Available online at www.aexpbio.com

ISSN : 2348-1935

RESEARCH ARTICLE

Annals of Experimental Biology
2015, 3 (2):15-20

Detection of PfATPase-6 and PFTCTP genes of *Plasmodium Falciparum* in patients receiving artemisinin combination therapy In Kano, Nigeria

D. B Maikaje[1] , Y. A. Umar[1], Y. Sadiq[2]*, S. S. Usman[3] F. Sarkinfada[4] and B. A Darma[5]

[1] *Department of Biological Sciences, Nigerian Defence Academy Kaduna, Nigeria. P.M.B. 2109*
[2]*Department of Pharmacy Technician, School of Health Technology Kano, Nigeria P.O.BOX 11549*
[3]*Jordan University of Science and Technology, Department of Biochemistry and Molecular Biology.*
[4]*Department of Medical Microbiology and Parasitology, Bayero University, Kano, Nigeria. P.M.B 3011*
[5]*Department of Obstetrics and Gynaecology Murtala Muhammad Specialist Hospital Kano, Nigeria*
*Correspondence: yahayasadiq3@gmail.com

(Received: 22/04/15) *(Accepted:16/07/15)*

ABSTRACT

Phusion blood direct Polymerase Chain Reaction (PCR) Kit was used to detect P. falciparum adenosine triphosphatase-6 (PfATPase-6) and P. falciparum translationally controlled tumor suppressor protein (PfTCTP) genes of the malaria parasite for artemisinins in 20 treated patients with Artemisinin Combination Therapy (ACT). Amplification was observed in at least 1 gene locus in 80% of blood sample assayed for PCR. The gene coding for the enzyme targeted by Artemisinin which is a drug used in the treatment of Malaria was detected in the blood of treated patients by Polymerase Chain Reaction. Amplification was observed in at least 1 gene locus in 80% of blood sample assayed. Successful amplification in patients in whom there was P. falciparum parasitemia after full treatment with ACT was detected in the gene loci PfATPase-6,1 (35%), PfATPase-6,2 (15%); and PfATPase-6,3 (60%). No amplicons were detected in PfATPase-6,4 and PfTCTP genes.

Key words: Polymerase Chain Reaction (PCR), *Plasmodium falciparum* and Artemisinin Combination Therapy (ACT).

INTRODUCTION

Plasmodium falciparum is one of the 5 species of *Plasmodium* parasite that causes malaria in human [1]. The current recommended treatment for malaria is Artemisinin and its derivative [2] in combination with another drug in a fixed dose combination therapy, referred to as Artemisinin Combination Therapy (ACT). In the mode of action of artemisinin, studies had demonstrated the involvement of the enzyme *P. falciparum* adenosine triphosphatase-6 (PfATPase-6) encoded by *P. falciparum* adenosine triphosphatase-6 (PfATPase-6) gene [3] and *P. falciparum* translationally controlled tumor suppressor protein (PfTCTP) which is encoded by *P. falciparum* translationally controlled tumor suppressor protein (PfTCTP) gene [3]. The aim of this study was to detect PfATPase-6 and PfTCTP genes in patients' blood that were receiving treatment with an ACT using Phusion blood direct PCR Kit.

MATERIALS AND METHODS

Study Area
Three Primary Health Care (PHC) centres in Kano State - Nigeria were selected for the study: **Gwagwarwa** PH**C** in Nassarawa Local government Area (L.G.A.), **Rurum** PHC centre in Rano L.G.A and **Sani Marshall Memorial hospital** in Kura L.G.A located in the urban, rural and semi-urban areas of the State respectively

Sample size:
A total of 122 patients were used in the research based on Slovin's formular (1960).

Inclusion criteria: Any malaria patient that attended the PHC centres with clinical symptoms of malaria like fever, headache, chills, loss of appetite, vomiting and general body malaise was selected for the study.

Exclusion criteria: Patients that attended the PHC centres with no fever or any symptoms of malaria were excluded from the study. For example pregnant women for antenatal care, Patients for wound incision and dressing, follow up hypertensive and diabetic patients etc.

Study Design:
Blood samples were collected from suspected malaria patients and analyzed for malaria parasite (MP) using microscopy. Four different Artemisinin-based Combination Antimalarials comprising *Artemeter-Lumefantrin, artesunate, Artemether and Dihydroartemisinin + Piperaquine* were used for therapeutic interventions on patients who tested positive for malaria parasite. In order to maintain uninterrupted supply chains of the drugs used in this study, the project supplied 3 out of 4 drugs administered to the patients free of charge while *Artemether-Lumefantrine* was provided to the patient by the malaria control programme of the state.

The malaria positive patients were treated using one of the so far mentioned artemisinins on the first visit to the health facility (Day 0). The patients were re-tested after completing 3 days dose of the drug. Patients who tested positive for malaria parasites at the second visit (first 3 days of treatment) and third visits (28/42 days of treatment) were given another regimen of ACT treatment accordingly.

Whole blood sample taken from each parasitemic patient that came back to the PHC centres after full treatment with ACT [6] and those with 3% parasitemia on first visit to the PHC centres were used in carrying out PCR for the detection of PfATPase-6 and *Pf*TCTP genes. This PHC based cross-sectional survey was carried out from March to April 2014.

Ehical Consideration:
Ethical approval to carry out this study was obtained from Medical Research Committee, Hospital Management Board Kano; and Kano State PHC Management Board. The participant/parent has signed an informed consent form that was ethically approved by the Kano State Medical Research Committee.

Sample Collection and Handling
One milliliter (1 ml) of patient's blood was withdrawn by venous puncture from a total of 20 participants with at least 2% parasitaemia on first, second and third visits respectively and transferred to vacutainer tube containing 0.2 ml citrate-phosphate-dextrose-adenine (CPD-A) buffer [5] and then kept in cold boxes and later taken to Biotechnology laboratory for PCR.

Molecular Determination of PfATPase-6 and PfTCTP genes using PCR
The blood samples were tested in two batches of 10 blood sample per batch. Each blood sample was tested with all the primers (Table 1) for the two genes (PfATPase-6 and PfTCTP) independently [that is, 4 primers of PfATPase-6 + 1 primer of PfTCTP, making a total of 5 primers]. The primer pairs were designed for genotyping the *Pf*TCTP *gene* [GenBank:DQ141561] and coding sequence of *pfATPase6gene*[Gen-Bank:EF564342],)**[5].**

Table 1: The primer sequences and the PCR reaction conditions for the amplification of *Pf*ATPase-6 and *Pf*TCTP genes.

Gene	Primer sequence 5' ⇒3'	PCR Product size (bp)	PCR reaction conditions*
***Pf*ATPase-6** atp6-1F	Tcatctaccgctattgtatgtgg	777	98°C 5' 1 cycle 98°C 1"62°C 5" followed by 40 cycles (98°C 1"; 62°C 5"; 72°C 30"); 72°C 1' 4°C hold
atp6-1R	Attcctcttagcaccactcct		"
atp6-2F	Tcaccaaggggtatcaacaa	692	"
atp6-2R	Tggcataatctaattgctcttcc		"
atp6-3F	Atgtatagctgttgtaatcaacctaga	822	
atp6-3R	Tcactatatggatcagcttcatca		
atp6-4F	Ccagtacattgaatgaaaatg	605	"
atp6-4R	Acgtggtggatcaataatacct		
Pftctp			
pftctp-1F	Atgaaagtatttaaagacgtt	462	"
pftctp-1R	Ttcttctcctttataataagaat		"

**Is obtained using Thermo Scientific Phusion Blood Direct PCR Kit, as a modification of Bethel et al., 2011.*

The research used Phusion blood Direct Kit for amplifying the PfATPase-6 and PfTCTP genes. The Pipetting instruction of each component of the PCR Phusion blood Direct Kit and its Cycling protocol was described hereunder.

Different 0.2 ml PCR tubes were labeled 1-50. Five 2 ml Eppendorf tubes were also labeled 1-5. All tubes containing PCR components (Table 2) were carefully mixed by shaking for 30 seconds using an electric shaker to ensure homogeneity of the contents of each reagent.

An aliqoute of 10 µl of "Reverse and Forward PfATPase-6,1, PfATPase-6,2, PfATPase-6,3 and PfATPase-6,4 Primers" were respectively added to the labeled 1-4 Eppendorf tubes above; and also 10µl of PfTCTP Reverse and Forward primers were added to the 5th one**.** Likewise the other components of the PCR Master Mix (Table 2) were all added to each of the Eppendorf tubes 1-5.

Table 2: The Composition of PCR reaction mixture for the amplification of *Pf*ATPase-6 and *Pf*TCTP genes.

Component content of each PCR reaction tube (x1) in µL	Content of each eppendorf tubes 1-5 for 10 blood samples (x10) [Batch 1] in µL	
2x Phusion blood PCR buffer (includes dNTPs ∞$MgCl_2$)	25	250
Forward Primer	1	10
Reverse Primer	1	10
Phusion blood II DNA Polymerase	1	10
50mM $MgCl_2$	1.5	15
50mM EDTA	2.0	20
DMSO	2.5	25
H_2O	13.5	135
Whole blood	2.5	.....
Total reaction volume	**50 µL**	**225 µL**

The PCR Master Mix was set up at room temperature (25^{o}C). 47.5µl was pipetted from each eppendorf tubes 1-5 into 50 labeled 0.2ml PCR tube with each set of 5 PCR tube consisting of different primers for testing 1 blood sample (Appendix 1). To each PCR tube, 2.5 µl of a test blood sample was added to make 50 µl mixture (Table 2).These were then carefully mixed by vortexing for 3 second and then transferred into the thermocycler (9700 Applied Biosystems). The PCR reaction run and cycling protocols is presented in Table 3.

Table 3: PCR cycling protocol* designed for the primers used in the amplification of the *Pf*ATPase-6 and *Pf*TCTP genes.

STEP*	Temp.(^{o}C)	Time	No. of Cycles
Cell lyses	98^{0}C	5 min	1
Denaturation	98^{0}C	1s	
Annealing**	62^{0}C*	5s	40
Extention	72^{0}C	30s	
Final extention	72^{0}C	1min	
	4^{0}C	Hold	1

** 3-Step protocol was selected because of the low-Tm primer pairs*

***Primer annealing temperature was obtained using Tm Calculator and instructions on website: www.thermoscientific .com/pcrwebtools.*

At the end of the amplification cycles, the PCR tubes were removed and centrifuged at 1000 × g for 3 minutes in a refrigerated centrifuge. 15µl of the DNA from each tube was withdrawn and run in an agarose gel electrophoresis.

Agarose gel electrophoresis:
Two percent (2%) Agarose gel used for this study was prepared by dissolving 2g of agarose powder in 100 ml of 1× TBE buffer**.** Two drops of ethidium bromide were added to the prepared 2% agarose gel and casted on electrophoretic tray. A drop of loading dye was spotted on fifty (50) different places of cleaned dried Glass. The amplified DNA solution from each PCR tube was pipetted, mixed with one loading dye on the glass and finally loaded on the electrophoretic tray. New pipette tips were used for each DNA sample. The loading process was repeated for all the amplified DNA samples in the PCR tubes. The DNA marker of 1000 base pairs was loaded in the first well of the agarose gel. The electroporetic tank was then connected to an electric power source (90v) and allowed to run for 30 minutes.

After the completion of the electrophoresis, the gel was removed and placed in Bio-rad Gel Doc^{tm} XR^{+} connected to a computer for analysis. The location of each amplicon on the gel plate was documented.

RESULTS

Plates of Agarose Gel electroporesis.
The typical agarose gel pictures of the bands of PfATPase-6 and PfTCTP genes amplified in this research were presented in Plate 1. The Plate have 2 rows and the 1st well in all the plate consist of the 1000 base pairs (bp) DNA Ladder.

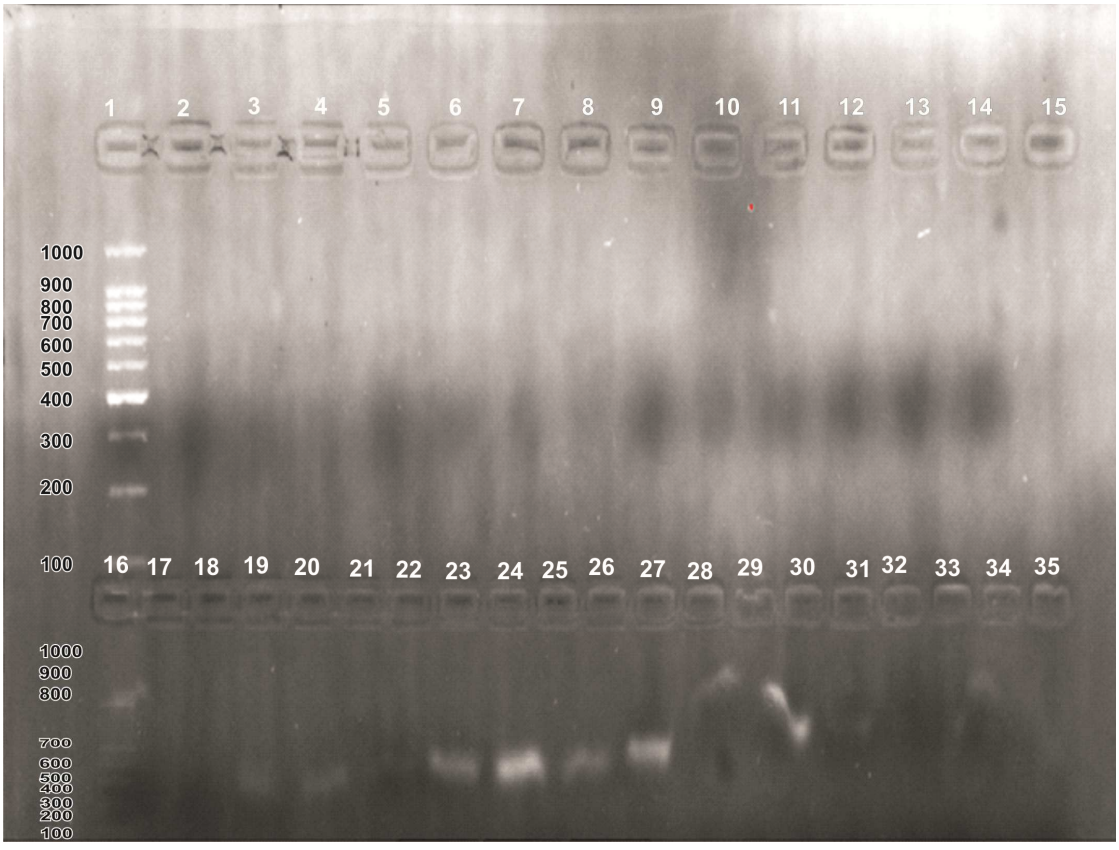

Plate 1: Agarose gel picture showing bands of *Pf*ATPase-6 genes detected in relation to DNA ladder.

The first row in the Plate has 15 wells and no bands of PfATPase-6 and TCTP genes analyzed were observed. Whereby, the second row consists 20 wells making a total of 35 wells (15+20) for the whole Plate. The wells containing bands of PfATPase-6 gene in relation to the DNA ladder in this row are number: 19, 20 (440 bp), 23, 24, 25 and 26 (605 bp), 27 (692bp) 28 (822 bp) 29 (777 bp) and 30 (692 bp) (Plate 1a).

Amplified gene locus and the type of ACT administered to the patients.

Out of the 20 blood samples that were run for PCR, seven (35%) were successfully amplified for PfATPase-6,1, three (15%) for PfATPase-6,2 and twelve (60%) for PfATPase-6,3. No amplicons were detected in PfATPase-6,4 and PfTCTP genes (Table 4). Amplification was observed in at least 1 gene locus in 80% of blood sample assayed for PCR. Amplification of any of the 5 gene loci is not detected in 20% of these patients. The results of the amplified gene locus and number of patients treated with each of the ACT and the type of ACT administered was presented in the table 4.

Table 4: **Amplicons detected in each gene locus and the ACT administered.**

DETAIL		GENE LOCUS INVOLVED IN THE AMPLICATION				
		PfATPase-6,1	**PfATPase-6,2**	**PfATPase-6,3**	**PfATPase-6,4**	**PfTCTP**
NUMBER OF BLOOD SAMPLES INVOLVED IN THE AMPLIFICATION.	GW	3	3	6	0	0
	KR	1	0	3	0	0
	RN	3	0	3	0	0
	Total	**7**	**3**	**12**	**0**	**0**
TYPE OF ACT ADMINISTERED	**A-L**	3	1	5	**AL**	**AL**
	DP	1	0	4	**DP**	**DP**
	ASNT	1	1	2	**ASNT**	**ASNT**
	ATM Inj.	2	1	1	**ATM Inj.**	**ATM Inj.**

GW= Gwagwarwa Primary Health Centre Kano Metropolis (urban),KR= Sani Marshall memorial Hospital in Kura, Kura L.G.A (Semi-Urban) and RN= Rurum PHC centre in Rano L.G.A.- (Rural area).

The samples of blood from each of the 3 study areas, the type of ACT administered to each patient, genes that were successfully amplified in the research work and Position of Primers/PCR tubes loaded on the gel, primers that shows bands of genes; and the band size were illustrated in Figure 1.

Figure 1: Samples of blood from each of the 3 study areas, the type of ACT administered, position of Primers/PCR tubes loaded on the gel and primers that shows bands of genes.

BLOOD SAMPLE	TYPE OF ACT ADMINISTERED TO THE PATIENT	PRIMERS / PCR TUBES				
		PfATPase-6,1	**PfATPase-6,2**	**PfATPase-6,3**	**PfATPase-6,4**	**PfTCTP**
1	**A-L**	1*	11*	**21***	31	41
2	**D-P**	2*	12*	**22***	32	42
3	**A-L**	3*	13	**23***	33	43
4	**D-P**	**4**	**14***	**24***	34	44
5	**A-L**	**5**	**15***	**25***	35	45
6	**D-P**	**6**	**16***	**26***	36	46
7	**ARTESUNATE**		**17***	**27***	37	47
8	**ATMT INJ**	**8***	**18***	**28***	38	48
9	**ATMT INJ**	**9***	19	29	39	49
10	**D-P**	**10***	20	30	40	50
11	**A-L**	**51****	**61****	**71****	81	91
12	**A-L**	**52****	62	**72****	82	92
13	**D-P**	**53****	63	**73****	83	93
14	**ARTESUNATE**	54	64	**74****	84	94
15	**ATMT INJ**	55	65	75	85	95
16	**ARTESUNATE**	56	66	76	86	96
17	**A-L**	**57****	67	77	87	97
18	**ARTESUNATE**	**58****	68	78	88	98
19	**ATMT INJ.**	**59****	69	79	89	99
20	**ATMT INJ.**	**60****	70	80	90	100

***Are primers that shows bands of PfTPase-6 genes. **Yellow** = Sample from Rano L.G.A, **Red** = Sample from Nassarawa L.G.A, **Purple** = Sample from Kura L.G.A.*

DISCUSSION

These loci detected and amplified by PCR, especially in patients in whom there was *P. falciparum* parasitemia after full treatment with **ACT** may suggest that the parasite may generate the enzyme (*Pf*ATPase-6) which is not being inhibited by the drug or it generates the enzyme from other/extra source(s) apart from the loci quoted in this research. This is probably because the artemisinins used may be adulterated or fake as it was reported by Johnbull *et al*., (2013) [7] that counterfeit antimalarial drugs circulate in Nigeria. On the other hand, unsuccessful amplification of the locus *Pf*ATPase-6,4 and *Pf*TCTP gene may indicate that, the two genes were either not detected due to technical error, or due to the presence of mutation in the DNA template. Therefore, this non detection of the genes in parasitemic patients indicates that; the target of the drug (the enzyme) is not present; hence, resistant strains of *P. falciparum* might emerge. This claim can only be confirmed by DNA sequencing of the amplicons detected. Some of fragments are of the correct sizes and tally with that of Bethel *et al.,* (2011).

In contrast to many literatures such as Bethel *et al*., (2011) that used conventional method of PCR which starts with DNA extraction prior to amplification to detect PfATPase-6 and PfTCTP genes, the current research used direct PCR kit and detect these gene loci *Pf*ATPase-6,1, *Pf*ATPase-6,2, and *Pf*ATPase-6,3.

CONCLUSION

The gene coding for the enzyme targeted by Artemisinin which is a drug used in the treatment of Malaria was detected in the blood of treated patients by Polymerase Chain Reaction. Amplification was observed in at least 1 gene locus in 80% of blood sample assayed. Successful amplification in patients in whom there was *P. falciparum* parasitemia after full treatment with ACT was detected in the gene loci *Pf*ATPase-6,1 (35%), *Pf*ATPase-6,2 (15%); and *Pf*ATPase-6,3 (60%). No amplicons were detected in PfATPase-6,4 and PfTCTP genes.

Acknowledgement
The authors appreciate Y.Y. Pala of the Department of Vetenary Public Health, Ahmadu Bello University (A.B.U) Zaria-Nigeria; for his immense support.

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