IN-VITRO AND MOLECULAR STUDIES ON THE RESISTANCE OF *P. falciparum* TO ANTIMALARIAL DRUGS IN OGUN STATE, SOUTHWESTERN NIGERIA

BY

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FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY

(Ph.D) IN MICROBIOLOGY

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CERTIFICATION

We certify that this is an original research study by OLASEHINDE Grace Iyabo, for the award of Ph.D in Microbiology in the Department of Biological Sciences, Covenant University, Ota, Nigeria.

Dr. Olusola Ojurongbe (Supervisor)	Signature……..	Date……
Prof. L. O. Egwari (Co-Supervisor)	Signature…….	Date……
Dr. E. O. Fagade (Co-Supervisor)	Signature……	Date……..

DECLARATION

We declare that this thesis entitled "*IN-VITRO* AND MOLECULAR STUDIES ON THE RESISTANCE OF *P. falciparum* TO ANTIMALARIAL DRUGS IN OGUN STATE, SOUTHWESTERN NIGERIA" which is an original work of Olasehinde, Grace Iyabo (Matriculation Number CUGP050156) has been examined and found to have met the requirements of the Covenant University for the award of The degree of Doctor of Philosophy (Ph.D). We therefore recommend the work for the award of the Ph.D. degree in Microbiology.

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DEDICATION

This Ph.D work is dedicated to The Almighty God, The giver of all knowledge and wisdom.

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Grace

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ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
*pf*ATPase	*P. falciparum* Adenosine Triphosphatase 6 genes
SERCA	Sarco/endoplasmic reticulum calcium-dependent
DELI	Double-site Enzyme-linked Lactate dehydrogenase Immunodetection
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HEPES	*N*-(2-hydroxyethyl)piperazine-*N´*-(2-ethanesulfonic acid)
HPLC	High-performance liquid chromatography
HRP II	Histidine-rich protein II
IC50	50% inhibitory concentration
LDH	Lactate dehydrogenase
MIC	Minimal inhibitory concentration
NAD	Nicotinamide adenine dinucleotide
PABA	P*ara*-aminobenzoic acid
PCR	Polymerase chain reaction
Pfcrt	*P. falciparum* *C*hloroquine resistance transporter gene
PCR	Polymerase chain reaction
*pfmdr*1	*P. falciparum* multidrug resistance gene 1
RPMI	Roswell Park Memorial Institute
TDR	Special Programme for Research and Training in Tropical Diseases
Tween 80	polyoxyethylenesorbitan monooleate
vs	versus
WHO	World Health Organization
DMSO	Dimethyl sulphoxide
MSP1	Merozoite Surface Protein 1
MSP2	Merozoite Surface Protein 2
GLURP	Glutarmate Rich Protein
QT-NASBA	Quantitative Nucleic Acid Sequence Based Amplification
BSA	Bovine Serum Albumin
WBC	White blood cell(s)
TCM	Tissue Culture Medium

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ABSTRACT

The widespread of drug resistant *Plasmodium falciparum* has led to a rise in malaria-associated mortality most especially in sub-Saharan Africa. *In-vitro* and molecular studies were carried out in order to determine the resistant pattern of *P. falciparum* to antimalarial drugs and some local antimalarial herbs in Ogun State, Southwestern Nigeria. Prevalence of *falciparum* malaria was determined by microscopic examination of Giemsa-stained blood samples of patients who presented with fever in selected State Hospitals in Ogun State. Antimalarial drug sensitivity of one hundred (100) *P. falciparum* isolates to chloroquine, amodiaquine, mefloquine, quinine, sulphadoxine/pyrimethamine, artesunate and three local antimalarial herbs: *Momordica charantia (Ejirin,) Diospyros monbuttensis (Eegun eja)* and *Morinda lucida (Oruwo)* was determined using the *in-vitro* microtest (Mark III) technique. For molecular studies and genotyping, DNA was extracted from patient blood using the QiaAmp DNA Blood Minikit extraction method. Nested Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphisms (PCR/RFLP) were used for the detection of *P. falciparum* chloroquine resistance transporter (*Pfcrt*), *P. falciparum* multidrug resistance 1 (*pfmdr1*), *P. falciparum* dihydrofolate reductase (*Pfdhfr*), *P. falciparum* dihydropteroate synthase (*Pfdhps*) and *P. falciparum* sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) *PfATPase*6 genes. Genetic diversity of the isolates was determined using merozoite surface proteins 1 and 2 (msp1 and msp2) and Glutamate rich Protein (Glurp). Structured Questionnaires were administered to patients or/and parents of infants to determine the factors that could lead to the development of drug resistance by the parasite in the study population. Out of 4066 subjects screened during the period of study, 2550 (61.1%) were positive. Highest prevalence (72%) was recorded in children 1-5 years while the same group also had the highest parasitaemia of 1080. All the isolates tested were sensitive to Quinine, Mefloquine and Artesunate**.** Only 51% of the isolates were resistant to chloroquine, 13% to amodiaquine and 5% to sulphadoxine pyrimethamine respectively. Highest resistance to chloroquine (68.9%) was recorded among isolates from Yewa zone while highest resistance to amodiaquine (30%) was observed in Ijebu zone. Highest resistance to sulphadoxine and pyrimethamine was recorded in Yewa and Egba zones respectively. A significant positive correlation was observed between the responses to artemisinin and mefloquine (P=0.001), artemisinin and quinine (P=0.05), Quinine and mefloquine (P= 0.01). A significant negative correlation was observed between the responses to chloroquine and mefloquine (P=0.05). For the local herbs highest

antiplasmodial activity was obtained with the ethanolic extract of *Diospyros monbuttensis* (IC_{50} = 32 µg/ml). *P. falciparum* isolates analyzed during this study have demonstrated highly diverse nature of field isolates in respect of *msp-1* (block 2) and *msp-2* (central repeat region, block3). All the three reported families of *msp-1*(K1, MAD20 and RO33) and two of *msp-2* (FC27 and 3D7) were observed among the isolates. Proportion of isolates with K1 family was 68% with 4 alleles in the range of 100 to 300 basepairs (bp). Proportion of isolates with MAD20 family was 40% and a total of 3 alleles were observed within 100 to 300 bp. RO33 proportion was 20% and the family was observed to be monomorphic with an allele size of 200 bp. In *msp-2* the proportion of FC27 family was 76% and that of 3D7 was 56%. Proportional Prevalence of FC27 and 3D7 families was significantly different ($\chi 2 = 16.5$, $P = 0.002$). Eighty percent of the isolates harbor the genes that code for Glutamate rich protein with size ranging between 700 and 900bp. *Pfcrt* (K76T) *Pfmdr1 (mdr 1) Pfdhfr (*S108N*)*, and *Pfdhps (K540E)* resistant genes were detected among the isolates while resistant SERCA*PfATPase*6 gene which codes for artemisinin resistance was not detected in the population. The questionnaire study showed that 24.6% of the patient visit hospitals for treatment, 12.0% use local healers while 25.0% buy antimalarial drugs without prescription. It was also observed that some use more than one method in their management of malaria. Those who combined antimalarial drugs with traditional medicine from local healers were found to be 17.4%. Only 18% of the sample population used Insecticide treated mosquito nets, 42.3% use window and door nets while 13% do not employ any mosquito preventive method. Continuous use of the current antimalarial drugs increases the chance of resistance developing to those drugs. Control of drug use and reducing exposure of parasites to the drugs are most effective where the parasite is still sensitive to the drug. Molecular methods are most effective for monitoring the spread of resistant strains of *P. falciparum*.