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In-Vitro Examination Utilizing the Nested Multiplex PCR for Identificationand Speciation of Human Non-*falciparum* *Plasmodium* Species in Owerri, Imo State Nigeria

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Abstract

An all-inclusiveapproach to parasitological testsis important for achieving theWorld Health Organization malaria goals, hence, precise conclusion is a significant factor in the continued effort to eradicate malaria. Microscopy has been considered the gold standard for the diagnosis of malaria parasite, yet, it has underserved in its obligation to distinguish blended contaminations when one of the Plasmodium species is available at very low levels (<100 parasites/mL), or altered by hostile to malarial medication. It isalso an intensified technique that requires adequate training of staff to ensure accurate result outcomes. Molecular techniques have presented better options that enables the distinction and recognition of malaria parasites at very low levels and in mixed parasitaemia. Blood samples were received from 300 persons and examined carefully under oil immersion (x100) to identify trademark highlights of malaria parasites and with nPCR. DNA extraction was done utilizing the fast gDNA small prep and measurement with a Nano-drop 1000 spectrophotometer. Microscopic results showed 14 positive cases for non-falciparum malaria parasitewhere occurrence for *P. ovale*, *P. malariae*, and *P. vivax* were 92.9%, 7.1% and 0% respectively. PCR results showed that malaria parasite infection prevalence among the sampled participants (male and female)examined for this study was also factually significant (P>0.05). Plasmodium species determined from the 93 PCR positive cases were *P. ovale* (25.6%), *P. malariae* (1.1%), and *P. vivax* (73.3%). The most noteworthy mean was (2.45±0.00) while the least mean was (2.14± 0.00). Prevalence of *P. vivax* malaria parasitaemia was measurably significant (P<0.05) among various age groups. The age-group 1-8 years had the most significant occurrence of*P. vivax* malaria parasites prevalence 13(21.7%) while the age group 49-56 years and 65-72 years had none 0 (0%). The outcomes of this investigation showed that the assessment of thick and thin blood smears by microscopy were insufficient for the diagnosis of malaria in the study area, hence the need for molecular diagnostic methods should be highly considered. Also, molecular diagnostic techniques furtherdemonstrate the incidence of *P. vivax* malaria parasite in the study area.

Keywords: *Plasmodium* Species, *P. malariae*, Nested, Multiplex, nPCR, *P. Ovale. P. vivax*, Microscopy

Background of the study

Malaria is a mosquito-borne malady of humans brought about by parasitic unicellular protozoans of the family Plasmodium [1]. The presence of stale water spots was one of the favouredplaces by Plasmodium carrying mosquitoes. Other favourable areas include flooded nurseries, swamp-like grounds, run off from agribusiness, and seepage issues from street development which had prompted the expansion of standing water [1].

Precise determination is a significant stratagem in the battle against malaria causing parasite and timely access to an accurate parasitological test is important in achieving the WHO Global Technical Strategyfor Malaria 2016-2030 [2]. Giemsa-recoloured blood spreads and microscopic examination has been considered as the reference best quality [3] for the analysis of malaria parasite for over a century, but this method neglect to recognize mixed infections, when one of the *Plasmodium* species is available at very low levels (<100 parasites/mL), or changed by hostile to malarial medication therapy[4][5].Also, it is a work intensive processthat requires well-trained personnel [6]. Notwithstanding, molecular diagnostic technique have been proficientin the detection and identification of malaria parasites with low and mixed parasitaemia [7]. As indicated by the WHO, PCR was resolved as a more explicitprocedure than every other procedure. It does, in any case, require particular and expensive hardware and reagents, just as research facility conditions that are regularly not accessible in the field [8].

Lately, there have been demonstration of the Immunochromatographic test strip which depends on three antigens; Plasmodium histidine-rich protein (HRP)- 2 (pHRP-2), Plasmodium lactate dehydrogenase (pLDH) and Plasmodium aldolase, for the diagnosis of malaria. This technique has central indicative impediments; in particular (i) none of the three antigens is explicit for *Plasmodium ovale*,*Plasmodium malariae*, or *Plasmodium knowlesi*; (ii) there are variations of *P. falciparum* in Africa that don't deliver the 2 most regular kinds of HRP (*P. falciparum* HRP [pfHRP] 2 and pfHRP-3), which implies that malaria quick symptomatic tests (MRDTs) in view of identification of those antigens would not be helpful in the study area [9]. (iii) cross-responses with a pfHRP-2 measure have been accounted for from patients with *Schistosoma mekongi* disease (with no cross-response with a pLDH examine) [10]; (4) cross-responses with certain tests have been accounted for in patients with rheumatoid factor or other flowing auto-antibodies [10]; (5) patients with elevated levels of *P. falciparum* parasitemia may give bogus positive outcomes with pLDH examination intended to identify *P. vivax* [11]; (6) in contrast to microscopy, MRDTs can't be utilized to decide the extent of parasitemia; and (7) on the grounds that pHRP-2 isn't cleared from blood for as long as 30 days after treatment, MRDTs that test for this antigen ought not be utilized to screen reaction to treatment [12][13[14]. Despite the fact that pLDH and aldolase are cleared rapidly from blood after treatment, gametocytes are not killed with standard antimalarial treatment and continue creating every one of the 3 antigens. Subsequently, testing for these 2 antigens likewise, ought not to be utilized to screen reaction to treatment [13]. However, in Nigeria the utilization of the *Plasmodium* histidine-rich protein 2 (pHRP-2) have been used as the main Immunochromatographic quick test, which cannot recognize different types of plasmodium and are not explicit to P. *falciparum* HRP [pfHRP]-2 and pfHRP-3.This implies that MRDTs dependent on location of those antigens would not be valuable in directing these strains of *Plasmodium falciparum.* Data on the nature and degree of hereditary dependent variety inside non-falciparum plasmodium species is basic in understanding the pathology of malaria, the procurement of invulnerability, the spread of medication opposition, the state of transmission, event of occurrence of mixed infection and the improvement of immunizations against the parasite [3].

This work reports on the molecular methodology of non-falciparum Plasmodium species and the advancement of malaria immunization for endemic zones. The genetic variation of non-falciparum Plasmodium species strains predominant in Owerri and Imo state was also studied.

Methodology

Sample Size/Selection

Three hundred (300) randomly selected patients with clinical symptoms of malaria were enrolled into the study. The sample size was determined by epiinfo™ with an estimated population size of 127,213 [15], at 95% confidence level, 5% confidence limit and an expected frequency of 32.2%.

Ethical approval

Ethical approval was sought for and received from the Ethical Committee of the Health facility while patients' consent was determined using consent form that was duly signed by patients/patient's guidance.

Sample collection

Venous blood samples were collected into EDTA bottles which were labelled with information such as: Name of the Patient, Investigation, Date, Sex, Age, Laboratory, and Hospital Numbers and screened for the presence of malaria parasites [16].

Microscopic examination

Method as described by Cheesbrough [17] was adopted. A modified thin film slide was flooded with 10% Giemsa solution for 10 minutes and two drops of buffered distilled water of pH 7.1 was added and left for further 10 minutes, the slide was then washed thoroughly under slow running tap water to enable differentiation (the colour appeared salmon pink).The slide was left to dry and the back of the slide were cleaned with cotton wool. The thick film was flooded with 3% Giemsa solution and allowed to stand for 30minutes. The slides were then washed using clean water and the back of the slides were wiped with cotton wool and placed in a draining rack to air dry. Immersion oil was spread to cover about 10mm in diameter in the areas of the film. Both the thick and thin smears prepared were examined microscopically under oil immersion with the (x100) objective lens for characteristic features of malaria parasites [17]. All of the blood smears were examined by an experienced WHO certified microscopist according to the WHO competency assessment protocol [2].

Malaria parasite Density Calculation

$$\text{Parasites}/\mu l = \frac{Number\ of\ parasites\ counted\ X\ 8000\ WBC}{Number\ of\ White\ Blood\ Cells\ (WBCs)}$$

[2].

DNA extraction and quantification

The DNA was extracted using the quick gDNA mini prep DNA extraction kit supplied by Inqaba Biotechnological, South Africa [18][19]. DNA quantification was done on a Nano-drop 1000 spectrophotometer [18].

Nested Amplification for Plasmodium Species

The nPCR amplification strategy was used for genotyping the 18S ssrRNA genes (*Pfmdr1* gene) of *Plasmodium* on thermal cycler, in which specific primers were used, as described by Snounou*et al*. [4].

Primers for PCR amplification reactions:

Plasmodium genus-specific:

Forward:
rPLU6 5' TTAAAATTGTTGCAGTTAAAACG 3'
and
Reverse:
rPLU5 3'CCTGTTGTTGCCTTAAACTTC 5'

Primary Amplification:

The primary amplification was performedusing primers rPLU6 and rPLU5 and the machine program conditions adopted.

Secondary Amplification:

Secondary amplification was performed using 2µl of the primary amplicon which was transferred into a second tubewith fresh reagents and genus specific primers. And 0.5µl of the clones used as control.

The species-specific primers used were:

***P. vivax*:**

Forward:
rVIV1-
CGCTTCTAGCTTAATCCACATAACTGATAC

Reverse: rVIV2 -
ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA

***P. malariae*:**

Forward: rMAL1 -
ATAACATAGTTGTACGTTAAGAATAACCGC

Reverse: rMAL2 -
AAAATTCCCATGCATAAAAAATTATACAAA

***P. ovale*:** Forward: POVA FWD –
CTGTTCTTTGCATTCCTTATGC

Reverse: RVS COMMON –
GTATCTGATCGTCTTCACTCCC

Table 1: Schematic representation of the *Plasmodium* ssrRNA genes and nPCR protocol

Species	PCR Product	Primer	Sequence	Reaction
Plasmodium genus-specific	1.6 –1.7kb	rPLU6 rPLU5	TTAAAATTGTTGCAGTTAAAACG CCTGTTGTTGCCTTAAACTTC	Nested 1
P. malaria	145bp	rMAL1 rMAL2	ATAACATAGTTGTACGTTAAGAATAACCGC AAAATTCCCATGCATAAAAAATTATACAAA	Nested 2
P. ovale	226bp	POVA FWD RVS COMMON	CTGTTCTTTGCATTCCTTATGC GTATCTGATCGTCTTCACTCCC	Nested 2
P. vivax	121bp	rVIV1 rVIV2	CGCTTCTAGCTTAATCCACATAACTGATAC ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	Nested 2

Inqaba Biotechnological South Africa.

PCR Products Analysis by Gel Electrophoresis

Method described by Dahm [19] was adopted and modified. The PCR products was confirmed and analysed on a 2% agarose gel electrophoresis with 0.5µg/ml of ethidium bromide.

Data Analysis

The data was obtained using EPI DATA to develop the questionnaires and analysed using MINI TAB Version 10. The data generated from this study were presented using descriptive statistics. Chi-square was used to obtain level of significance ($p<0.05$), analysis of variance (ANOVA) and student's t-test. All the values were reported as mean ± standard error of the mean (SEM) and the results were considered significant at p-value of less than 0.05($p<0.05$) i.e. at 95 % confidence level.

Results

The sex of the patients in relation to non-falciparum malaria diagnosis (Fig. 1), showed that 144 (48%) of the total sampled population enrolled in the study, were males and 152 (52%) females with a mean of 1.52 ± 0.5 (Fig. 1). Sex distribution was statistically significant among the studied population with ($T=52.61$, $P= 0.000$, $p< 0.05$) and a cumulative average of 48 and 100 respectively.

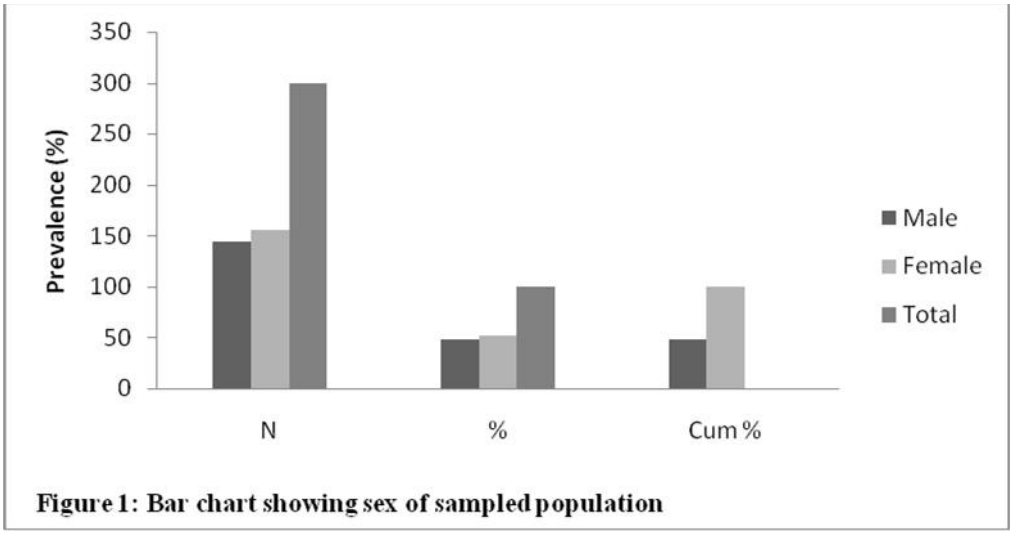

Figure 1: Bar chart showing sex of sampled population

From the microscopy result, Plasmodium species isolated from the 14 positive cases were *P. ovale* (92.9%), *P. malariae* (7.1%), and *P. vivax* (0%) (Fig. 2). The highest mean was (± 2.45) while the least mean was (± SD 2.14). The results were significant (P=0.0000 p<0.05 and T=15.00) (Fig. 2).

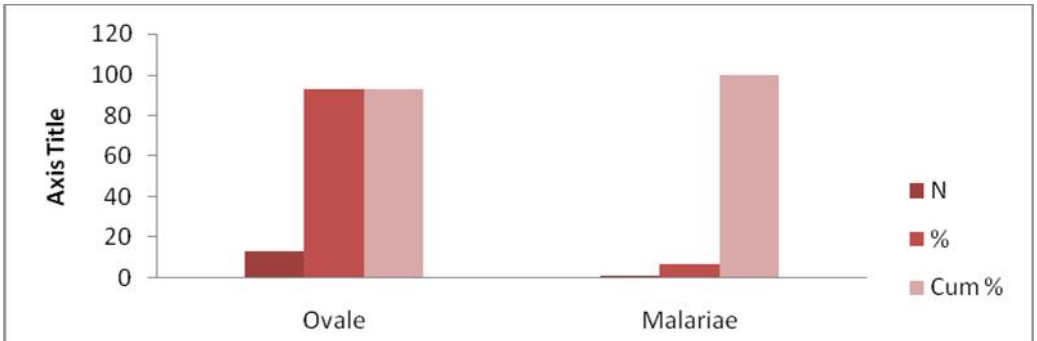

Figure 2: Bar chart showing the result of species of non-falciparum plasmodium malaria isolated from microscopic investigation of sampled population.

PCR results for non-falciparum malaria parasite showed that of the 300 sampled patients, 93 (31%) were positive, while 207 (69%) were negative. This result was statistically significant (P=0.0000, p>0.05 and T= 166.17) (Fig. 3).

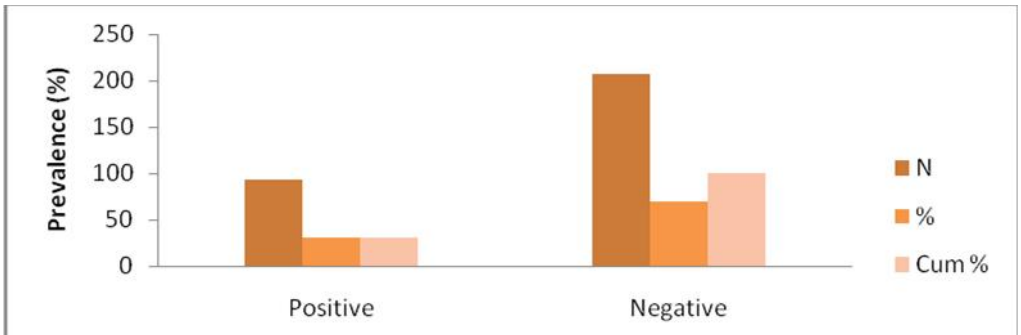

Figure 3: Bar chart showing the result of species of non-falciparum plasmodium malaria identified from PCR of sampled population.

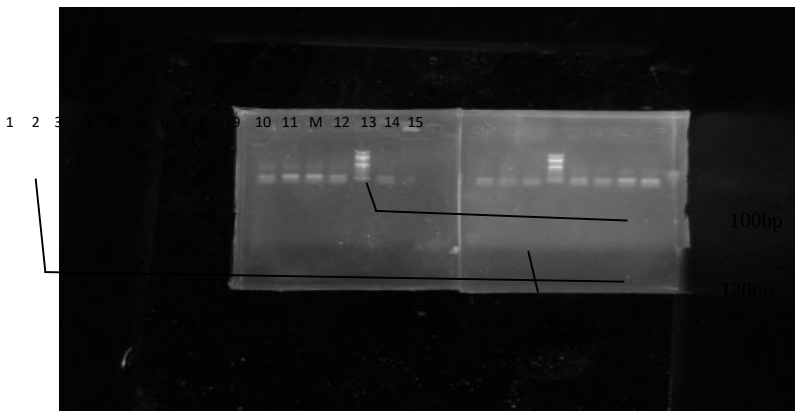

Fig. 4:Agarose gel electrophoresis picture showing *Plasmodium vivax* (120bp) isolates. Lane 1-5, 9-15represent the isolates, Lane 6-10 represents no amplification, while lane M represents Quick- Load 100bp molecular ladder.

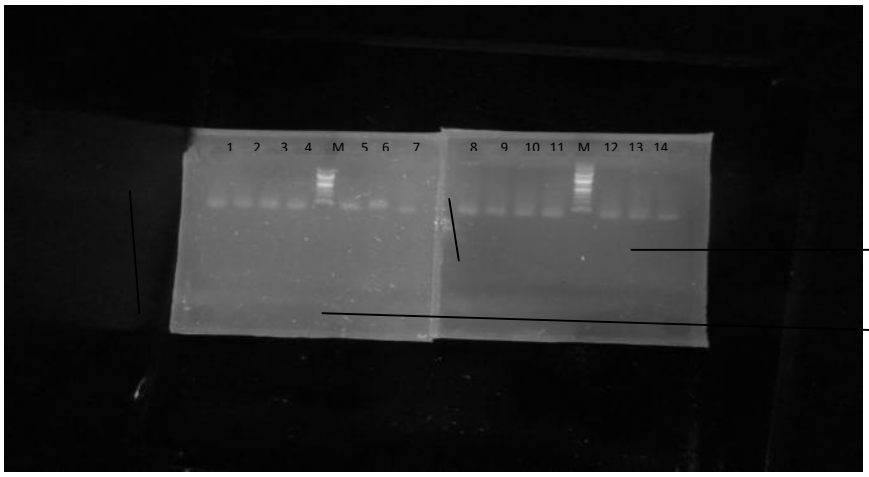

Fig. 5:Agarose gel of *Plasmodium ovale* showing spurious amplification Lane 1-14, while M represents the quick load 100bp molecular ladder

Malaria prevalence among the sexes was statistically significant (P>0.05), malaria parasitaemia was slightly higher among the males than the females (Fig. 6).

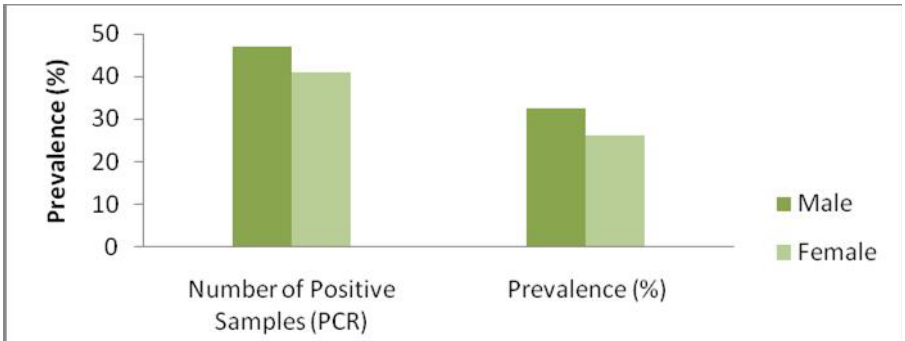

Fig. 6: Bar chart showing sex prevalence of non-falciparum malaria

P. vivax had the highest percentage occurrence of 73.3% of the 93 positive cases identified from PCR. Others were *P. ovale* and *P. malariae* with 25.6% and 1.1% as shown in (Fig. 7). The statistical results were significant (P=0.002 p<0.05 and T=57.15).

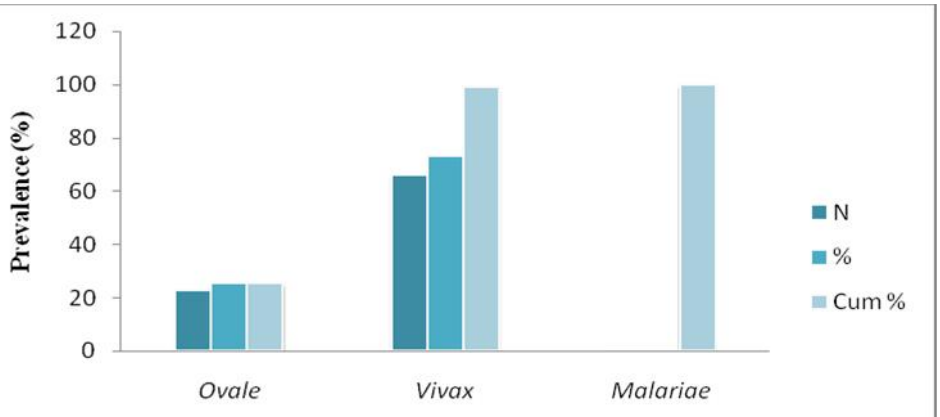

Fig. 7: Bar chart showing species of non-falciparum malaria identified from PCR

The age-group 1-8 years had the highest *P. vivax* malaria prevalence 13(21.7%) while the age group 49-56 years and 65-72 years had the least 0 (0%) as shown in Fig. 8. The other age groups 17-24, 9-16, 33-40, 25-32, 41-48, 57-64, 73-80, and 81-88 had a prevalence in descending order of 10(16.7%), 9(15%), 9(15%), 8(13.4%), 5(8.3%), 2(3.3%), 2(3.3%) and 2(3.3%) respectively. The statistical results were significant (P=0.011 p<0.05) (Fig 8).

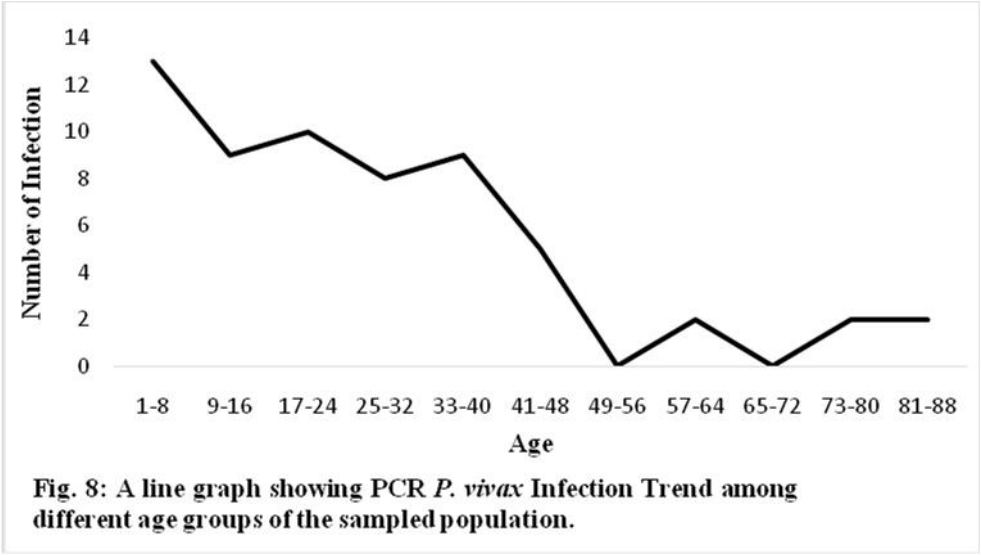

Fig. 8: A line graph showing PCR *P. vivax* Infection Trend among different age groups of the sampled population.

The age-group 1-8 years had the highest *P. ovale* malaria prevalence 12(46.2%) while the age group 33-40 years and 41-48 years had the least 0 (0%) as shown in Figure 13. The other age groups 9-16, 49-56, 57-64, 17-24, 25-32, 65-72, 73-80, and 81-88 had a prevalence in descending order of 3(11.5%),3(11.5%), 3(11.5%), 1(3.8%), 1(3.8%), 1(3.8%), 1(3.8%) and 1(3.8%) respectively. The statistical results were significant (P=0.017 p<0.05) (Fig. 9).

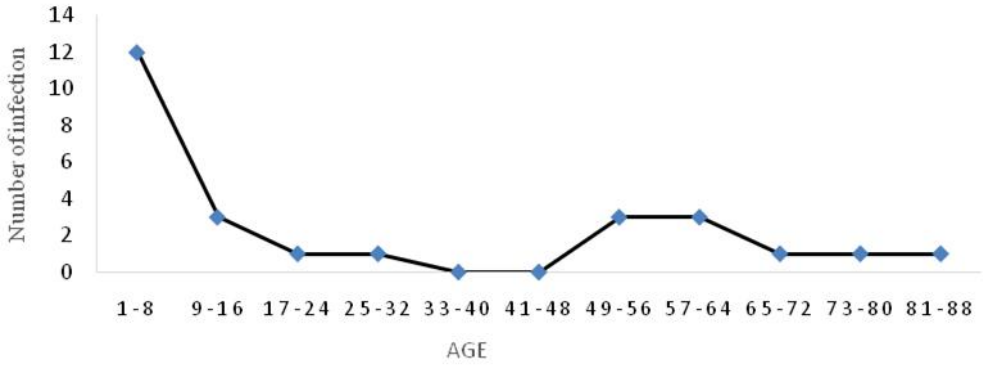

Fig. 9: Line graph showing PCR *P. Ovale* infection trend per age group of the sampled population

Except age groups 1-8 and 33-40 that reported positive results of 1(50%) each (Fig. 10), the results for the other age groups tested negative for *P. malariae*.

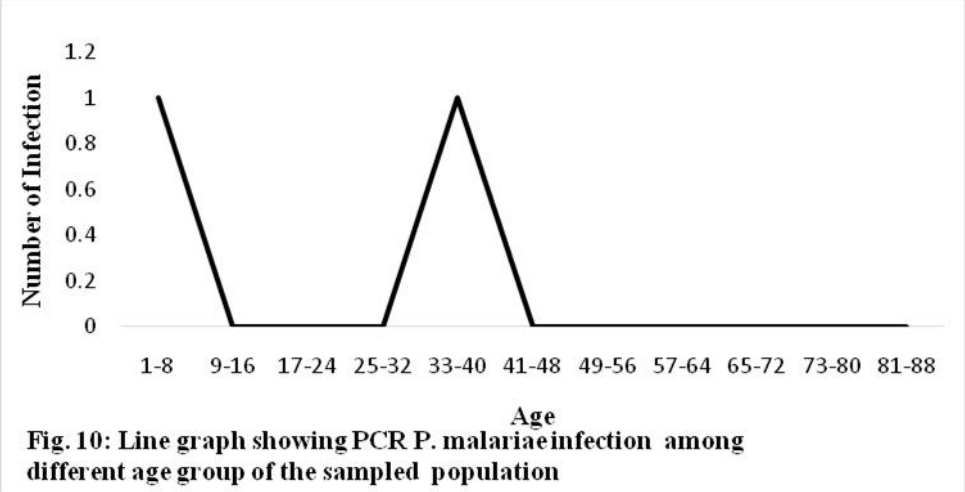

Fig. 10: Line graph showing PCR P. malariae infection among different age group of the sampled population

Fig. 11 compared the percentage prevalence of *P. vivax*, *P. malariae*, and *P. ovale* among the study population. The result was statistically significant (P=0.000, p<0.05).

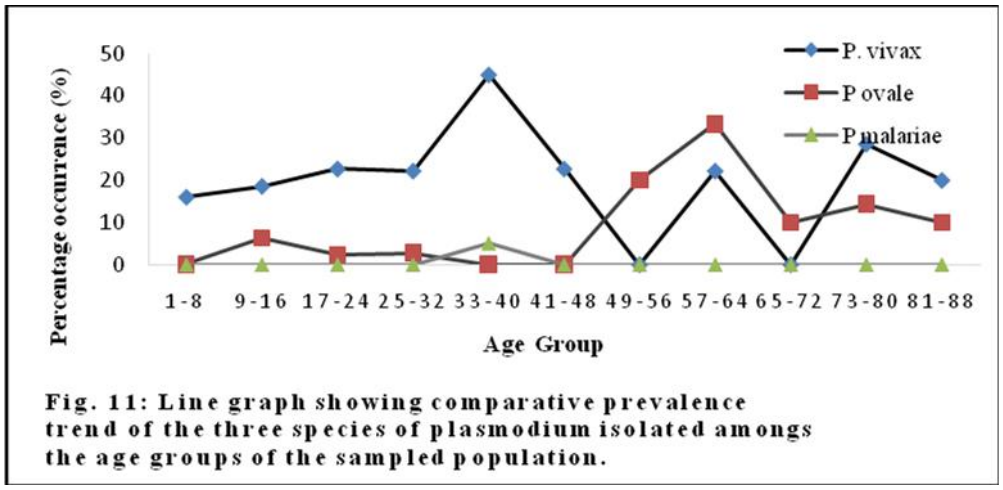

Fig. 11: Line graph showing comparative prevalence trend of the three species of plasmodium isolated amongs the age groups of the sampled population.

Fig. 12, shows prevalence of the percentage of the sample population infected with mixed non-*falciparum* plasmodium species. Among the total sampled population, 8(2.9%) were infected with *P. vivax* and *P. ovale* co-infection, 1(0.4%) was infected with *P. vivax* and *P. malariae* co-infection while 289(96.8%) were negative.

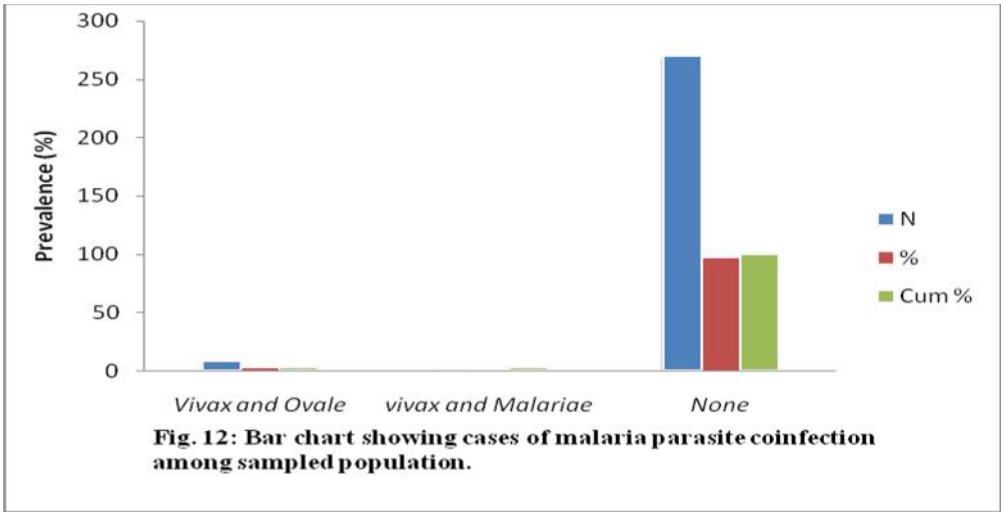

Fig. 12: Bar chart showing cases of malaria parasite coinfection among sampled population.

Discussion

The likely high frequency of misdiagnosis by microscopy which is the adopted method for malaria diagnosis in Nigeria could be a direct result of the indistinguishable initial blood stages of parasite species as well as low degree of parasitaemia as an outcome of either rivalry between the two species at the platelet level [20], cross species insusceptibility [21] or both. Moreover, microscopists regularly are slanted to recognize just one specie based on pre-considered thoughts of the malaria epidemiological setting of the area, leaving the uncommon species undetected. The Rapid Detection Test (RDT) based on the immunochromatographic location of antigens by monoclonal antibodies is utilized notwithstanding alongside microscopy [22], however these techniques need precision and particularity for the recognizable proof of non-*falciparumplasmodium*like *P. knowlesi* [23]. These strategies in spite of being fast, straightforward and simple to decipher, most occasions just distinguish explicitly *P. falciparum* infection.

The aftereffects of this investigation demonstrated that the assessment of thick and thin blood smears by microscopy were inadequate for the conclusion of malaria in this area. This examination concurs with the possibility that precision diminishes with microscopic tests as parasitaemia falls under 100 parasites/ml and false negatives are missed [3]. In this manner underlining that nPCR is more delicate and explicit than microscopy, as it has been accounted for somewhere else [3].

Malaria parasitaemia was somewhat higher among the males (P>0.05) than the females (Fig. 1). This concurs with the outcome acquired by Mendel and White [24]; Pelletier *et al*. [25]; Malcom [26]. Studies have indicated that females have better insusceptibility to malaria and assortments of other parasitic infections and this was credited to hormonal and genetic factors [24].

Prevalence of *P. vivax* malaria parasitaemia was measurably noteworthy (P<0.05) (Figs. 2-10) among various age groups, this disagrees with Wobo *et al.* [27] who revealed the nonattendance of *P. vivax* malaria in the south east and Erhabor*et al*. [28] who detailed that the parasite isn't found among indigenous Nigerians. In spite of the fact that this could almost certainly be because of the recognizable proof strategy (microscopy) utilized. The presence of this parasite among a populace viewed as Duffy-negative (FY*BES/*BES) genotype concurs with Menard *et al.* [29] who announced that *P. vivax* is equipped for causing blood stage disease among Duffy-antagonistic individuals and this may be on the grounds that the parasite has discovered a method of tainting human erythrocytes without the Duffy antigen. The presence of *P. ovale* and *P. malariae* in this area is in concurrence with the reports of Molineaux *et al*. [30] and May *et al*. [31] who revealed the presence of, *P. malariae* and *P. ovale* in Garki, Northern Nigeria and Ibadan, South-western Nigeria individually.

To test the potential outcomes of mixed infection, microscopically inspected slides were compared to malaria infection recognized by PCR examine (Fig. 11). Misdiagnosis of these slides can be credited to human mistake; or the outcome of either insensitivity of microscopy-based finding, differential pathogenic conduct of the two parasite species or both. For instance, in *P. vivax*-contaminated patients, the greater part of the existence cycle stages ring, trophozoite, schizont and gametocytes flow in human fringe blood, yet in *P. falciparum* contamination, just ring-stage and gametocytes are available in peripheral circulation. Immature blood stages (e.g., ring stages) of *P. falciparum* and *P. vivax* look fundamentally the same as under the magnifying Microscope, and ignoring one species on this premise is very conceivable [32]. Along these lines, if *P. vivax* gametocytes are not various in the example, it builds the opportunity of diagnosing a mixed infection as a solitary infection of P. falciparum, which may clarify the current outcomes.

This study plainly uncovers an extremely high percentage of mixed malaria infections due to *P. ovale* and *P. vivax* and *P. falciparum* and *P. vivax* in Nigeria, like what has been found in numerous other malaria-endemic areas (for example in South America and Africa in general) [33][34]. It has been hypothesized that a higher generally speaking prevalence of *P. vivax* and *P. ovale* is related with less mixed-species infections [35], with either *P. ovale* or *P. vivax* initiating cross-species invulnerability in the host [21].

Shockingly, an average of 59% of unadulterated *P. vivax* infections was identified in tests gathered in *P. falciparum* endemic regions. Despite the fact that factors liable for such a high pace of unadulterated *P. vivax* infection in *P. falciparum*-endemic territories are obscure, this may be a result of the cyclic

occasional changes of infection by various malaria parasites in a specific Nigerian region [36].

Further, an effect of eco-topographical minor departure from the prevalence and transmission of the parasite species [37] can't be precluded. The example of infection (single⁄mixed) is additionally dictated by the event and the capacity of the vector species to be infected by different parasite species at the same time [32].

Conclusion

Precise identification of the malaria parasite species is significant for fruitful treatment, yet additionally to plan and create powerful malaria control measures and exact malaria-epidemiological checking. Wrong malaria finding is a serious general wellbeing concern; incapably treated *P. vivax* infections can prompt backslides, and undiscovered *P. malariae* and *P. ovale* infections can turn extreme and lethal. Further, misidentification of malaria parasites could stretch the parasite leeway time and lead to recrudescence and medication obstruction.

Authors' contributions

Umeh, S.I. and Braide, W. supervised the entirety of this study, while Chibundu, N.O. conducted the sampling, interview and survey, analysed the data and wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that there are no competing interests.

Availability of data and material

Data will not be shared.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of Federal Medical Centre Owerri and St. David Hospital Owerri, Nigeria while Medical Health participants gave written consent to participate in the refreshed training.

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