was positive by microscopy and RDT was 56 years. The detection of the plasmodium parasite in subjects above 20 years proves that individuals of all ages in endemic countries such as Nigeria can be affected by the parasite.

The study compared the sensitivity of Rapid Diagnostic Test methods to conventional microscopy for the diagnosis of malaria in developing countries. Microscopy has always been the gold standard for malaria diagnosis, even in the advent of newer methods such as RDTs, Polymerase Chain Reaction (PCR) and even Loop-Associated Isothermal Amplification (LAMP). While 23 (9%) of the samples were positive to both RDT and Microscopy, three [3 (1.2%)] other samples were positive to RDT only and four [4 (1.6%)] others were positive to Microscopy while being negative to RDTs. This is similar to the results gotten by Ibrahim *et al.* (2014) where RDT picked 33 out of 35 blood samples positive to malaria parasite by microscopy. Some healthcare professionals attribute cases in the latter statement to be as a result of reduced sensitivity of the RDTs, and further stated that this could be due to self-therapy and therapeutic intervention before samples are collected for diagnosis. The parasites are inactivated by the drugs and hence are not detected by microscopy, but the antigens are detected by the RDTs. On the other hand, the RDT would show negative to non-falciparum malaria since the antibodies present are specific for the Histidine-Rich Protein 2 (HRP-2) of *Plasmodium falciparum* only. In addition, the work done by Christina *et al.* (2017) on the Impact of *Plasmodium falciparum* isolates lacking HRP-2, stated that it is possible for RDTs to yield False-negative in the presence of *Plasmodium falciparum* isolates lacking HRP-2. Hence, it is true that RDTs can achieve more than 94% Sensitivity and Specificity to malaria parasites stated by Ibrahim *et al.* (2014). Currently, the PCR technique is the most sensitive and specific method of malaria diagnosis.

According to the work done by Leann *et al.* (2016) which stated that the ATP6 gene is important in the *Plasmodium falciparum* parasite as it codes for an important calcium transporter. This serves as a protein target for the Artemisinin group of drugs. A mutation in the *PFATP6* gene can lead to an increase or a decrease in their susceptibility to Artemisinin and its derivatives. Thirteen [13 (65%)] out of 20 samples that were subjected to molecular amplification using Polymerase Chain Reaction technique were positive for the *PFATP6* gene. Despite the presence of the *PFATP6* genes in the study isolates, specific mutations that could confer resistance to Artemisinins were not further investigated.

4.2 Conclusion

This study was able to show validity in our methods of diagnosis and rule out clinical diagnosis in the management of malaria. Our RDTs have increased sensitivity as well as specificity to their different targets. Despite its effectiveness, the resistance to Artemisinins continues to be on the increase and has been linked to genetic mutations of key genes such as *PFATP6*. Malaria endemic countries such as Nigeria

should seek out preventive measures to reduce the incidence of this dreaded infection.

References

[1] Abba K., Kirkham A., Olliano P. L., Deeks J., Donegan S., Garner., Yemisi T. (2014). Rapid Diagnostic Tests for Diagnosing Uncomplicated Non - falciparum or *Plasmodium vivax* Malaria in Endemic Countries". *Cochiane Database of Systematic Reviews.*

[2] Caraballo H. (2014). Emerging Department Management Of Mosquito-borne Illness: Malaria, Dengue, and West Nile Virus. *Emerging Medicine Practice.* 16 (**5**).

[3] Cecile D., Pascal S., Lydia M., Elodie H., Sylvie P., Denis F., Ahmed A., Alexander W., Julie B., Nicodeme W., Casimir D., Ludovic A., Andre B., Ambaliou S., and Ermanno C. (2014) The ears of the African elephant: unexpected high seroprevalence of Plasmodium ovale and Plasmodium malariae in healthy populations in Western Africa. *Malaria Journal* **13**:240

[4] Christina T., Noella U., Stephen R., Emil I., Jean P., Jean M., Corine K., and Donald J. (2017) False-negative malaria rapid diagnostic tests in Rwanda: impact of *Plasmodium falciparum* isolates lacking hrp2 and declining malaria transmission. *Malaria Journal*. **16**: 123

[5] Eric A. A., Tobias O. A., Judith K. A., Regina N. M., Andre N., and Clarisse N. Y. (2012). Severe and uncomplicated falciparum malaria in children from three regions and three ethnic groups in Cameroon: prospective study. *Malaria Journal* **11**: 215

[6] Feng L., Richard C., Meihua Z., Abhinay R., Lorenz VS. (2017). Emerging of Indigenous Artemisin-Resistant *Plamodium falciparum* In Africa". *The New England Journal of Medicine*. **376**:991-993.

[7] Ibrahim S., Jerome B., Babatunde I., Akinjide O., Bridget O., Olanrewaju O., Samuel A. O., Emmanuel A., and Joseph O. E. (2014) Reliability of Rapid Diagnostic Tests in the diagnosis of malaria amongst children in two communities in South West Nigeria. *Malaria Journal* **13**: 10

[8] Kesinee C., Kamolrat S., and Nicholas P. (2007). Laboratory diagnosis of malaria infection- A short review of methods. *Australian Journal of Medical Science* 27 (**1**): 11-15

[9] Lalremuruata A., Ball M., Bianucci R., Welte B., Neilich A. G., Ken J. E., Pusch C. M. (2013). "Molecular Identification of Falciparum Malaria and Human Tuberculosis Co-infections in Mummies from the Faywin Depression (Lower Egypt)". *PLOS ONE.* 8 (**4**):60307.

[10] Leann T., Judith S., Nina F., Stuart A., and David A. (2016). Artemisinin action and resistance in *Plasmodium falciparum. Trends in Parasitology* 32(**9**): 682-696.

[11] Rosauro V., Valerie M. C., Antonio S., Lola M., Lena S., Kevin C. K., and Quique B. (2018). Adjunctive therapy for severe malaria: a review and critical appraisal. Malaria Journal **17**:47

[12] Samina N., Madiha S., Gul S., Kashif S., Arshi N., and Tahir S. (2017). Laboratory Diagnosis of Malaria: Comparison of Manual and Automated Diagnostic Tests. *Canadian Journal of Infectious Diseases and Medical Microbiology*

[13] Tatfeng Y. M. and Dennis E. A (2010). Age-related cytokine profile in uncomplicated Plasmodium malaria infection. Colombia Medica, 41(4): 323-327.

[14] Wilson M. L. (2012). "Malaria Rapid Diagnostic Tests". *Clinical Infectious Diseases.* 54 (**11**):1637-1641.

[15] World Health Organization (2017). World Malaria Report. Geneva.