Molecular screening of S769N mutation for PFATPASE in subjects with plasmodiasis in Owo

Hassan A.O, Ademujumi O.G and Popoola A.O*

Department of Medical Laboratory Sciences, Achievers University, Owo, Ondo State, Nigeria

*Corresponding author: popoolaolayiwola@gmail.com

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) PfATPase6 gene mutation. Results: The result showed that resistant PfATPase6 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
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° and longitude 5.58681° 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 Artisinin 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. The filters were air dried and packed in sealed plastic bags and stored at 4°C for use within a few hours.

The filters were then pressed gently at the bottom of the tube several times, using a new pipette tip for each punch and heated at 97°C for 5 minutes. The punches were then 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 a 15μL reaction volume containing 1× PCR buffer, 1mM MgCl2, 125μM dNTP, 125 mM 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. The nest 2 or secondary amplifications was done in a 20μL reaction volume containing 1× PCR buffer, 1mM MgCl2, 125μM dNTP, 125 mM 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'-GGG 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 TCA CAA TTA GAC TCA TC-3'.

The filters were then 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 a 15μL reaction volume containing 1× PCR buffer, 1mM MgCl2, 125μM dNTP, 125 mM 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. The nest 2 or secondary amplifications was done in a 20μL reaction volume containing 1× PCR buffer, 1mM MgCl2, 125μM dNTP, 125 mM of each primer (F/R), and 1X dream Taq green DNA polymerase. The final concentration of the PCR mixture for the nest 1 or primary amplification was 1× PCR buffer, 2mM MgCl2, 125 μM dNTP, 25 mM 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. The nest 2 or secondary amplifications was done in a 20μL reaction volume containing 1× PCR buffer, 1mM MgCl2, 125μM dNTP, 125 mM of each primer (F/R), and 1X dream Taq green DNA polymerase. 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 PfATPase6 gene in this region presently.

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 PfATPase6 gene in this region presently.

**Sample size determination**

One hundred thousand samples were studied. The sample size was determined using the formula N = 4pq/l², 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, 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). 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. The punches were then 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 an eppendorf tubes and heated at 97°C for 15 minutes to elute the DNA.

**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 MgCl2, 125 μM dNTP, 25 mM 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. The nest 2 or secondary amplifications was done in a 20μL reaction volume containing 1× PCR buffer, 1mM MgCl2, 125μM dNTP, 125 mM of each primer (F/R), and 1X dream Taq green DNA polymerase. 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'-GGG 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 TCA CAA TTA GAC TCA TC-3'.

Secondary PCR products were digested at 37°C by XbaI restriction enzymes- Thermo fisher: as recommended by the manufacturer.[8,13] The cycling profile consisted of initial denaturation at 98°C for 5 minutes followed by 35 cycles at 98°C for 1 second, 59.6°C for 5 seconds and 72°C for 15 seconds and a final extension at 72°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 PfATPase6 gene in this region presently.

**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 PfATPase6 gene in this region presently.
Table 1: Age distribution of the study participants

<table>
<thead>
<tr>
<th>Age</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 15</td>
<td>254 (25.4)</td>
</tr>
<tr>
<td>16 – 30</td>
<td>233 (23.3)</td>
</tr>
<tr>
<td>31 – 45</td>
<td>221 (22.1)</td>
</tr>
<tr>
<td>46 – 60</td>
<td>151 (15.1)</td>
</tr>
<tr>
<td>61–75</td>
<td>141 (14.1)</td>
</tr>
</tbody>
</table>

Figure 2: Category of antimalaria regimen used by study participants
Figure 3: DNA bands of *falciparum* sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) *PIATPase6* gene in primary amplification. +C – positive control, -C – negative control, S - sample

Figure 4: DNA bands of *PIATPase6* 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 \textit{in vitro} but instead results from a complex series of genetic and epigenetic events affecting multiple pathways.\[17\] \textit{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 \textit{P. falciparum} by microscopy were subjected to PCR genotyping for the \textit{PFATPase} codon S769N mutation. All the 100 (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 \textit{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.

REFERENCES


Submit your valuable manuscripts to Michael Joanna Publications for:

- User-friendly online submission
- Rigorous, constructive and unbiased peer-review
- No space constraints or colour figure charges
- Immediate publication on acceptance
- Unlimited readership
- Inclusion in AJOL, CAS, DOAJ, and Google Scholar

Submit your manuscript at www.michaeljoanna.com/journals.php


Conflict of Interest: None declared
Submit your next manuscript to any of our journals that is the best fit for your research.

International Journal of Medicine and Biomedical Research
Scope: UMBR publishes cutting edge studies in medical sciences
Editor-in-Chief: Sofola A. Olusoga, MBBS, PhD, FAS
Deputy Editor: Lehr J. Eric, MD, PhD, FRCSC
URL: www.ijmbr.com
E-mail: editor@ijmbr.com
Pissn: 2277-0941, eISSN: 2315-5019

International Journal of Ethnomedicine and Pharmacognosy
Scope: UEP publishes novel findings on the use of complementary and alternative medicine in the management of diseases
Editor-in-Chief: Dickson A. Rita, B.Pharm, GCAP, PhD, MPSGh, MCPA
Deputy Editor: Kuete V., PhD
URL: www.ijepharm.com
E-mail: editor@ijepharm.com
Pissn: 2437-1262, eISSN: 2437-1254

International Journal of Infectious and Tropical Diseases
Scope: UIJD publishes interesting findings on infectious and tropical diseases of public health importance
Editor-in-Chief: Yang Z., PhD
Deputy Editor: Liping L.P., MD, PhD
URL: www.ijitd.com
E-mail: editor@ijitd.com
Pissn: 2384-6607, eISSN: 2384-6585

Reasons to publish your manuscript with Michael Joanna Publications:
• User-friendly online submission • Rigorous, constructive and unbiased peer-review • No space constraints or coloured figure charges • Immediate publication on acceptance • Authors retain copyright • Inclusion in AJOL, CAS, CNKI, DOAJ, EBSCO, Google Scholar, and J-Gate • Unlimited and wide readership • Member of COPE and CrossRef

Editorial Director
Professor Sofola A. Olusoga,
Department of Physiology,
University of Lagos,
Nigeria.
Tel: +234(0) 7093848134
Email: enquiry@michaeljoanna.com
www.michaeljoanna.com