International Journal of Medicine and Biomedical Research
Volume 5 Issue 1 January – April 2016
www.ijmbr.com

Original Article Open Access

Molecular screening of S769N mutation for *PF*ATPASE in subjects with plasmodiasis in Owo

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Received: 12.08.15; Accepted: 08.03.16; Published: 09.03.16

ABSTRACT

Background: The emergence and spread of drug-resistant *Plasmodium falciparum* is a major problem of public health concern. Due to the high incidence of parasite resistance to antimalarial drugs, artemisinin-based combination therapies (ACTs) have now replaced those failing drugs in order to combat the growing drug resistance. **Aim**: This study is undertaken to detect resistant strains of *P. falciparum* to artemisinin in infected human blood in Owo. **Methods**: A total of 1000 participants took part in the study. *Falciparum* malaria was confirmed by microscopic examination of Giemsa-stained blood samples from patients who presented with fever at Federal Medical Centre, Owo, Ondo state, Nigeria. Molecular methods were employed to detect a marker of resistance of *P. falciparum* to artemisinins: Parasite DNA was extracted from patient blood using Tris-EDTA buffer-based extraction method. Amplification was carried out by Nested Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphisms (PCR/RFLP) for the detection of *falciparum* sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) *PfATPase*6 gene mutation. **Results**: The result showed that resistant *PfATPase*6 gene which codes for artemisinin resistance was not present in the population studied. **Conclusion**: Since there was no resistant gene detected in the population studied, it could be said that artemisinin resistance has not yet developed in this area and subsequent monitoring of resistance pattern to artemisinin based therapy is advised in order to detect and prevent its spread.

Key words: Artemisinin, pfATPase, falciparum malaria, S769N mutation, polymorphism, drug resistance

INTRODUCTION

Malaria generally occurs in tropical and subtropical areas, it is commonly associated with poverty and represents a major burden to economic and social development.[1] The World Health Organization (WHO) estimates that there are over 200 million cases of malaria each year with 80% of cases and 90% of deaths estimated to occur in the African region.[1]

Effective malaria control is hampered by increasing resistance of malaria to the available drugs.[2] However, resistance to currently available anti- malarial drugs has seriously reduced the effectiveness of the

drugs.[3] In 2006, the World Health Organization officially recommended that Artemisinin (ART)-based combination therapies (ACTs) be adopted as first-line treatment of uncomplicated malaria caused by *Plasmodium falciparum*.[4] This recommendation came in response to the global spread of resistance to the former first-line anti-malarial Chloroquine (CQ) and Sulfadoxine–Pyrimethamine (SP).[5] Following increased CQ and SP resistance, artemisinin and its derivatives gradually became the mainstay of *falciparum* malaria therapy.[5] Besides, previous experiences with other anti-malarial drugs showed that resistance appears after long-term use.[5]

Reports of the reduced Artemisinin sensitivity of *P. falciparum* parasites in known foci in Cambodia and Thailand and new suspected foci in Myanmar and Vietnam may suggest a decline in ACT efficiency.[6,7] Previous work discovered a large region in one of the parasite's chromosomes that was linked to resistance, by looking for genetic variants in the parasite associated with their response to artemisinin.[8] Therefore, due to the essential role of ACT in malaria control and elimination, it is crucial to have a regular surveillance of resistance to determine whether resistance to artemisinin has developed or not [8].

Since the first disclosure of artemisinin to the rest of the world in 1979, several more potent derivatives have been synthesized, including Arthemether, Artemotil, and Artesunate.[9] These artemisinin derivatives possess several important pharmacological characteristics, including rapid onset of action, short half-life, activity against the broadest range of stages in the life cycle of the malaria parasite and an excellent safety profile.[9] Importantly, Artemisinin derivatives can kill gametocytes, the sexual stage of parasites, in the human circulation system.[10] Artemisinins have been widely used in parts of China since their initial discovery in the 1970s.[11] In endemic regions in Southeast Asia, Artemisinins have been deployed in the form of ACTs since the 1990s.[11] Meanwhile, in many endemic regions of Africa, the introduction of ACTs was delayed until the mid-2000s due to financial challenges, a lack of political will, and logistical problems in ACT implementation.[11] Since then, ACTs have been widely adopted in most endemic regions, along with other control measures and the use of ACTs has undoubtedly contributed to a substantial reduction in malaria burden.[11]

Molecular markers are recommended as the earliest technique to detect emerging drug resistance, therefore the identification and monitoring of genes and mutations, which correlate with resistance to artemisinin and its derivatives are essential for the evaluation and monitoring of ACT.[12]

ACTs have become the standard for treatment of malaria in sub-Saharan Africa. All ACTs combine a short-acting artemisinin with a long acting partner drug, and continued success of these regimens depends on activity of both component drugs. Prolonged circulation of artemisinin partner drugs suggests that selection of resistance to these agents may occur readily.[13] Thus, the need for regular and comprehensive surveillance including molecular tests, of resistance is a high priority.[13] Only limited information on clinical resistance to artemisinin has been reported in Nigeria. Therefore, as ACT become widely used in sub-Saharan Africa, predicting the emergence and spread of resistance is necessary for planning malaria control and instituting strategies that might delay the emergence of resistance.[14] This study is undertaken to detect resistant strains of *P. falciparum* to artemisinin in infected human blood in Owo.

METHODOLOGY

Study area

The study was conducted at Federal Medical Centre, Owo. Owo is a town in Ondo state, situated at the South-Western Nigeria, latitude 7.1962^{o} and longitude 5.58681^{o} at an elevation/altitude of about 400 meters. It is at the southern edge of the Yoruba hills, and at the intersection of roads from Akure, Kabba, Benin City and Siluko.[15]

Malaria is present throughout the year with a marked increase during the raining season (that is April to October). Approval for the study was obtained from Federal Medical Centre, Owo and ethical clearance (FMC/OW/380/VOL.XXV/59) was issued by the Ethical and Research Committee. Suspected malaria carriers attending Federal Medical Centre, Owo were screened for *P. falciparum* parasitaemia. The initial screening for *P. falciparum* was conducted using microscopic observation of thin and thick Giemsa-stained blood films and those who tested positive for *P. falciparum* were recruited in the study. Samples were analysed at Institute of Advance Medical Research and Training, Ibadan.

Blood sample collection procedure

Blood samples were collected from 1000 malaria individuals who have been treated with Artimisinin in the last 48 months at Federal Medical Centre, Owo. No age restriction was applied. After confirmation of *P. falciparum* infection with different parasite densities, by microscopic observation of thin and thick Giemsa-stained blood films, 1 ml of venous blood was collected into vacutainer tubes containing EDTA, a sub-sample of this was spotted onto Whatman No 3 filter paper.[16] The filters were air dried and packed in sealed plastic bags and stored at 4^{o}C until further analysis.[16] Patients with severe disease, co infection, infection with other malaria species, anti malarial-drug use within 48 hours before enrollment, or known allergies to ACT were excluded. The protocol was reviewed and approved by the Ethical and Research Committee, Federal Medical Centre, Owo and all participants provided informed consent.

Sample size determination

One hundred thousand samples were studied. The sample size was determined using the formula $N = 4pq/l^2$; where N= sample size; P = prevalence from previous study q = 1-p and l = permissible error (5% of p)

Extraction of parasite DNA

Tris-Edta buffer-based extraction

Tris-EDTA buffer, composed of 10 mM Tris, pH 8.0 (Tris-base plus Tris-HCl) and 0.1 Mm.[17] EDTA in distilled water, was prepared and kept at room temperature. Punches of same dimension (4 mm in diameter) were taken from individual dried blood spots (four punches from each spot) using a sterile biopsy punch (Kai Industries Co., Ltd., Oyana Seki City, Japan).[17] Each filter paper punch was placed in an eppendorf (Hamburg, Germany) tube, soaked in 65 µL of TE buffer, and incubated at 50°C for 15 minutes.[17] The punches were than pressed gently at the bottom of the tube several times, using a new pipette tip for each punch and heated at 97°C for 15 minutes to elute the DNA. The DNA extract was taken into eppendorf tubes and was kept at 4°C for use within a few hours.[7,8]

Polymerase Chain Reaction

The fragment of *PfATPase6* gene was amplified by nested PCR with specific oligonucleotide primers, followed by two separate amplifications. The final concentration of the PCR mixture for the nest 1 or primary amplification was 1× PCR buffer, 2mM $MgCl_2$, 125 µM dNTP, 25 nM of each primer (F/R), and 1X dream *Taq* green DNA polymerase. One microliters of DNA template was added to a reaction volume of 19µL.[19] The nest 2 or secondary amplifications was done in a 20µL reaction volume containing 1× PCR buffer, 1mM MgCl2, 125µM dNTP, 125 nM of each primer (F/R), and 1X dream *Taq* green DNA polymerase.[26] The product of the first amplification was used as the template for the second PCR (1µL/reaction). For primary amplification, the primers were designed using primer 3 plus software and are FW-Ope1 5'-GGA AGA GGT TAT TAA GAA TGC-3' and RV-Ope2 5'-GCT TCA ACA TTT CCT TCA TC-3'. For secondary PCR amplification the primers are FW-Ope3 5'-TAT TAG ATA TGA AAC ATA AAA ATC-3 and RV-Ope4 5'-GGA GTT TTA TTA CCA ACA CTC AAT TCA-3.[19] Secondary PCR products were digested at 37 ^{o} C by XbaI restriction enzymes- Thermo fisher: as recommended by the manufacturer.[8,13] The cycling profile consisted of initial denaturation at 98^{o} C for 5 minutes followed by 35 cycles at 98^{o} C for 1 second, 59.6^{o} C for 5 seconds and 72^{o} C for 15 seconds and a final extension at 72^{o} C for 1 minute using ABI 9700. The PCR products were subjected to electrophoresis on 2% Bio-Rad gel (Bio-Rad document system, Rockland), stained with ethidium bromide, and visualized by ultraviolet transillumination. Restriction digests were loaded in 15µl volumes per lane. Band sizes were measured using Syngene gel imaging analysis software.

RESULTS

Of the total 1000 participants recruited for the study, 64% were female and 36% male (figure 1). Participants were between 1-75 years (table1). Participants were on different antimalaria drugs; 40% were on artesunate, 26% and 9% on coartem and lonart respectively. Twenty five percent were however treatment naive before sampling (figure 2).

Parasites DNA extracted from blood spot on FTA CARDS using Tris-EDTA based buffer extraction method were amplified with primers specific for PfATPase6 gene. The first round of amplification resulted produced a 285bp amplicon and the secondary amplification produced an amplicon of 147bp. All the 1000 (100%) isolates carried the wild type allele, which shows that there are no S769N mutations for *Pf*ATPase6 gene in this region presently.

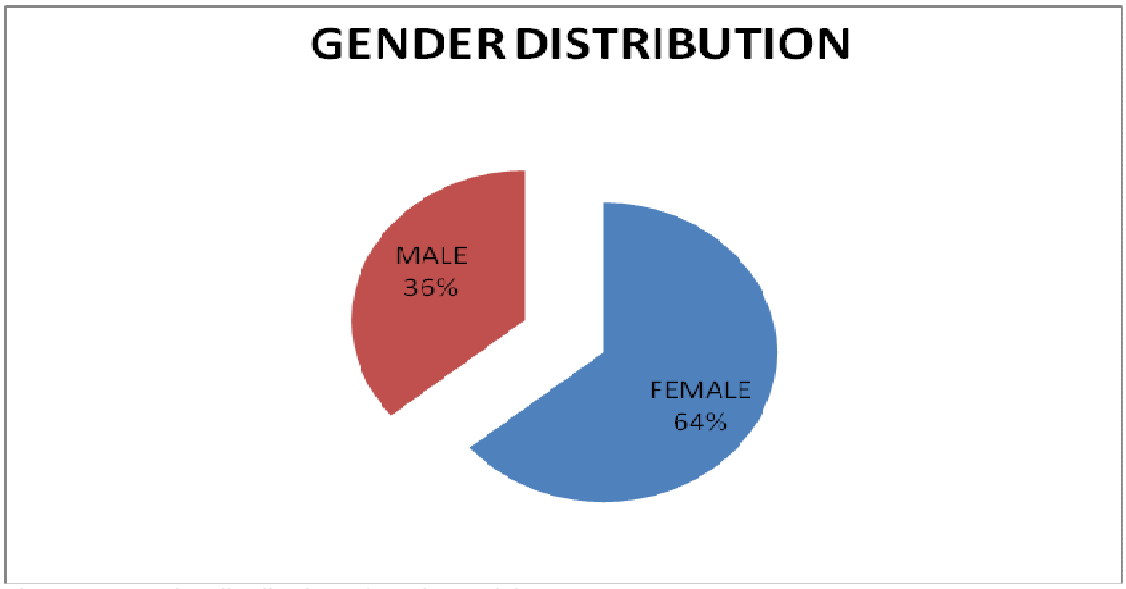

Figure 1: Gender distribution of study participants

Table 1: Age distribution of the study participants

Age	No (%)
1 – 15	254 (25.4)
16 – 30	233 (23.3)
31 – 45	221 (22.1)
46 – 60	151 (15.1)
61–75	141 (14.1)

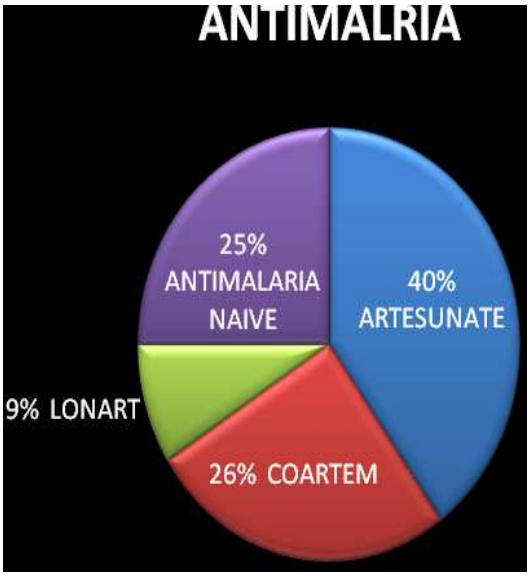

Figure 2: Category of antimalaria regimen used by study participants

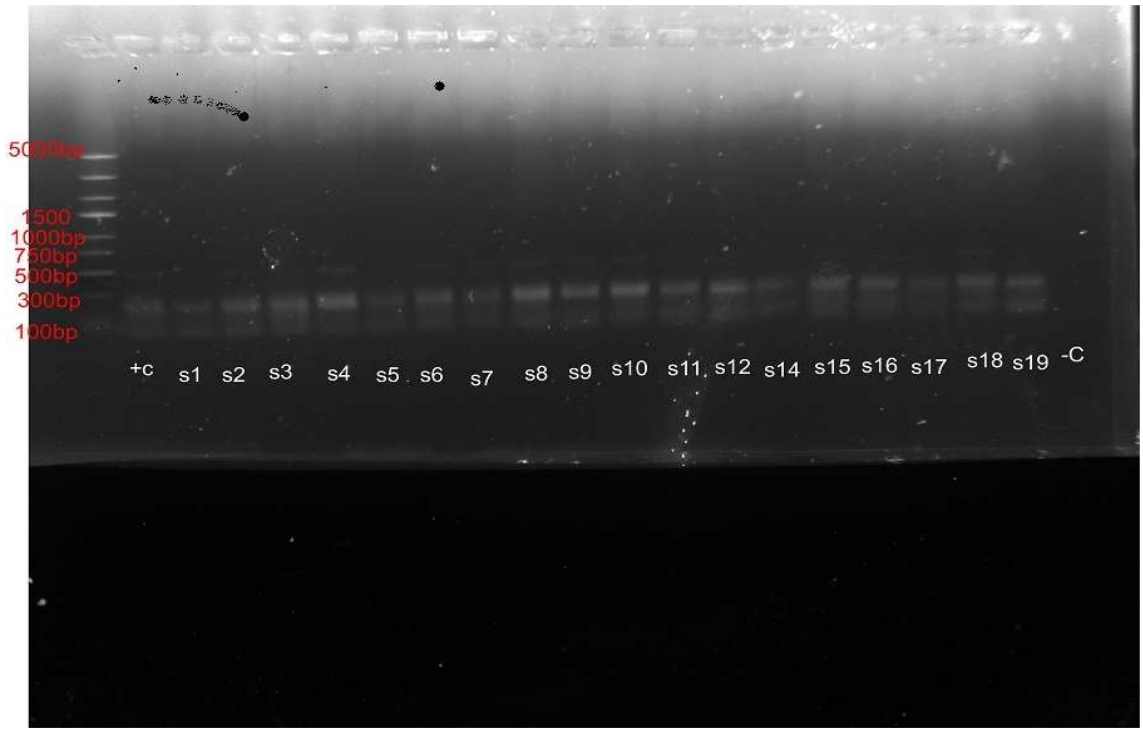

Figure 3: DNA bands of *falciparum* sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) *PfATPase*6 gene in primary amplification. +C – positive control, -C – negative control, S - sample

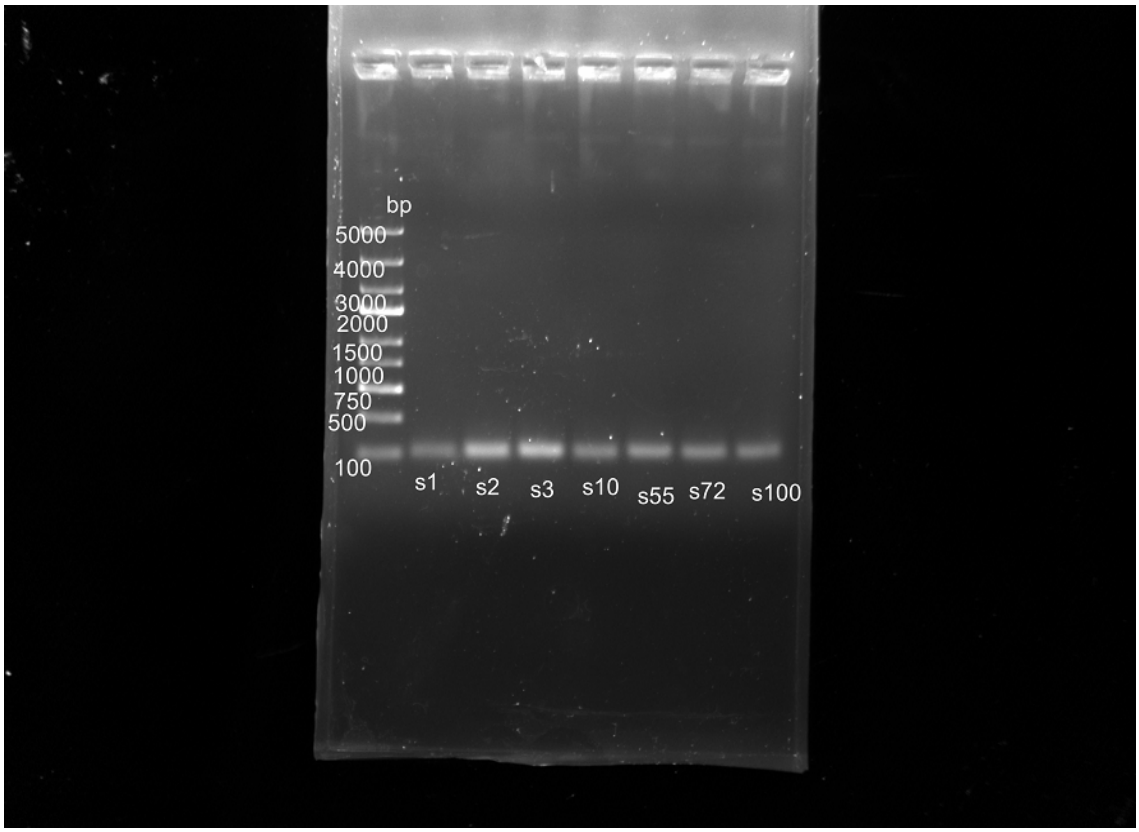

Figure 4: DNA bands of *PfATPase*6 gene in the secondary amplification

DISCUSSION

Anti-malarial drug resistance is the single most important threat to global malaria control. Understanding artemisinin molecular basis is thus essential for determining treatment strategies, mapping the spread of resistance and guiding elimination.[20,21] Mutations or amplification of genes encoding transporters or target enzymes have been identified as resistance mechanisms to other anti-malarial drugs.[18] It is possible that resistance to Artemisinin is unlike these classical mechanisms *in vitro* but instead results from a complex series of genetic and epigenetic events affecting multiple pathways.[17] *Plasmodium falciparum*, which causes the most life-threatening malaria syndromes, has developed resistance to almost every class of anti-malarial compounds.[1] Strong evidence has shown that resistance to artemisinin may depend on single nucleotide polymorphisms in the drug's putative chemotherapeutic target known as SERCA type ATPase protein.[26] It has been shown that residues S769N, L263E, E431K and A623E are associated with resistance to artemisinins. A total of one hundred percent samples that were positive for *P. falciparum* by microscopy were subjected to PCR genotyping for the *PfATPase* codon S769N mutation. All the 1000 (100%) isolates carried the artemisinin sensitive wild type allele, S769. This observation is similar to those documented in previous studies[23,24] in several African countries where artemisinin and its derivatives are used as first line of treatment of uncomplicated malaria. This study is also in accordance with findings from Kefas and colleagues who reported no S769N or A623E in 1205 subjects studied in Tanzania, similarly the findings from a Kenyan study conducted by Laura *et al.* in 2011.[22,25,26] Observed drug failure in most previous studies could have been due to re-infections and not resistance to ACTs.[27,28]

CONCLUSION

Since there was no artemisinin resistant gene detected in this study, it was therefore concluded that the drug still remains effective antimalaria in this area. As there are no confirmed molecular markers of resistance to ACTs, continuous analysis of malaria parasites resistance to drugs offers the best means of tracking emerging resistance to ACTs. Educational campaigns should be targeted to further create awareness on the severity of the infection, to train communities on how to prevent malaria, and to inform on the importance of early diagnostic and compliance of treatment. Also, further screening of clinical samples from endemic areas for other genes that could confer resistance to ACTs and active surveillance of clinical response to ACT therapy will help establish the levels of efficacy of ACTs.

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doi: http://dx.doi.org/10.14194/ijmbr.5.1.4
How to cite this article: Hassan A.O, Ademujumi O.G and Popoola A.O. Molecular screening of S769N mutation for *PF*ATPASE in subjects with plasmodiasis in Owo. Int J Med Biomed Res 2016;5(1):28-34

Conflict of Interest: None declared