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ABSTRACT:

Malaria is a life-threatening disease caused by protozoan *Plasmodium* species. *Plasmodium falciparum* is the deadliest species. Reducing and eliminating malaria burden are linked to most of the Sustainable Development Goals (SDG), central to SDG3 targeting the end of malaria by 2030. This study was aimed at assessing the Management of malaria and prevalence of *P. falciparum kelch-13* among febrile patients in selected Government Hospitals in Nigeria. Malaria patients (399) attending outpatient clinics of the Hospitals between August, 2019 and January, 2021, were enlisted in the study, following ethical approval and informed consents. Blood (5mL) was collected from patients for microscopic and molecular investigation of malaria parasite. DNA extraction, PCR amplification, BLAST, and alignment were performed. *Plasmodium* resistance to Artemether/lumefantrine was determined by PCR amplification of extracted DNA using *Kelch-13* gene primer. Data obtained were subjected to One-way Analysis of Variance and Linear Regression. The VapA gene primer amplified 55 (68.75%) out of the 80 DNA extracts tested. Twenty-five strains of *P. falciparum* belonging to 3 clades phylogenetically were identified and they showed evolutionary relationships with others. *Plasmodium falciparum* resistant *Kelch-13* gene was detected in 70% of the isolates. This study observed a high prevalence of resistant gene to ACT drugs in the study area. Monitoring the effectiveness of ACTs must be done routinely to ensure timely changes in National treatment policies.

Keywords: *Febrile, Hospitals, kelch-13, Malaria, Molecular, Plasmodium falciparum, Resistant.*

INTRODUCTION:

Plasmodium falciparum is a malaria-causing protozoan parasite and it's one of the several species of *Plasmodium.* Infected female Anopheles mosquitoes, known as malaria vectors, transmit malaria parasites by biting their prey at night and in the early morning hours [1]. The parasite is classified into Kingdom - Protista, Phylum - Apicomplexa, Class - Aconoidasida, Order - Haemosporida, Family - Plasmodiidae, *Genus - Plasmodium and Species - falciparum* [2].

Almost 94% of all global malaria cases were reported in the WHO's African Region in 2019, which is estimated at 215 million cases. The consistent efforts and increased awareness of malaria control and prevention measures have resulted in a 29% reduction in malaria mortality rates worldwide between year 2000 and 2019 [3]. Malaria continues to be a major problem in Sub-Saharan Africa. Nearly all malaria deaths occurred in the sub-Saharan region, where 90% of cases and 92% of deaths occurred in 2015 [3]. There are certain groups of people who are more susceptible to malaria infection; immune-compromised individuals, such as those with HIV/AIDS, pregnant women, infants under the age of five, and immigrants are all included in this category. Most of the world's cases were concentrated in 29 countries, with Nigeria accounting for 27% of the total [3].

In spite of the fact that there were 204 million fewer malaria cases reported in the WHO African Region in 2000 than there were in 2019, the incidence of malaria decreased from 363 to 225 cases per 1000 population at risk during this time period, highlighting the difficulty in interpreting changing disease transmission in a rapidly growing population in the region [3]. The population of the WHO African Region grew from 665 million in 2000 to 1.1 billion in 2019. More than seventy-eight percent of this region's cases of malaria have decreased from about 18 cases per 1000 people at risk in 2000 to about four cases today [3]. From around 25 deaths per 100,000 people at risk in 2000 to 12 in 2015 and 10 in 2019, the global malaria fatality rate has been steadily declining [3]. As a whole, only 31 countries were responsible for 95% of the global malaria deaths [3].

Malaria deaths in 2019 were estimated to be 43% in Nigeria (23%), followed by the DRC (11%), Tanzania (5%) and Mozambique (4%). This year, Nigeria had the highest number of global malaria cases (27%), and deaths (23%), [3]. 33 million pregnancies were estimated in the WHO African Region in 2019, of which 36% (12 million) were malaria-infected during pregnancy [4]. East and Southern Africa had the lowest prevalence of malaria exposure during pregnancy (24%).

A key element in malaria prevention and treatment is the availability of effective antimalarial medications. Resistance to Artemisinin-based combination therapies, for example, puts at risk the global effort to lessen malaria's impact. As of now, all malaria-endemic countries recommend Artemisinin combination therapy (ACT), with Artesunate (injectable) as the main treatment for severe malaria.

There is a link between the prevalence of *Pf-Kelch13* genetic variations and Artemisinin resistance [5].

As PCR is far more accurate than microscopy and can detect 5 parasites/L of blood with 100% accuracy and equal specificity, it's the most important research technology at the moment [5]. For studying strain variation, mutations, and parasite genes involved in treatment resistance, PCR techniques are extremely useful [5]. Rapid real-time PCR (Real-time PCR, Quantitative Nucleic Acid Sequence Based Amplification, QTNASBA) techniques are emerging as high-throughput genotyping systems [5]. Multiple marker molecular research can provide insight into the emergence of drug resistance patterns in the field, which can be used to develop malaria control methods. Point mutations in the *P. falciparum* Chloroquine Resistant Transporter *(PfCRT)* and *PfMDR1* genes have been linked to Chloroquine resistance in *P. falciparum isolates, whereas dhfr and dhps have been linked to Sulfadoxine-Pyrimethamine resistance* [6, 7].

Many African countries now use ACTs to treat *P. falciparum* malaria, including Artemether/Lumefantrine and Amodiaquine/Artesunate [8]. Worldwide, *P. falciparum* (malaria parasite) treatment resistance is a serious public health concern [8, 9]. ART-resistant parasites were not linked to the five mutations in some regions, such as Africa [10]. Because of this, it serves as an example of the complexity of the mechanism of *P. falciparum* medication resistance [11]. The only current alternatives for lowering malaria morbidity and death, particularly in Africa, are chemoprophylaxis and chemotherapy in the absence of effective and practicable preventive interventions. As a result, the rising incidence of *P. falciparum* variants resistant to therapies poses significant problem to treating and controlling malaria. Phylogenetics studies the evolutionary relatedness among various groups of organisms through molecular sequencing data and morphological data matrices. In phylogenetics, DNA sequencing methods are used to analyze the observable heritable traits [12]. It also makes use of a phylogenetic tree which is a diagram to show the hypothetical evolutionary histories and relationships of groups of organisms based on the phylogenies of different biological species. The phylogenetic tree has been used to understand biodiversity, genetics, evolutions, and ecology of organisms [12].

This study assessed the prevalence of *Plasmodium falciparum* infection in the South-Western part of Nigeria, the predisposing factors to malaria occurrence, the Artemisinin-based combination therapy resistance in *P. falciparum*, and the molecular variations of *P. falciparum* in the area.

METHODOLOGY:

Study Area and Demographic Data Collection:

The study was carried out in selected Government Hospitals in Ondo and Ekiti States, Nigeria. Ondo State lies between longitudes 4"30" and 6" East of the Greenwich Meridian, 5"45" and 8" 15" North of the Equator. This means that the state lies entirely in the tropics while Ekiti State lays South of Kwara and Kogi State, East of Osun State and bounded by Ondo State in the East and in the South Ekiti State Ethical clearance and approval:

Prior to the commencement of the study, approval was obtained from Selected Government Hospitals, Ekiti State Teaching Hospital, Ado-Ekiti (EKSUTH/A76/2019/04/009), Federal Teaching Hospital, Ido-Ekiti **(**ERC/2018/08/02/131B) and Ondo State Ministry of Health (OSHREC/24/07/19/154). Written informed consent was obtained from each adult participant and the parent or guardian of each child examined.

enjoys tropical weather with two awesome seasons. These are the rainy season (April–October) and the dry season (November–March). This study was carried out from August 2019 to January 2021.

Study Population and Sample Size:

This was a cross sectional Hospital-based study. A total of 399 consented individuals were enlisted into this study. The sampled size was calculated using the following equation:

$n = t^2 \times p(1-p)/ m^2$
Where n = the required sample size, t = confidence level at 95% (standard value of 1.96),
p = estimated prevalence of the infection in the project area which is 71% [13]
m = margin of error at 5% (standard value of 0.05).

$n = 1.9622 \times 0.71(1-0.71)/ (0.05)^2$

$= 3.924 \times 0.2059/0.0025 = 323.180.$

Data Collection:

A structured questionnaire was administered to a total of 399 consented participants from the selected Hospitals in order to collect information on their age, sex, occupation, education, response, and management of malaria. The questionnaire was face validated, pretested, and tested for reliability before administration [13, 14].

Sample Collection and Examination**:**

With the assistance of a trained Medical Laboratory Technician, a sterile syringe was

used to collect 2.0mL of blood from each of the subjects. The blood was put into an EDTA bottle and used for thin and thick blood film preparations. Thin and thick smears of the samples were prepared on sterile slides and subsequently stained with Giemsa stain. The smears were viewed under an x100 objective lens of the light microscope to detect the presence of malaria parasites [13, 15].

Molecular characterization of *Plasmodium falciparum*:

The DNA were extracted using commercial kit (Norgen Biotek Corp, Canada) and the purified genomic DNA samples were then eluted into separate properly labeled microcentrifuge tubes and stored at 4^{o}C until required for analysis.

Plasmodium falciparum species specific primers, vacuolar proton adenosine triphosphate (vapA) gene (Table 1) was used to amplify the genomic DNA and was followed by sequencing, blast, alignment and phylogenetic tree analysis using MEGA - X [16].

Molecular Identification of Antimalarial Resistant Genes (Nested PCR):

The gene fragment of *Plasmodium falciparum kelch-13* resistance gene (*Pfk-13*- PCR- F- CGG AGT GAC CAA ATC TGG GA; PCR- R- GGG AAT CTG GTC GTA ACA GC) as shown in table 1 was amplified by nested PCR protocols reported previously [17]. The products of restriction digestion were separated by electrophoresis on a 2% agarose gel and detected by staining with ethidium bromide which were sized 1- kilo base pair (2000bp) molecular weight marker (New England Biolabs, Beverly, MA). The bands were identified at 2000bp for successful amplifications. The experiment was done at the (Bioscience Centre, International Institute of Tropical Agriculture, Ibadan, Nigeria).

Table 1: Primer Sequence

Primer Name	Primer Sequence	Amplicon size (bp)
Pfk-13- PCR F- PCR- R- [16]	5'- CGG AGT GAC CAA ATC TGG GA -3'; 3'- GGG AAT CTG GTC GTA ACA GC- 5'	2000
VapA (vacuolar adenosine trisphosphate) L08200.1- F-, R- Bunmi Obagaye, 2016- unpublished)	5'CTTCTTATTACGGAGCAAAATGACA 3' 3'CCACAACCAAATGCACCAGG 5'	750

Statistical Analysis:

The data were analyzed using IBM Statistical Product and Service Solutions (SPSS) version 23 which was used to determine the differences in prevalence of malaria by age, sex, occupation, education, frequency, response and management of malaria. The results were expressed as mean ± standard error of mean (SEM). Significant differences were established by the one-way analysis of variance (ANOVA). Mean values with $p<0.05$ were considered statistically significant.

A total of 80 samples were extracted and amplified using vacuolar Adenosine trisphosphate (vapA) gene primer. About 55 (68.75%) out of the amplified samples showing clear bands at 750bp were sequenced and analyzed for the molecular detection of *Plasmodium falciparum* as shown in Plate 1.

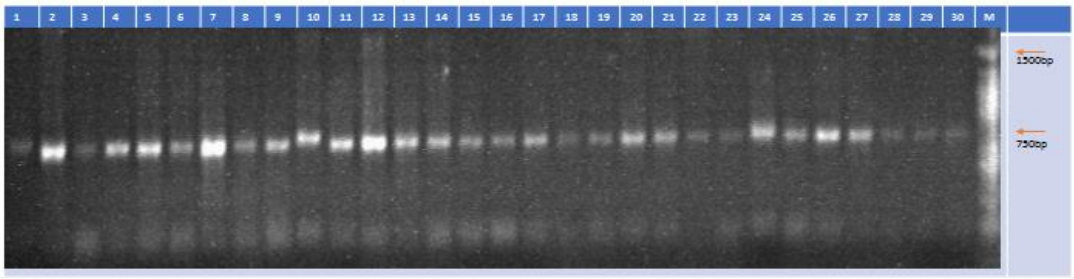

Plate 1: Amplified *P. falciparum* using vacuolar adenosine trisphosphate (vapA) gene
Key: M showing the ladder

equence Blast of Isolated *Plasmodium falciparum:*

Twenty- five (25) strains of *P. falciparum* were identified following sequence blast of isolates as shown in Table 2. Isolates 3, 11, 17, 18, 19 and 20 showed 100% homology to the nearest relatives compared with Isolates 10, 23a, 36, and 42 recorded 99% homology relationships. Isolates 13, 22, 26a, 28a, 34, 23b, 24, 25b, 26b, 27, 28b, 29 and 30 showed 98% homology while isolate 25a showed 97% relationship with existing isolate (Table 2).

Table 2: Nearest relatives of isolated *Plasmodium falciparum* following sequence blast

S/N	Isolate code	Nearest relatives	Accession no	Homology
1	6	*Plasmodium falciparum* Strain BAK5	DQ135202.1	100%
2	10	*P. falciparum* Strain BK71	KY337745.1	99%
3	13	*P. falciparum* Strain 0957	AM116507.1	98%
4	17	*P. falciparum* Strain BAK27	DQ135134.1	100%
5	20	*P. falciparum* Strain BK543	KY331903.1	100%
6	22	*P. falciparum* Strain 0886	AM116315.1	98%
7	23a	*P. falciparum* Strain BK820	KY337016.1	99%
8	25a	*P. falciparum* Strain 445	KP086191.1	97%
9	26a	*P. falciparum* Strain BK959	KY332967.1	98%
10	28a	*P. falciparum* Strain 37F3-17	KC678236.1	98%
11	34	*P. falciparum* Strain F1 EMP-1-	MT017271.1	98%
12	36	P. falciparum Strain Bk622	KY333674.1	99%
13	42	*P. falciparum* Strain BK 753	KY335857.1	99%
14	11	*Plasmodium falciparum Strain TH166.12*	MT060894.1	100%
15	15	*P. falciparum Strain PO19*	DQ135473.1	98%
16	18	*P. falciparum Strain DO-39*	KC887581.1	100%
17	19	*P. falciparum Strain PIK20*	HQ733544.1	100%
18	23b	*P. falciparum Strain GC27*	KX849839.1	98%
19	24	*P. falciparum Strain GC38*	KX850025.1	98%
20	25b	*P. falciparum Strain G3st25*	KX850309.1	98%
21	26b	*P. falciparum Strain GC19*	KX850077.1	98%
22	27	*P. falciparum Strain BK1 309*	KY330890.1	98%
23	28b	*P. falciparum Strain GC20*	KX850697.1	98%
24	29	*P. falciparum Strain GC11*	KX851219.1	98%
25	30	*P. falciparum Strain G3st10*	KX851127.1	98%

Plasmodium falciparum resistant *Kelch-13* gene was detected in 70% of the isolates (Plate 2) after the gel- electrophoresis amplification of the resistant gene.

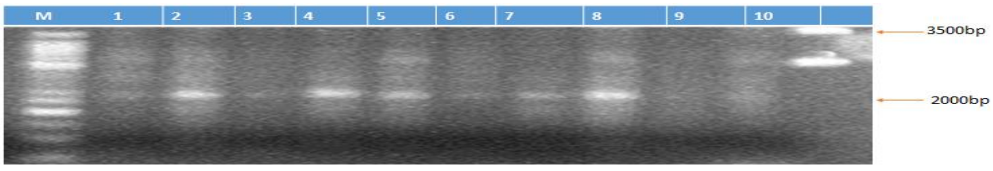

Plate 2: Amplified *P. falciparum* resistant *Kelch - 13* gene
Key: M showing the ladder

Phylogenetic Evolutionary Tree Analysis:

Evolutionary analyses were conducted in MEGAX. Figure 1 showed the phylogenetic tree of the evolutionary relationship of *P. falciparum* isolates from Ekiti and Ondo States. Twenty (25) strains belonging to three clades were identified phylogenetically.

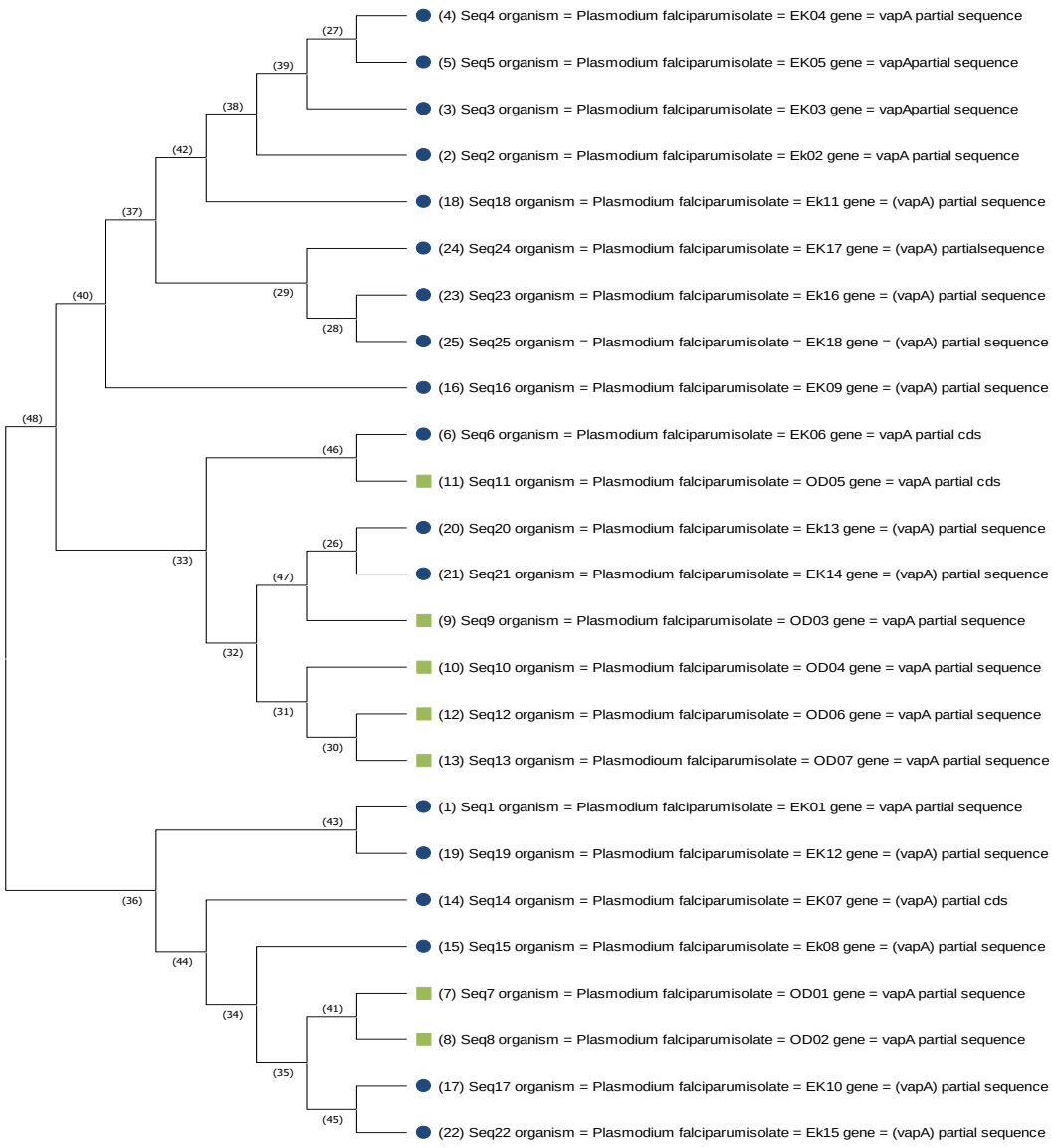

Figure 1: Phylogenetic Tree showing Evolutional Relationship of *P. falciparum* isolates from Ekiti and Ondo States Key: Ekiti - Blue, Ondo – Green

The isolates showed distinct evolutionary relationship when compared to other parts of Nigeria found on National Centre for Biotechnology Information (NCBI) as shown in Figure 2 and 3.

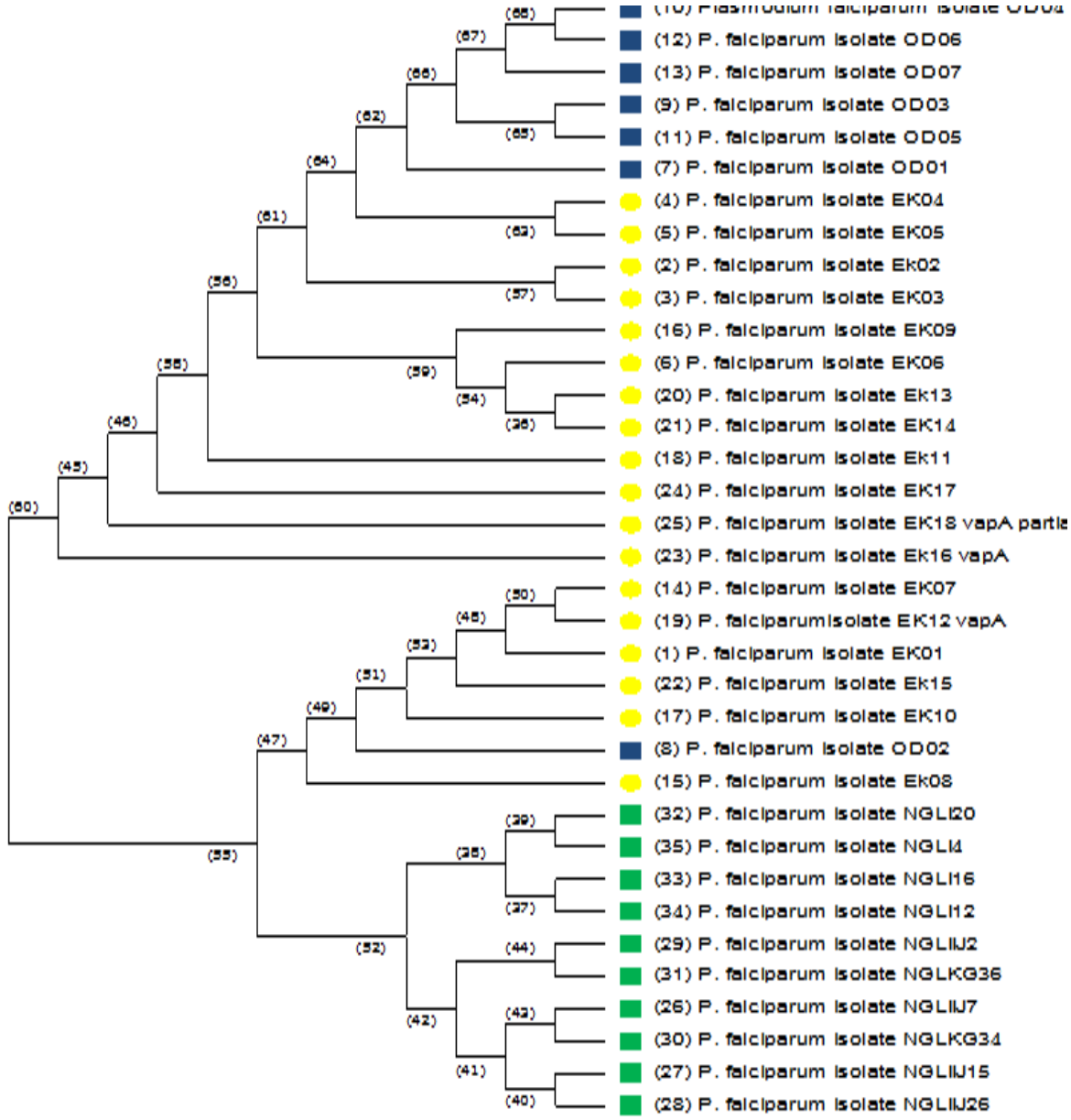

Figure 2: Phylogenetic Relationship of *P. falciparum* isolates from Ekiti, Ondo and other parts of Nigeria.
Key: Ondo - Blue, Ekiti – Yellow, Other parts- Green

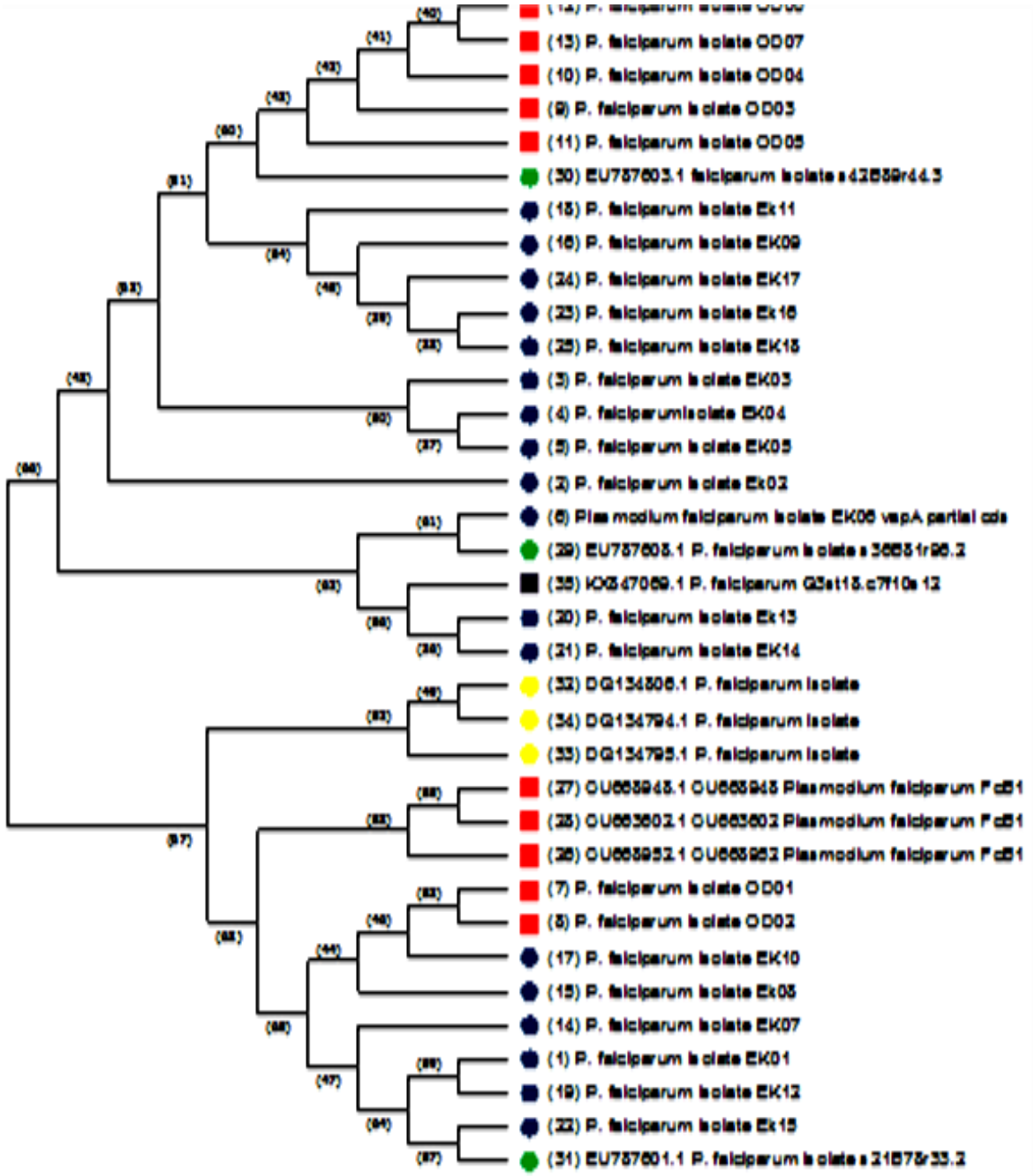

Figure 3: Phylogenetic relationship of *P. falciparum* isolates from Ekiti, Ondo and other parts of the World.
Key: Ondo - Red, Ekiti – Blue, Uk- Green, New Guinea – Yellow, France – Orange, South America – Purple.

DISCUSSION:

The efficacy of ACTs is being monitored in most malaria-endemic countries. There have been some reports of delayed parasite clearance during routine therapeutic efficacy studies of ACTs conducted in Africa. However, these reports have not been consistent over time. Artemether-Lumefantrine and Artesunate-Amodiaquine are the first-line treatment policies used in most African countries, with some countries adding Dihydro - Artemisinin-Piperaquine [11].

This allele was not previously associated with clinical or *in-vitro* resistance to Artemisinin but a study associated it with prolonged parasite clearance in Ugandan children who had severe malaria and were treated with intravenous Artesunate [18]. All the participants in this study tested positive for *Plasmodium falciparum* infection.

The emergence and spread of ACT-resistant isolate in Nigeria is a matter of concern. Looking at the molecular evolutionary pattern in this study, twenty - five different strains of *P. falciparum* were identified and diagnostically, the specific resistant gene primer *K-13* used confirmed the resistance of *Plasmodium falciparum* to ACTs in the study area. Previous research work near the study area [19] confirmed the emergence of resistance to ACTs drugs.

Artemisinin- based Combination Therapy (ACTs) was introduced in 2005 in Nigeria with Artemether–Lumefantrine (AL) as first-line treatment for uncomplicated malaria and Artesunate + Amodiaquine as the alternative [20]. There is no alternative to the current regimen Artemisinin Combination Therapy for the severe uncomplicated malaria. It is essential to monitor the efficacy of the Artemisinin derivative through molecular surveillance study [17]. Recent study aimed to track the research questions raised for the surveillance of *falciparum* infections by the PCR-based sequencing analysis of *Pfkelch13* gene propeller polymorphism [17].

Twenty- five strains of *P. falciparum* belonging to three (3) clades phylogenetically were identified in this study. The isolates from the two States were evenly distributed within the three clades, showing evolutionary relationships. *P. falciparum* resistant *Kelch 13* gene was detected in 70% of the isolates.

This study, though limited to a number of field-sampled malaria parasite isolates and sampled only from Southwestern part of Nigeria, could enlighten on the evolutionary basis of Artemisinin- based combination therapy (ACT) resistance of *P. falciparum*, infection in Nigeria. Considering ACT in different combinations (with Artemisinin being the first-line drug) is the only surviving antimalarial in the control program of

many endemic countries, non-observance of validated AA mutations of *Pfk13* gene with no association among other mutations of other genes conferring resistance to partner drugs is, in fact, welcome news concerning malaria public health situation in Nigeria [21]. However, considering the historical migration of CQ-resistant parasites to Africa from other different malaria-endemic countries e.g., Southeast Asian countries [22] regular and expanded molecular surveillance with a large sample size on the mutations of the *Pfk13* gene are needed to monitor the prevalence of ACT-resistant *P. falciparum* in Nigeria.

CONCLUSION:

Artemisinin Combination Therapy remains the most effective treatment for uncomplicated *falciparum* malaria. Most patients with delayed parasite clearance are cured, as long as the partner drug remains effective.

The Monitoring of the effectiveness of the recommended ACT drugs must be done routinely to ensure timely changes in National treatment policies. More work must be done to study the antigenic shift pattern of *P. falciparum* in their intermediate hosts. In order to prevent antimalarial drug resistance, Plasmodium falciparum antimalarial drug sensitivity should be closely monitored, and compliance with antimalarial drug use should be encouraged in the study area.

Acknowledgement

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