CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1STUDY AREA

This study was carried out in two communities - Badagry (Lagos State) and Alajue (Ede, Osun State), both located in South Western Nigeria (Fig. 3). Badagry ($6^0$25'N $2^0$53'E), is a coastal town with an area of 170m^2 and a human population of 241,093 (2006 census). It was founded on a Lagoon off the Gulf of Guinea with its protected harbour leading to the town. Badagry is also a border town with the Republic of Benin. The predominant occupation of the native Badagry people is fishing and agriculture [Coconut plantations were first established in the vicinity in the 1880s, and modern Badagry exports coconuts, copra, coir (coconut husk fibre), fish, vegetables, and cassava to Lagos]. Artemisinin-based combination therapy resistance reports has mostly been in border towns, this informed the choice of Badagry in this study

Ede ($7^0$40'N $4^0$30'E), is an ancient Yoruba town, located in the guinea savannah zone. It has a total area of 130m^2 and a human population of 159,866 (2006 census). The predominant occupation of the people of Ede (especially Alajue village) is farming. Alajue enjoys rainfall ranging from 45 to 55 inches annually.It is based on production of food crops, such as yam, cassava, cocoyam, potato, maize, guinea corn, cowpea and cash crops like cocoa, palm oil, kola-nut, coconut and varieties of fruits. Trading in textiles and imported goods is also common in the town others include carpentry, blacksmithing and welding.Alajue (control site) is an inland village in Ede, Osun state with much reduced human movement and high malaria report from the state malaria control program.

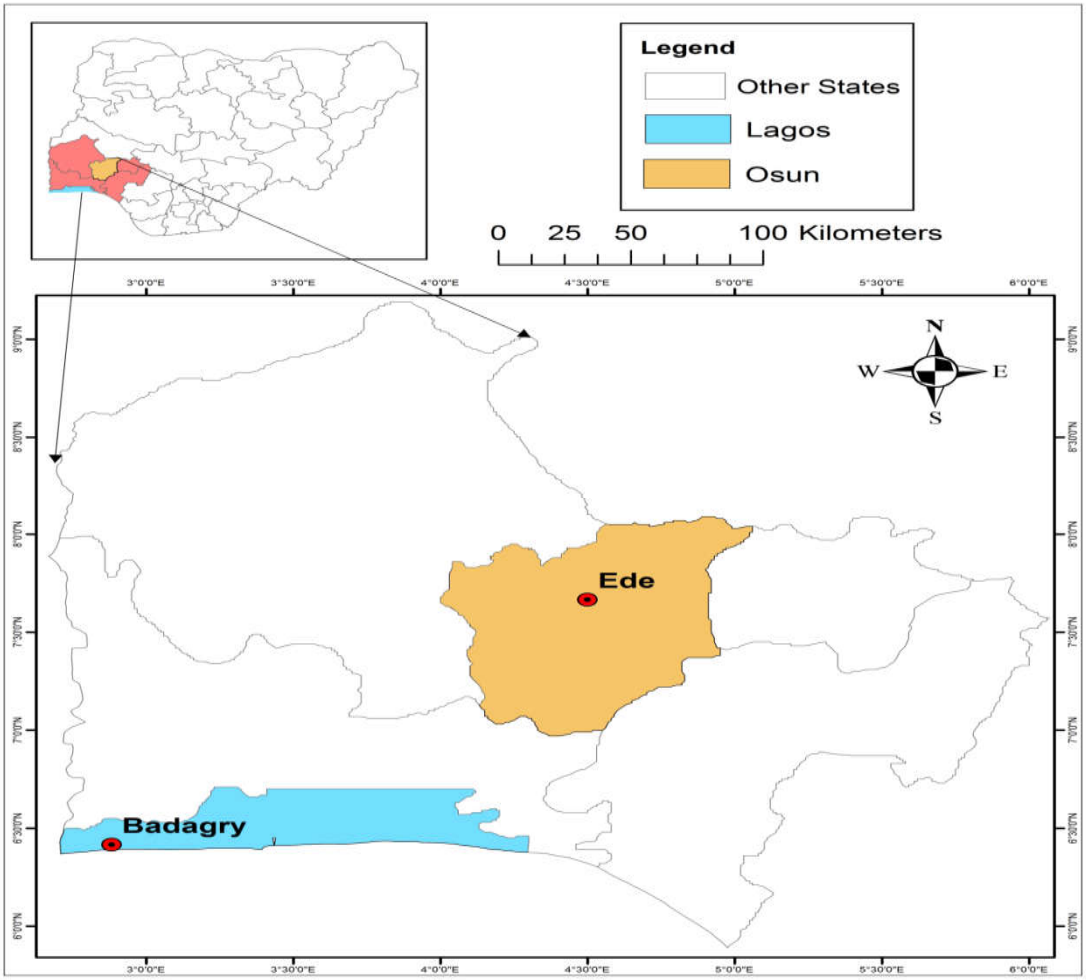

Figure 3: Map of study communities showing Lagos and Osun States

3.2 STUDY PARTICIPANTS /COLLECTION OF SAMPLES

A minimum of 50 patients were required for this study according to the WHO guideline (WHO, 2009), however a total of 83 patients were recruited in the course of this study and 33 patients lost due to follow-up. Recruitment of patients and follow-up at Badagry was done at Badagry general hospital while Alajue primary health centre was the site for Osun state. The consent of the participant, parent or guardian was first obtained; thereafter they were tested with malaria Rapid Diagnostic Test (RDT) kit.

Patients were eligible to participate in the study if they had symptoms compatible with acute uncomplicated malaria and levels of pure *P. falciparum*parasitemia of >2,000 asexual forms/μL, a body (axillary) temperature of >37.4°C or a history of fever in the 24 to 48 h preceding presentation, the absence of other concomitant illnesses and no history of antimalarial use in the 2 weeks preceding presentation. Patients with severe malaria, severe malnutrition, serious underlying diseases (renal, cardiac, or hepatic diseases), and known allergies to the study drugs were excluded from the study.

ArtemetherLumefantrine (LOKMAL by Emzor Pharmaceuticals Ltd, Nigeria) was given orally to patients who tested positive to malaria parasite by RDT; each tablet of AL contains 20 mg of artemether and 120 mg of lumefantrine. Patients' blood samples (5ml) were collected for malaria parasite examination, full blood count and liver function test. Samples were also collected into RNAlater and on 3MM Whatman filter paper (which was air-dried). This was done on day 0 only (before ACT treatment). Follow-up with clinical and parasitological evaluations was done daily on days 1 to 3 and then on days 7, 14, 21 and 28 (Fig. 4). Blood was spotted on filter papers on days 0, 3, 7, 14, 21 and 28 and at the time of treatment failures for parasite genotyping.

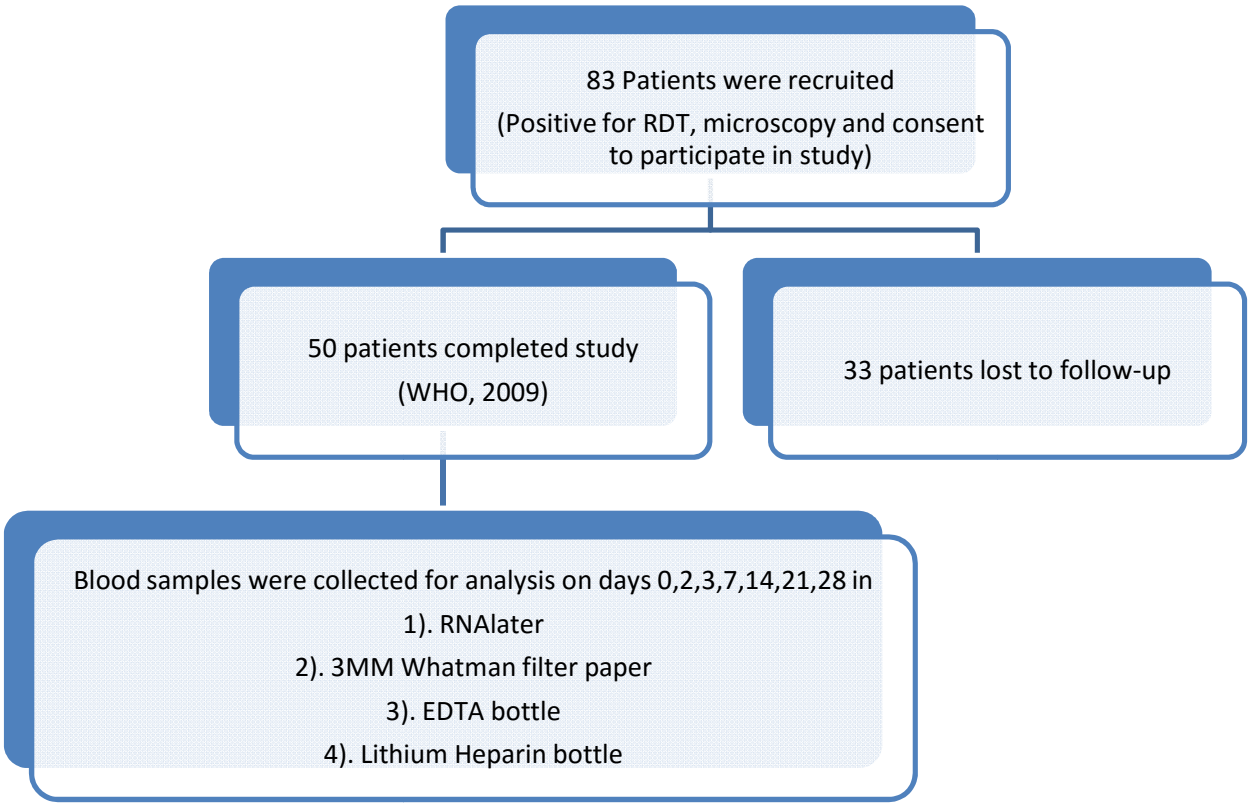

Figure 4: Flow chat of sample collection

3.3 KNOWLEDGE, ATTITUDE AND PRACTICE ABOUT ANTIMALARIAL DRUG

A descriptive cross sectional survey was carried out to assess antimalarial drug preference among people in the selected communities. A semi-structured questionnaire was used for data collection (Appendix 1). The sample size (n) was determined by using the formula developed by Cochran in 1963 and the total sample size required for the study was 135 individuals after adding a 20% contingency.

$n = Z\alpha^2pq/d^2$

where n = the desired sample size, Z = the standard normal deviate usually set at 1.96, which corresponds to 95% confidence interval, α = level of significance, set at 0.05, p = the proportion in the target population estimated to have a particular characteristic. 50% can be used if there is no reasonable estimate. q = 1.0 – p; d = degree of accurancy desired, set at 0.05.

Inclusion criteria for study were those that gave their consent to be part of the study, who have had malaria or are currently diagnosed of malaria and had used (or currently using) antimalarial drugs for treatment and, aged 2 years and above. Informed consent form was given to the respondent before filling the questionnaire (Appendix 2).

3.4 PARASITOLOGICAL EXAMINATION

The thick and thin films (blood spots) were prepared and allowed to dry. The thin film was then fixed with methanol. The slide was allowed to dry before staining with 10% Giesma solution. This was left for 10 minutes, rinsed off with water, allowed to dry and examined microscopically using X100 oil immersion objective lens.

3.5 BLOOD ANALYSIS

3.5.1 Full Blood Count (FBC)

Mindray BC 3200 haematologyautoanalyzer was used for Full Blood Count (FBC). The machine was switched on and allowed to initialize.Whole blood was mixed in EDTA vercutina bottle for a minute and then inserted into the machine. An aspiration button was used to suck the sample into the autoanalyzer via capillary action. Results were automatically displayed and printed out.

3.5.2 Liver Function Test (LFT)

Vital Scientific Selectra Junior chemistry autoanalyzer was used for the LFTs namely Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP). . The machine was switched on and allowed to boot. When standby was displayed, all reagents were then placed on the board in the machine. On sample position 1, put distilled water on cuvette to blank machine and samples to be analyzed are placed in other positions. On the system, sample handling is double clicked to select the test to be carried out (AST, ALT and ALP) and then start measurement is clicked. The results were displayed automatically and printed out.

3.6 MOLECULAR ASSAYS

3.6.1 Expression Studies

3.6.1.1RNA Extraction

PureLink^{TM} RNA Mini Kit (Invitrogen, San Diego, CA, United States of America) that uses enzymatic lysis and silica membrane extraction was used. Total RNA was isolated from whole blood samples stored in RNAlater following the manufacturer's protocol with minor modifications (Appendix 3).

3.6.1.2 DNase treatment

Genomic DNA was digested with DNase I (Fermentas, Ontario, Canada). Briefly, 2µl of DNase I and 5µl of reaction buffer were added to the RNA sample and incubated at 37^0C for 30 minutes. Inactivationof the DNase I enzyme require 5µl of 25 mM EDTA added to the mixture and incubated at 65^0C for 10 minutes. The concentration and purity of the total RNA was spectrometrically determined using a NanoDrop 1000TM (Thermo Scientific).

3.6.1.3 cDNA Synthesis

Invitrogen RNA Reverse Transcriptase kit was used for cDNA synthesis (Appendix 4). The manufacturer's instructions were followed and the extracted cDNA synthesized were quantified using NanoDrop 1000 Spectrophotometer.

3.6.1.4 Gene expression quantification

The synthesized cDNA was quantified using qPCR method. qPCR run was performed on a CFX 96 (Bio-Rad) with the following cyclic parameter: 95^0C for 10 minutes, 49 cycles of 95^0C for 15 seconds and 60^0C for 90 seconds. qPCR products were analyzed by melting curves for unspecific products or primer dimer formation. Relative fold increase of specific mRNA transcripts in samples comparing with parasite cultures (3D7), was calculated using $2^{-\Delta\Delta Ct}$ method, (Livak and

Schmittgen, 2001), where 2 stands for the 100% reaction efficiency (the reaction efficiency was determined experimentally and thus 100% efficiency was replaced by the real efficiency) and ΔCt =Ct (housekeeping gene)-Ct (target gene). The data analysis was based on at least 3 independent experiments.

3.6.2 SEQUENCING ASSAY

3.6.2.1 DNA Extraction

Genomic DNA was extracted from filter papers blood spots. Briefly, a disc of the filter paper blood spot (approximately 6 mm in diameter) was punched out using a methanol-flamed puncher and placed in 1.5ml centrifuge tubes using clean and flamed forceps. Jena Bioscience DNA extraction kit was used for extraction of DNA and manufacturer's instructions were followed in the extraction processwith minor modifications (Appendix 5). DNA extract was finally eluted using 50µl elution buffer and kept at 20°C until used. *Plasmodium* species were identified using genus-specific and species-specific primers in nested PCR based on small subunit ribosomal RNA (18s rRNA) gene. The extracted DNA was quantified using NanoDrop 1000 Spectrophotometer.

3.6.2.2 PCR Amplification

The extracted gDNA were used for amplification of 4 different genes (K13, *pfATPase*6, *pfcrt* and *pfmdr* 1), and the amplified genes were then sequenced.

The K13 and *pfATPase*6 genes were amplified using conventional PCR method. Amplification was done in a 15µl reaction mix using 7.5µl Q5 master mix (New England BiolabInc), 0.75µl of primer (10µM), 4.5µl of nuclease free water and 1.5µl of template. The following cyclic

parameters were used: 98^0C initial denaturation for 10 minutes, 35 cycles of 98^0C for 30 seconds, 60^0C for 30 seconds and 72^0C for 1 minute, final extension at 72^0C for 2 minutes 30 seconds. 6µl of product was taken for electrophoresis on 1.0% agarose gel (40 minutes at 100volts) stained with ethidium bromide and then visualised with Ultraviolet light. Positive samples were selected for sequencing(Appendix 6 and 7).

The *pfcrt* and *pfmdr*1 genes were amplified using nested PCR method. Amplification was done in a 15µl reaction mix using 1.5µl 10X thermopol reaction buffer, 0.25µl of dNTPs (10mM), 0.5µl of primer (10µM), 11.8µl of nuclease free water, 0.15µl of Taq DNA Polymerase (Thermo Fisher Scientific) and 1.0µl of template. The following cyclic parameters were used: 94^0C initial denaturation for 3 minutes, 30 cycles of 94^0C for 30 seconds, 60^0C for 30 seconds and 72^0C for 1 minute, final extension at 72^0C for 5 minutes. One µl of the PCR product was re-amplified with specified primers, using the following program: 3 minutes at 94^0C 30 cycles of 94^0C for 30 seconds, 56^0C for 30 seconds and 65^0C for 1 minute, final extension at 65^0C for 5 minutes. 6µl of product was taken for electrophoresis and positive samples were selected for sequencing(Appendix 8 and 9).

3.6.2.3 DNA sequencing

All PCR amplifications generating a single product were incubated with ExoSAP-ITR (USB, Cleveland, OH, USA) to remove excess primers and nucleotides. The cleaning was done by adding a 10µl reaction containing EXO (1µl), SAP (1µl), and nuclease free water (8µl) to 10µl PCR product. The cyclic condition is 37^0C for 1hour 15minutes and 80^0C for 20minutes.

EXO-SAP cleaning is followed by the addition of 7.5µl of sequencing mix containing 5X sequencing buffer (2µl), Big-Dye (0.5µl), 10mM Primer (0.64µl), nuclease free water (4.36µl) and 2.5µl EXO-SAP PCR product. A step sequencing cyclic condition(Appendix 10) is used for amplification.

Filtration of sequenced reaction product is done using G-50 microtiter plate. The G-50 powder is loaded on the plate, 300µl of distilled water is added and it is allowed to sit for 2hours. The plate is then centrifuged at 2300rpm for 5minutes and washed twice with 150µl of distilled water.

Sequenced reaction product, 11µl, is added to the G-50 plate and centrifuged at 2300rpm for 5minutes. The filtrate is placed on PCR machine and run at 90^0C for 20minutes with the machine lid open for complete evaporation of the solvent. To the dry tubes, 10µl of formamide is added, vortexed, purse centrifuged and placed in a thermal cycler at 90^0C for 2minutes.

An electronic plate plan is created and uploaded to the Applied Biosystems 313xl Genetic Analyzer with the samples for sequencing.This was done at the Molecular Diagnostic Laboratory of The Medical Research Council, The Gambia.

3.7 ETHICAL APPROVAL

Ethical approval for this study, (1RB/13/218,Appendix 11)was from the Institutional Review Board of the Nigerian Institute of Medical Research, Yaba, Lagos

3.8 DATA ANALYSIS

Descriptive statistics and one way ANOVA on IBM SPSS Statistics Version 20.0, descriptive statistics on Microsoft Excel 2010, sequence alignment on CLC Main Workbench Version 6.7.1

and translation of nucleotide sequence to amino acid sequences and editing of sequences(MEGA 7.0.4)were used in the analyses of data. A P-value of ≤ 0.05 was considered statistically significant in all comparison.