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Molecular Studies on *Plasmodium Falciparum* and *Schistosoma Haematobium i*n Obi and Oju Local Government Area of Benue State, Nigeria

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ABSTRACT

This study employed a molecular approach to detect, identify, and analyze *Plasmodium falciparum* and *Schistosoma* species co-infection using dried blood spots collected from individuals in Obi and Oju Local Government Areas. DNA was extracted using the QIAamp DNA Mini Kit protocol, followed by nested Polymerase Chain Reaction (PCR) targeting the 18S rRNA gene for *Plasmodium falciparum* and species-specific primers for *Schistosoma*. PCR-positive samples were further subjected to amplification of the *merozoite surface protein 2 (MSP2)* gene, and selected amplicons underwent sequencing using the Illumina platform. Sequence analysis and phylogenetic reconstruction revealed genomic variation in *P. falciparum*, which may contribute to the persistence of co-infections due to evolving resistance traits. Among 21 sequenced *P. falciparum* samples, only 7 were fully assembled and analyzed for genetic diversity. The study confirmed a notable prevalence of co-infection and highlighted the role of genomic variation in the resilience of these parasites. Findings underscore the necessity for integrated molecular surveillance, targeted treatment strategies, and improved public health interventions addressing water, sanitation, and hygiene (WASH) in endemic areas. Molecular diagnostic tools such as PCR remain crucial for early detection and control of parasitic diseases.

Keywords: Polymerase Chain reaction (PCR), DNA sequencing, co-infection, nucleotide transcription.

1. INTRODUCTION

Malaria is a life-threatening parasitic disease, prevalent in many African countries, south of sahara desert. plasmodium sp. are pathogens transmitted through the bite of infected female anophelines, causing malaria (Akpan *et al.*; 2018). They are a genus of protozoan (single celled) parasites that belong to the plasmodidae family. Four different species of plasmodium have been identified in humans: *P.falciparum, P.malariae, P.ovale and P.vivax.* occasionally, *P.knowlesi* which is majorly known to infect animals can also infect humans. Risk factors that can have significant impacts on plasmodium sp. transmissions includes population congestion, immunity, social and economic status (housing conditions), use of mosquito control measures, and environmental factors (De silva *et al.*; 2012). Prevalence and risk factors associated with plasmodium infection are used as indicators guiding public health interventions to track the progress of malaria control programs (Mabunda *et al.*; 2009)

To reduce the malaria burden and in accordance with the WHO recommendations, (WHO 2018) Nigeria implemented strategic plans for malaria control covering several areas of intervention including prompt and effective handling of cases, promotion of individual and collective protective measures, use of long-lasting insecticidal mosquito nets (LLINs), (Boisier *et al.*; 2002) intermittent preventive treatment and intra-home spraying, epidemiology, and fight against epidemics. However, despite repetitive and recent improvement in the coverage of malaria interventions, malaria remains a significant cause of morbidity and mortality in Africa, and Nigeria in particular (Messina *et al.*; 2011).

As earlier mentioned, various studies have shown that people who are vulnerable to malaria disease are pregnant women, children under five years of age, immune-compromised patients and travelers from non-endemic regions to malaria-endemic areas. Therefore, various studies assessing the efficiency of different modalities or strategies for malaria control in Nigeria mostly concentrated on these at-risk groups, most especially children under five years of age (Mutombo *et al.*; 2018, Doolan *et al.* microbiol Rev.2009, Philips *et al.*; 2009, Mfueni *et al.*; 2018). Adults were reported to have more mosquito bites and thereby constitute a potential infections reservoir (Mphaka *et al.*; 2011-2014; Papaioannou *et al.* ;2019 Goncalves *et al.*; 2017). In most of these researches this age group did not attract similar attentions as the at-risk groups. Considering this anomaly, this study aimed to investigate the carriage of plasmodium sp specifically in children and adults in Obi and Oju LGAs. We determined the prevalence, identified the parasite species,

assessed the parasite density in infected subjects or participants and identified the risk factors associated with plasmodium sp. infection in adults and children.

2. MATERIALS AND METHODS

DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit) Protocol

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from blood, both untreated and treated with anticoagulants, which has been spotted and dried on filter paper.

Things to do before starting

- Prepare an 85°C water bath for use in step 2, a 56°C water bath for use in step 3, and a 70°C water bath for use in step 4.
- Equilibrate Buffer AE or distilled water to room temperature (15–25°C) for elution in step 10.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions.
- If a precipitate has formed in Buffer AL or Buffer ATL, dissolve by incubating at 56°C.

Procedure

1. 3 punched-out circles from a dried blood spot was placed into a 1.5 ml microcentrifuge tube and 180µl of Buffer ATL.was added. (Cut 3 mm (1/8 inch) diameter punches from a dried blood spot with a single-hole paper puncher).

2. we Incubated at 85°C for 10 min and was briefly centrifuged to remove drops from inside the lid.

3. 20µl proteinase K stock solution was added and was mixed by vortexing, and incubated at 56°C for 1hr.This was briefly centrifuged to remove drops from inside the lid.

Note: The addition of proteinase K is essential.

3. 200µl Buffer AL was added to the sample and mixed thoroughly by vortexing, and was incubated at 70°C for 10 min. This was briefly centrifuged to remove drops from inside the lid. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.

4. Note: we were careful not to add proteinase K directly to Buffer AL. A white precipitate may form when Buffer AL is added to the sample. In most cases, the precipitate will dissolve during incubation.

5. 200µl ethanol (96–100%) was added to the sample, and mixed thoroughly by vortexing. This was briefly centrifuged to remove drops from inside the lid. It was essential that the sample and ethanol are mixed thoroughly.

6. The mixture from step 5 was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2ml collection tube (provided), and discarded the tube containing the filtrate. Each QIAamp Mini spin column was closed to avoid aerosol formation during centrifugation.

7. The QIAamp Mini spin column was carefully opened and 500µl Buffer AW1 was added without wetting the rim. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1min. The QIAamp Mini spin column was placed in a clean 2ml collection tube (provided), and discarded the collection tube containing the filtrate.

8. QIAamp Mini spin column was carefully opened and 500µl Buffer AW2 was added without wetting the rim. The cap was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3min.

9. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

10. This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. The QIAamp Mini spin column was placed in a clean 1.5ml microcentrifuge tube and discarded the collection tube containing the filtrate. QIAamp Mini spin column was carefully opened and 100µl Buffer AE or distilled water was added then Incubated at room temperature (15–25°C) for 1min, and then centrifuged at 6000 x g (8000 rpm) for 1min

PCR Condition

All PCR reactions were performed in 25µl of reaction volume. For the first PCR amplification (genus specific PCR) 2.5µl of extracted DNA was added to 25µL of reaction mixture including Q5 High-Fidelity 2X Master Mix (12.5µl) (New England Biolab), 10µM Forward Primer (1.25µl), 10µM Reverse Primer (1.25µl) of each primer (rPLU1 and rPLU5) and Nuclease-Free Water (7.5µl). DNA amplification was carried out under the following conditions: 98°C for 30sec, 35 cycles of 98°C for 10 se; 65°C for 30sec, 72°C for 30sec followed by final extension at 72°C for 5min.

The result of the first PCR amplification (genus specific PCR using primer set rplu6 and Rplu5(table 1) product was used as the DNA template for the Nested PCR (second PCR amplification for the species-specific using the primer set rFAL1 and Rfal2(table 2). For the nested species-specific PCR, 2.5µl of first amplification PCR product (genus-specific) was added to 25µl of reaction mixture including Q5 High-Fidelity 2X Master Mix (12.5µl) (New England Biolab), 10µM Forward Primer (1.25µl), 10µM Reverse Primer (1.25µl) of each primer (rFAL1 and rFAL2) and Nuclease-Free Water (7.5µl). (Using primer set in table 2). DNA amplification was carried out under the following conditions: 94°C for 3 min, 35 cycles of 94°C for 30 s; 58°C for 30 s, 72°C for 30 s followed by final extension at 72°C for 2min.

Molecular Detection and Identification of Malaria Parasites:

Considering the procedure by Snounou and colleagues (1993) *Plasmodium* species were detected and as well analyzed through the amplification of the 18S ribosomal RNA (fig 1) using the nested Polymerase Chain Reaction (PCR) with secondary primers which are specific to *P. falciparum* templates.

Extraction of DNA from samples collected on filter paper.

Parasite genomic DNA was extracted from blood samples collected on filter paper using the Qiagen extraction kit, following the manufacturer protocol. The purity and quality of the DNA were determined using the nanodrop 1000 spectrophotometer manufactured by Thermoscientific. The DNA samples were stored at -20 ºC until required for analysis.

Amplification of the 18SrRNA Gene of *Plasmodium falciparum*

The 18SrRNA of the *Plasmodium* sp was amplified using a BioRAD thermal cycler within the highly conserved regions of the small subunit (SSU) rRNA gene as described by Snounou *et al* (1993). Using the stated below primer (rFaL1 and rFAL2)

3.2 Second PCR amplification

All genus specific positive results were analysed to species level by species specific nested PCR using primers rFAL1 and rFAL2 (table 2) (*Plasmodium falciparum*).

For detection and identification of *Plasmodium falciparium* species, Nested -PCR was performed targeting the *Plasmodium falciparium* DNA, using the stated below primer

Third PCR amplification

All confirmed positive samples of *P. falciparum* were further subjected to Molecular detection of Merozoite surface protein (MSP 2) using the primer set in table 3.

Gel Electrophoresis

At the completion of the amplification, PCR products were resolved on l.5% agarose gel prepared by dissolving 1.5g of agarose powder in 100 ml of 1X Tris-borate-EDTA (TBE) buffer solution inside a clean conical flask. The 1.5% agarose solution was heated in a microwave oven for 2-3minutes and was observed for clarity which was an indication of complete dissolution. The mixture was then allowed to cool to about 500C after which 0.5µl of ethidium bromide was then added. It was allowed to cool further and then poured into a tray sealed at both ends with support to form a mould with special combs placed in it to create wells. The comb was carefully removed after the gel had set and the plate was placed inside the electrophoresis tank which contained 1X TBE solution, 5µl of amplicon was mixed with 1µl of loading buffer and the mixture was loaded to the wells of the agarose gel. The power supply was adjusted to 100 volts for 30 minutes. For each run, a 100 base-pair molecular weight DNA standard (size marker) was used to determine the size of each PCR product. The DNA bands were then visualized with a short wave ultraviolet trans-illuminator and photographed using gene gel bio-imaging system. The PCR product was then analyzed.

Table 1

Primer	Sequence	Bp
rPLU 6	TTAAAATTGTTGCAGTTAAAACG	1200
rPLU 5	CCTGTTGTTGCCTTAAACTTC	

SECOND STAGE

AMPLIFICATION OF GENES

<u>First gene Amplification</u>

Gene = rPLU5 and rPLU6

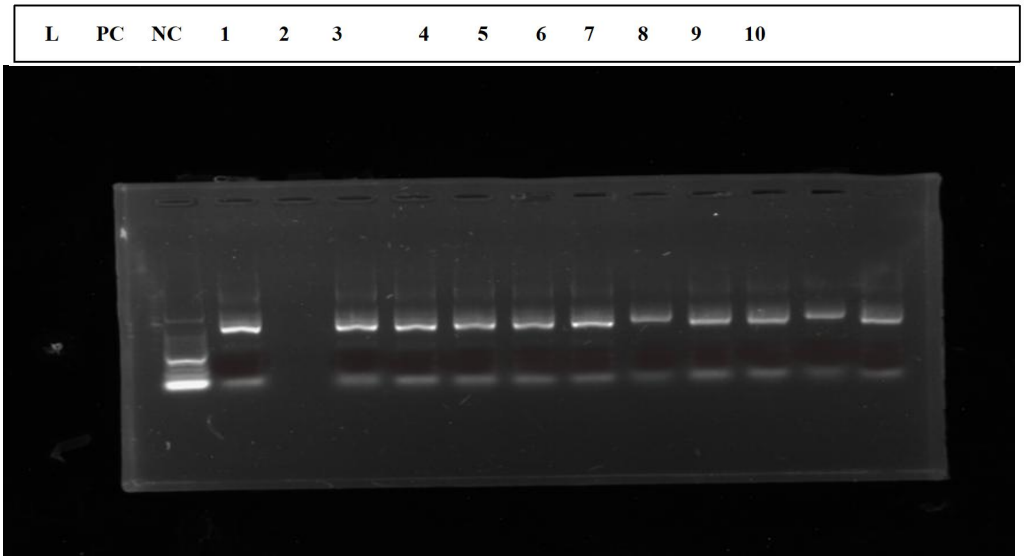


Fig 1: The representative result image of electrophoresis gel for the amplified samples of *P.falciparium* among the tested samples.

Key: L=DNA Ladder (100bp)

PC= Positive Control

NC= Negative Control

1-10 =these are the samples representatives

Expected band size= 12kb (1200bp)

Two genus-specific primers rPLU5 and rPLU6, were used for the first cycle of amplification.

Table 2

Primer	Sequence	Bp
rFAL 1	TTAAACTGGTTTGGGAAAACCAAATATATT	
rFAL 2	ACACAATGAACTCAATCATGACTACCCTC	205

Second amplification

Gene =*P. falciparium* (Fal 1 and Fal 2)

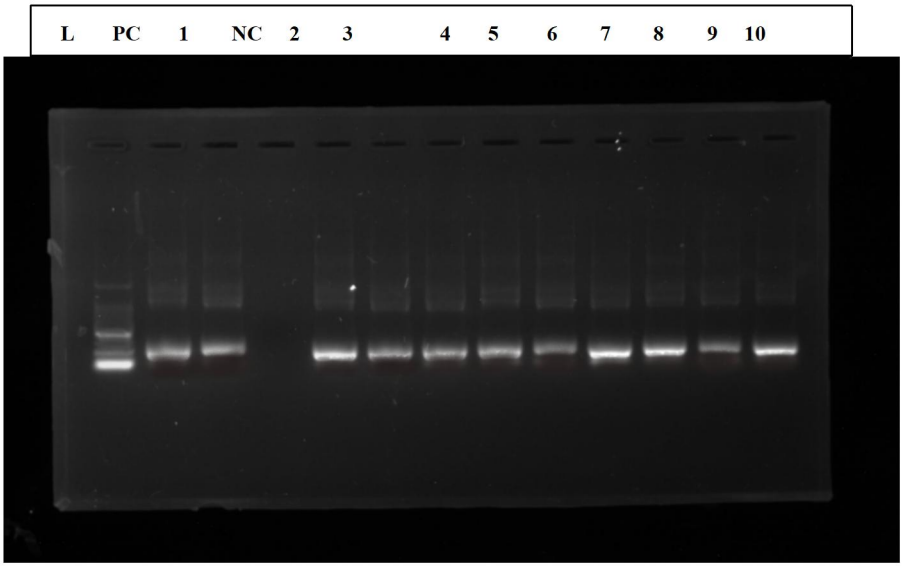


Fig 2: The representative result image of electrophoresis gel for the amplified samples of *P.falciparium* among the tested samples.

Key: L=DNA Ladder (100bp)

PC= Positive Control

NC= Negative Control

1-10 =these are the samples representatives

Expected band size= 205bp

An aliquot of the product thus obtained from the first amplification was used for a second amplification, in which each parasite species is detected separately using species-specific primers

Table 3

Primer	Sequence	Bp
Msp 2-1	ATGAAGGTAATTAAAACATTGTCTATTATA	
Msp 2-2	TTATATGAATATGGCAAAAGATAAAACAAG	

Third amplification

Schistomiasis **(ETIS 1 and ETIS 2)**

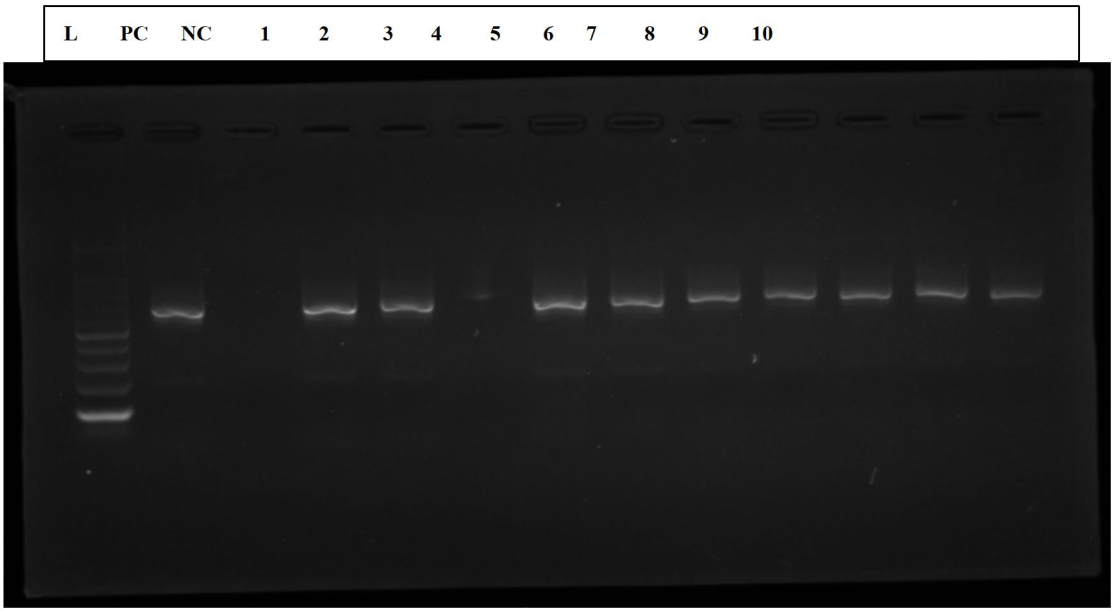


Fig 3: The representative image of electrophoresis gel for the amplified Schistosomiasis among the tested *P.falciparium* samples.

Key: L=DNA Ladder (100bp)

PC= Positive Control

NC= Negative Control

1-10 =these are the samples representative

The suspected *P.facilparium* samples were further checked for co-infection with schistosomiasis, thus the same DNA extract was also used for the amplification, in which the parasite species is detected separately using species-specific primers.

PCR RESULT Table 4

	PLU		**P.faciparium**		**Schistosomiasis**		**MSP**	
sample ID	**POS**	**NEG**	**POS**	**NEG**	**POS**	**NEG**	**POS**	**NEG**
570	Pos		Pos			Neg	Pos	
113	Pos		Pos			Neg		Neg
710	Pos		Pos			Neg		Neg
61	Pos		Pos			Neg	Pos	
29	Pos		Pos			Neg	Pos	
72	Pos			Neg		Neg		Neg
65	Pos		Pos		Pos		Pos	
32	Pos		Pos		Pos		Pos	
471	Pos		Pos			Neg	Pos	
939	Pos		Pos			Neg	Pos	
183	Pos		Pos			Neg		Neg
536	Pos		Pos		Pos		Pos	
746	Pos		Pos		Pos		Pos	
933	Pos		Pos			Neg	Pos	
774	Pos		Pos			Neg	Pos	
271	Pos		Pos		Pos		Pos	
444	Pos		Pos			Neg	Pos	
922	Pos		Pos		Pos		Pos	
173	Pos			Neg		Neg	Pos	
291	Pos		Pos			Neg		Neg
470	Pos		Pos			Neg	Pos	
818	Pos		Pos			Neg	Pos	
150	Pos		Pos			Neg		Neg
494	Pos		Pos			Neg		
890	Pos			Neg		Neg		
782	Pos		Pos		Pos		Pos	
780	Pos		Pos			Neg		Neg
568	Pos		Pos			Neg	Pos	
528	Pos		Pos			Neg		Neg
497	Pos		Pos			Neg		Neg
496	Pos		Pos		Pos			Neg
495	Pos		Pos		Pos		Pos	

DNA SEQUENCING

This is the process of determining the precise order of the four nucleotides bases (Adenine, Thymine, cytosine and guanine within a DNA molecule.

Every organism's DNA in a cell consist of a unique sequence of nucleotide. Determining the sequence can help scientists compare DNA between organisms which can help show how the organisms are related. This means that by sequencing a stretch of DNA, it will be possible to know the order in which the four nucleic acids occur in a DNA molecule.

DNA sequencing can be used to figure out how your genome affects your life.

DNA Sequencing Steps

1 Purify and copy the DNA

2. Read the sequence

3. Compare to other sequence.

Sequencing

Library Preparation

An amplicon sequencing was carried out as the sequencing method using Next Generation Sequencing machine (illumina). Library Preparation was done following the illumina XT library Kit, the primer for the preparation, MSP2 was used for the targeted regions.

The generated fast file was demultiplexed on the machine into different samples fast file per sample based on the barcodes of each sample. This was moved to the system for the bioinformatics analysis.

The pipeline for reference-based assembly was used on MEGA 11 for identifying the genetic diversity that may be present on Merozoite Surface Protein 2 (MSP2) gene among the confirmed positive *P.falciparium.*

Out of 21 positive samples sequenced, only 7 were fully assembled using a command-line tool (As shown in the image 1 and 2).

Also, the phylogenetic representative of the observed difference among the samples genetic sequenced against the Merozoite Surface Protein 2 (MSP2) gene sequence was drawn to show the changes (image 3).

Image 1: positive *P.falciparum*

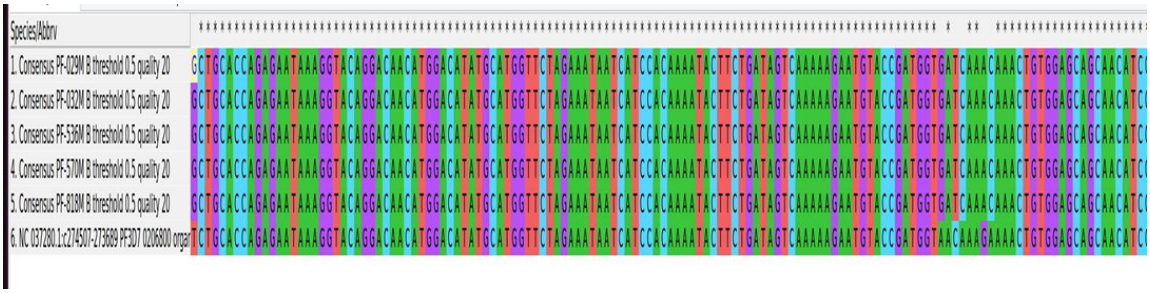


Image 2: positive *P.falciparum*

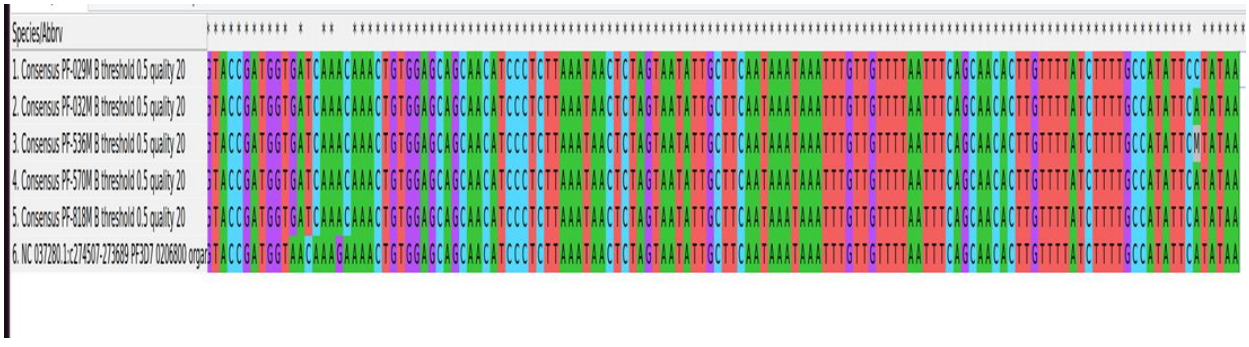
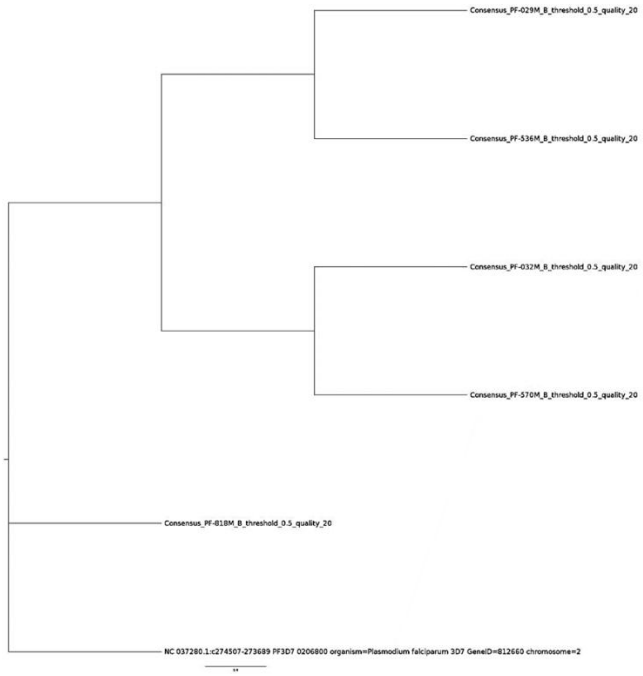


Image 3: Phylogenetic structure of the sample sequenced against MSP2



DISCUSSION

The reason for the persistence of coinfection was unraveled by the discovery by *Plasmodium falciparum* DNA sequencing where the phylogenetic representative of the observed difference among the sample's genetic sequences against the Merozoite Surface protein 2 gene sequence was obvious. The nucleotide transcription may account for reason for conferment of entirely new character on an organism as well as other genetic factors.

Following the procedure established by Snounou *et al.* (1993), PCR Procedures was a reliable method for molecular analysis and it was discovered that coinfection of malaria and schistosomiasis in Obi and Oju has been proven to be an established fact and not a mere speculation. Evidence from PCR procedures has proved that *Plasmodium falciparum* was the *Plasmodium* specie that dominated the coinfection status of the infected.

Though, percentage coinfection across the study area was not at epidemic stage yet, there was an urgent need to arrest the situation before it degenerates to epidemic proportion by planning an integrated method of treating both young and older folks who are the potential infection reservoir of malaria parasites. Also, the question of why coinfection persisted in the study area was addressed by the researcher. This was due to gradual changes (Variations) in the Phylogenetic structure of the parasite DNA and transcription of the nucleotides. *Plasmodium falciparum* was undergoing genomic structural changes which has conferred either new positive or negative behaviour (character) on the parasite. In this situation, it is resistance to hither to effective drugs which has resulted to persistence in the co-infection episodes. This could be seen in the Phylogenetic tree of the sequenced DNA of the parasite as evidenced in the differences in the molecular weight of the visualized DNA bands as well as the transcription of the nucleotides in the parasite DNA molecule. Out of 21 positive samples sequenced, only 7 were fully assembled using a command- line tool.

Conclusion

The use of molecular analysis strengthens the result of this study, as pcr assays are currently recognized as the most sensitive methods for the diagnosis and identification of plasmodium. (Mangold *et al.*; 2005, Cnops *et al.*; 2011, Kahindo *et al.*; 2020). Furthermore, the high prevalence among adults may be due to lack of access to prevention measures such as the use of insecticide-treated bed nets (ITBN) and treatment . Adults are most often asymptomatic or minimally symptomatic as many of them have acquired immunity. The unique findings of this study was that it has been able to unravel the secret/reason behind continuous persistence of coinfection of malaria and schistosomiasis in Obi and Oju LGAs. The overall contribution to knowledge was that coinfection of malaria and schistosomiasis could be resolved through addressing the problem of socio-environmental challenges viz WASH (water, sanitation and hygiene), housing as well as the discovery of the molecular studies through formulation of new drugs.

Competing interest

The authors wish to declare that there was no competing interest.

Consent

Participants gave their informed consent before the commencement of the research work.

Ethical approval

GOVERNMENT OF BENUE STATE OF NIGERIA

In replying please quote the number and date of this letter.

MOH/STA/204/VOL.1/248

Ref. No.

Ministry of Health & Human Services
P.M.B. 102093
Makurdi, Benue State.

Date: 7th September, 2023

Angwa Dickson Ikpe
Department of Biological Sciences,
Benue State University,
Makurdi.

Re: Request for permission to conduct research on "Epidemiological and Molecular Studies on Co-endemicity of Malaria and Schistosomiasis in Igedeland".

Your letter on the above subject matter dated 20th August, 2023 was received by the Research Ethical Committee. The Committee has gone through your proposal and wishes to inform you as follows:-

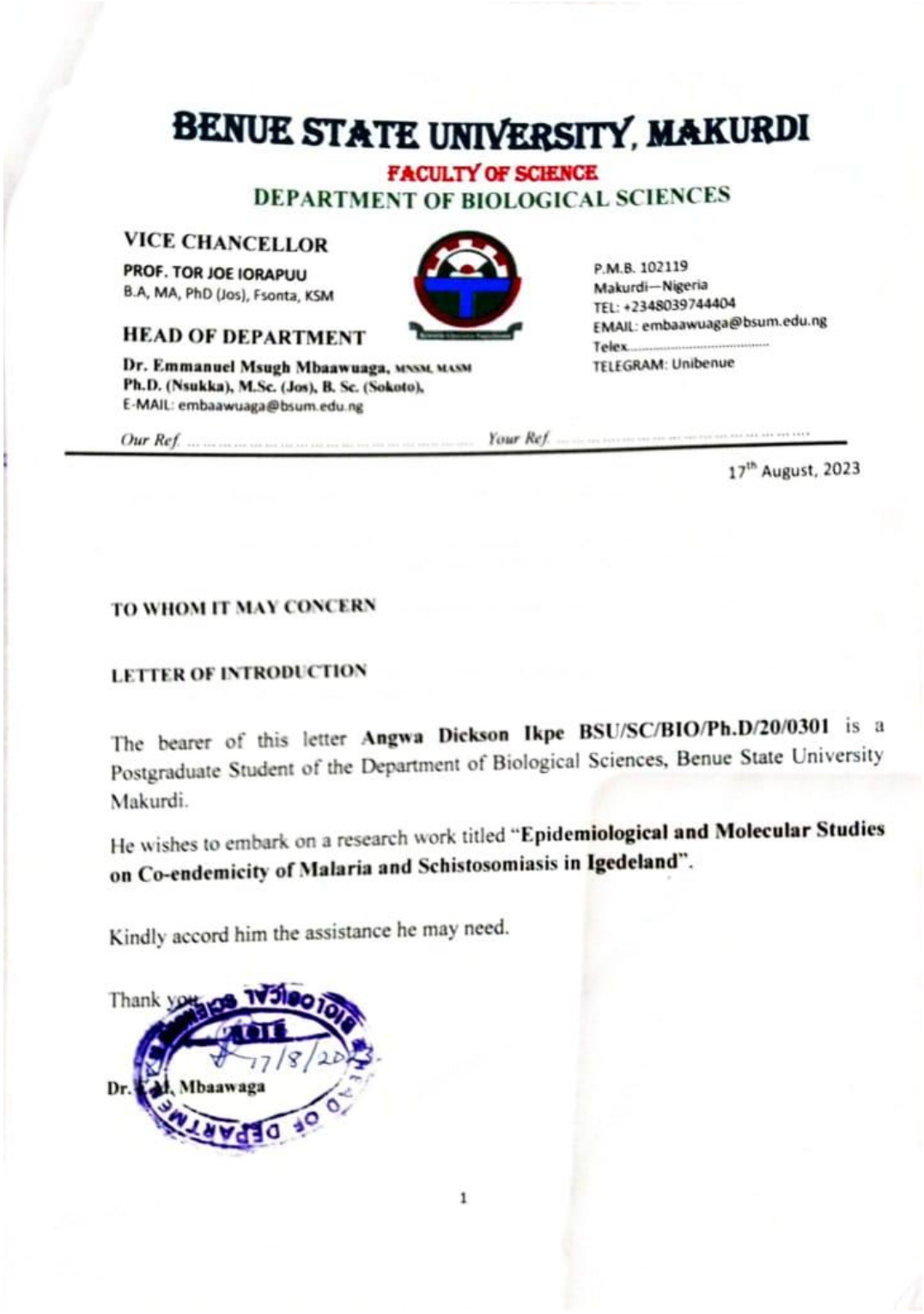
i. That you are hereby granted ethical approval to enable you proceed with the proposed study.

ii. You are however reminded that the research protocol cannot be changed without written permission from the ethical committee.

iii. You are requested to send the final copy of this research study to the Ministry for its information and necessary action.

Anweh Stephanie. U (Mrs)
For: Chairman
Health Research Ethical Committee

Ethical clearance was issued by the Benue State Ministry of Health and Human Services ethical committee.



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17th August, 2023

TO WHOM IT MAY CONCERN

LETTER OF INTRODUCTION

The bearer of this letter **Angwa Dickson Ikpe BSU/SC/BIO/Ph.D/20/0301** is a Postgraduate Student of the Department of Biological Sciences, Benue State University Makurdi.

He wishes to embark on a research work titled "**Epidemiological and Molecular Studies on Co-endemicity of Malaria and Schistosomiasis in Igedeland**".

Kindly accord him the assistance he may need.

Thank you.

17/8/20

Dr. [illegible], Mbaawaga

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