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RESEARCH ARTICLE

MOLECULAR IDENTIFICATION OF PLASMODIUM FALCIPARUM ISOLATES IN OWERRI MUNICIPALITY USING NESTED POLYMERASE CHAIN REACTION (nPCR)

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Abstract

As Public health seek ways to annihilate malaria, there has been special attention to abrogate low-density parasite reservoirs in carriers. This research was designed to detect *Plasmodium falciparum* using nested polymerase chain reaction (nPCR) as a diagnostic tool to test for malaria prevalence in Owerri municipality, and the result compared to that of microscopy. A total of 300 blood samples were collected from febrile patients who sought treatment from selected hospitals in Owerri municipal. Microscopy was carried out on Giemsa-stained blood smears of the samples. Extraction of *Plasmodium falciparum* DNA from the blood samples was carried out, and the DNA products were analyzed using nPCR with genus and specie specific primers in a two-step amplification of the *Pfmdr* gene. The PCR products were analyzed in ethidium bromide stained 2% agarose gel. The result from microscopy showed that 130(43.3%) samples were *P. falciparum* positive and 170(56.7%) samples were negative, while that of nPCR, showed 202(67.3%) samples were positive and 98(32.7%) samples were negative. Out of the 170 samples that were microscopically examined as negative, 72(42.35%) were positive with nPCR. This indicates that there is high level of false negative result by microscopy, which may either lead to negation for a deserved treatment or undeserved treatment. Accurate diagnosis of *Plasmodium falciparum* is of importance in the treatment of malaria. Microscopic examination of blood smears remains the "gold standard" for malaria detection and speciation. In spite of the fact that there is significant drop in the number of positive cases reported from microscopy, antimalarial drugs prescriptions are on the rise as patients exhibit symptoms of malaria. This makes it tough to confirm accuracy, sensitivity and specificity of light microscopy in diagnosis of malaria in epidemic areas. Polymerase chain reaction offers an alternative to microscopy having shown to have superior sensitivity and specificity. Diagnosis of malaria parasites by nPCR will serve as a useful complement to microscopy, albeit it's high cost and time consumption. In addition, negative patients suspected to have malaria should be subjected to PCR diagnosis to better drug use.

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Introduction:-

Malaria is a serious problem in Africa where one in every five (20%) childhood deaths is due to the effects of the disease [121]. The World Health Organization (WHO) reckon that Africa bears almost 90% of the global burden of malaria. Nigeria due to her population of over 180 million people bears the largest share of the burden, with over 300, 000 malaria related deaths annually, especially in children under 5years [162]. It is reported that every 30 seconds, a child dies from malaria. About 3.3billion people are at risk of malaria [121]. Many children who survive an episode of severe malaria may suffer from learning impairments or brain damage [165].

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Malaria is an infectious disease caused by the *Plasmodium* parasite and spread through mosquito bites [59]. Mosquitoes can be found in several parts of the world, the malaria-transmitting *Anopheles gambiae* mosquito is generally restricted to sub-Saharan Africa and some parts of Asia and Latin America [163]. There are currently five species of the genus *Plasmodium* known to infect humans: *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium knowlesi*. Of these five species that infect humans, *Plasmodium falciparum* is the most common in Africa, and is the species that causes the most severe disease [89].

There are still high cases of malaria globally, despite efforts to eliminate it. In 2015, there were 214 million cases of malaria and 438,000 deaths estimated, which primarily affected the sub-Saharan African population and children under 5 years of age [160]. Another challenge and focus in global health is to prevent drug resistance (notably Artemisinin combination therapy (ACT), the frontline treatment for *P. falciparum* infection) from becoming well established in Africa, where the consequences for childhood mortality could be disastrous [35].

The gold standard method for diagnosing malaria remains light microscopy of thick and thin stained blood smears [86]. Thick smears are 20–40 times more sensitive than thin smears for screening of *Plasmodium* parasites, with a detection limit of 10–50 trophozoites/ μ l [144]. Thin smears allow one to identify *Plasmodium* species (including the diagnosis of mixed infections), quantify parasitemia, and assess for the presence of schizonts, gametocytes, and malarial pigment in neutrophils and monocytes [144]. Diagnosis based on polymerase chain reaction for species-specific *Plasmodium* genome are the more accurate, sensitive and specific compared to microscopy, and are capable of detecting as few as 10 parasites/ μ l of blood [45]. WHO recommends that malaria be confirmed by parasite-based diagnosis before giving treatment [160]. In this research, Nested polymerase chain reaction was used to detect *P. falciparum* and the complicity of parasite populations in patients with malaria infections in Owerri municipal, Imo State, Southeastern Nigeria.

Materials and Methods:-

Study Area

Owerri municipal is a local government area in Imo State, Nigeria. It has an estimated population of above 127,213 as of 2006 (National and State Population Census, 2006) and is approximately 58 square kilometres in an area with latitude and longitude of 5° 28' 34.7160" N and 7° 1' 33.0708" E. Its elevation is around 71 meters in height which is equal to 233 feet. Owerri is bordered by the Otamiri River to the east and the Nworie River to the south [54]. Owerri has a tropical wet climate according to the Köppen-Geiger system. Rain falls for most months of the year with a brief dry season. The Harmattan affects the city in the early periods of the dry season and it is noticeably less pronounced than in other cities in Nigeria. The average temperature is 26.4 °C [54].

Ethical clearance and consent:

Scientific and Ethical clearance were obtained from St. David's Hospital, John Charles Hospital and St. Charles Hospital (all are within Owerri municipality). Volunteers were recruited with their consent and written informed consent were given to parents or guardians of children that participated in the study.

Study Population:

The blood samples from recruited participants were as a result of random sampling of the general population (those who sought treatment from the mentioned hospitals).

Sample Collection:

A total of 300 blood samples between April and May 2017 were collected from patients who sought treatment at St. David's Hospital, John Charles Hospital and St. Charles Hospital, Owerri, Imo State. The samples were collected with EDTA bottles and preserved in a refrigerator prior to the scheduled date for analysis.

Analysis of Samples

Microscopy:

Thick and thin blood film slides were prepared using Giemsa solution. The stained slides were examined under a light microscope using 100 $\times$ oil immersion. A smear was considered to be negative when no parasite was detected in the total area, where 200 white blood cells (WBCs) are observed [22].

DNA Purification (Extraction)

Genomic DNA was extracted using the quick gDNA mini prep DNA extraction kit supplied by Inqaba Biotechnological, South Africa. The procedures were performed at room temperature (15 – 30°C). All centrifugation steps were performed at 10,000rpm – 16,000rpm.

DNA Quantification

DNA quantification was done on a Nano-drop 1000 spectrophotometer. Two microliter of the extracted product (DNA) was placed on lower pedestal and the concentration read from the Nano-drop software on a computer system.

DNA Amplification by Nested PCR (nPCR)

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.* 1993 [133].

Components:

1X Master Mix (containing Four Deoxyribonucleotide triphosphates (dNTPs), Magnesium chloride (MgCl₂), Buffer and Taq polymerase), Forward and Reverse Primers, Template and Water.

Primers for PCR amplification reactions:***Plasmodium* genus-specific :**

Forward: rPLU6 5' TTAAATTGTTGCAGTTAAAACG 3' and

Reverse: rPLU5 3'CCTGTTGTTGCCTTAACTTC 5'

***Plasmodium* species-specific (*P. falciparum*) :**

Forward: rFAL1 5' TTAACTGGTTTGGGAAAACCAATATATT and

Reverse: rFAL2 3'ACACAATGAACTCAATCATGACTACCCGTC 5'.

Amplification:

The reaction mixture comprised of 4µl of template DNA, 1.5 mM magnesium chloride, 100nM dNTP, 0.4nm of each primer and Taq polymerase at 1unit/reaction to a total volume of 25µl with PCR water (water free of Dnase), these were setup alongside with 0.5µl 3D7 and DD2 standard lab clones that were used as control. The first and second PCR amplifications were carried out in 25 µl total volume respectively. The nPCR amplification required a total of six hours to complete both the first and second amplifications. The product from the first amplification (nested 1) served as the template for the second amplification.

Primary Amplification:

An initial amplification was performed using primers rPLU6 and rPLU5 under the following conditions:

Initial Denaturation: 94°C/3mins;

Denaturation: 94°C/1min;

Annealing: 60°C/2mins;

Extension: 72°C/1 min;

Final Extension: 72°C/5mins

× 30 – cycles

Secondary Amplification:

For the secondary amplification, 2µl of primary amplicon was transferred to a second tube with fresh reagents and inner primers rFAL1 and rFAL2. 0.5µl of the clones were used as control. Amplification conditions were:

Initial Denaturation: 94°C/3mins;

Denaturation: 94°C/1min;

Annealing: 50°C/1:45secs;

Extension: 68°C/1:30secs;

Final Extension: 68°C/7mins.

× 30 – cycles

PCR Products Analysis by Gel Electrophoresis

The PCR products were confirmed and analysed on a 2% agarose gel electrophoresis with 0.5µg/ml of ethidium bromide. The electrophoresis gel ran for 20 minutes at 80 volts on a horizontal electrophoretic tank submerged with 1× TAE buffer (Tris–borate-EDTA). DNA amplification products were visualized under ultraviolet light against a 100 base pair marker on a transilluminator and the results were documented using camera.

Statistical Analysis

Data entry and analyses were performed using EPI DATA™ 3.1 and MINITAB. The descriptive data was given as a mean with standard deviations, frequency counts, and percentages.

Results:-

Demography of the Study Population

A total of 300 patients were sampled for this study. The patients reside in Owerri municipal and were between the ages of 1-90 years. Among the patients, 151(50.3%) were Males while 149(49.7%) were Females (Table 4.2). Considering their educational status 107(35.7%), 64(21.3%) and 129(43%) had primary, secondary and tertiary education. Majority of the patients were students (61.7%) while 42(14%), 31(10.3%) and 13(4.3%) were civil servants, self-employed and pensioner/retirees respectively (figure 4.1).

Occupational Status of Patients

The stratification of malaria prevalence with respect to occupational status of the patients, detected using PCR and microscopy, was not statistically significant ($P=0.621$, $p<0.05$). (Table 4.1).

Table 4.1:- *falciparum* malaria prevalence with respect to occupation of the patients.

Occupation	N	%	PCR	Staining
Student	185	61.7	127(68.6%)	93(50%)
Civil servant	42	14	30(71.4%)	10(23%)
Self employed	31	10.3	15(48.4%)	7(22.6%)
Pensioner/Retired	29	9.7	20(69%)	16(55.2%)
Unemployed	13	4.3	10(76.9%)	4(30.8%)
Total	300	100	202(67.3%)	130(43.3%)

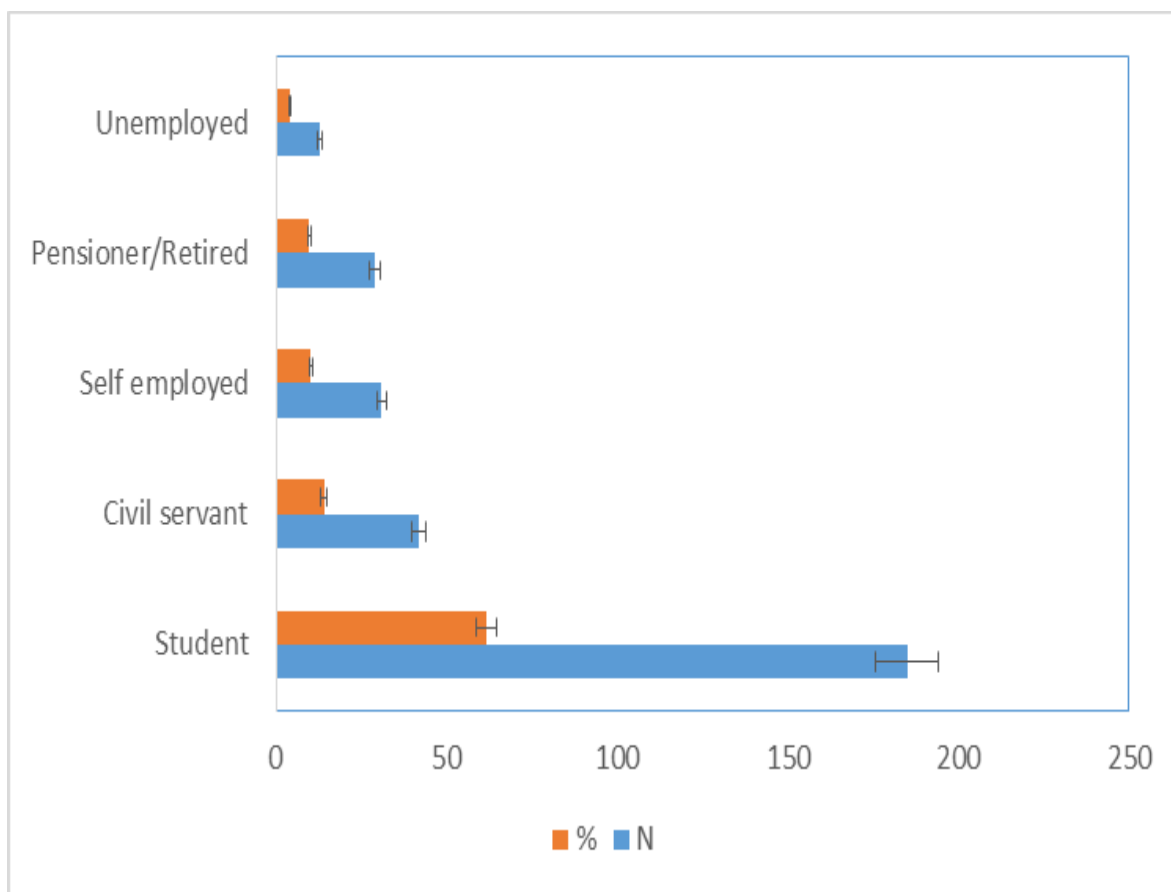


Figure 4.1:- Bar graph showing occupation status of the patients.

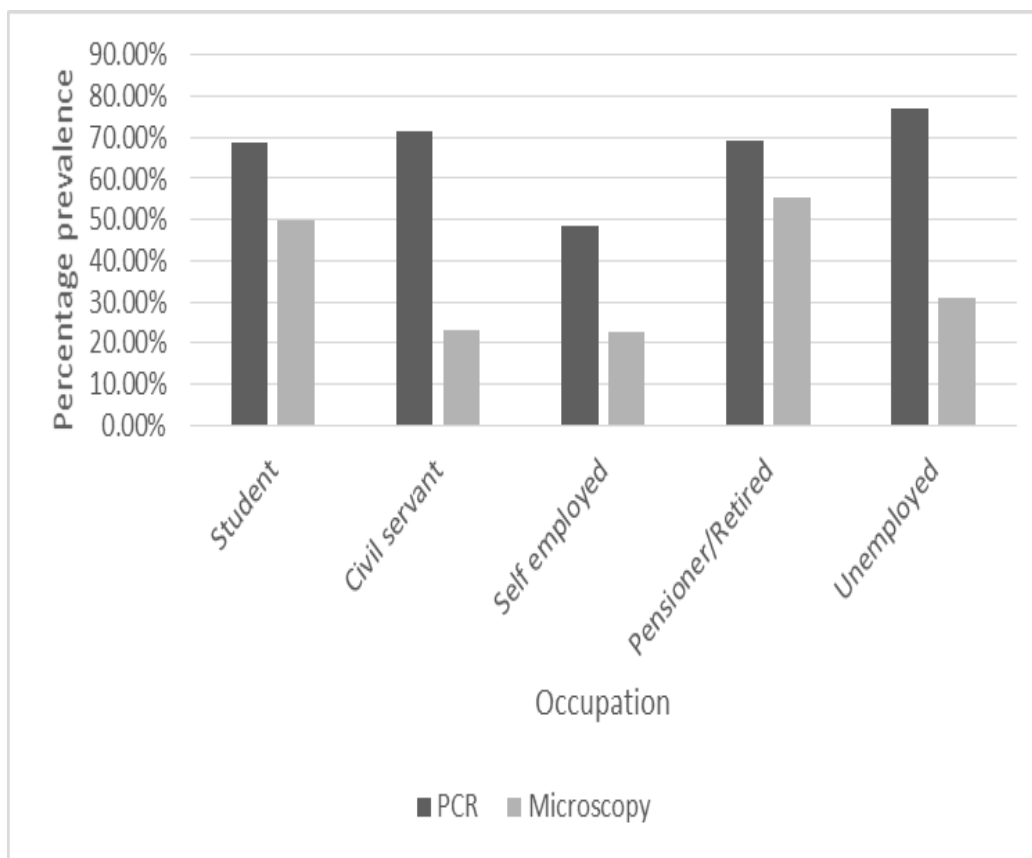


Figure 4.2:- Bar graph showing *falciparum* malaria prevalence with respect to occupation of patients as detected by nested PCR and microscopy.

Age distribution

The age-group 81-90 years had the highest malaria prevalence (100%) in both PCR and microscopy, while the age groups 41-50 and 51-60 years in microscopy and PCR respectively had the least 16.67% and 40% as shown in Table 4.2 and figure 4.3 below. The results were significant ($P=0.02$, $p<0.05$).

Table 4.2:- Prevalence of malaria parasite in infected persons of different age groups detected by Microscopy and Nested PCR.

Age	Male	Female	Total	Microscopy (positive)	nPCR (positive)
1-10	52	50	102	64(62.75%)	72(70.59%)
11-20	32	36	68	23(33.85%)	44(64.71%)
21-30	14	22	36	12(33.33%)	22(61.11%)
31-40	22	12	34	6(17.65%)	26(76.47%)
41-50	8	4	12	2(16.67%)	8(66.67%)
51-60	7	13	20	7(35%)	8(40%)
61-70	6	4	10	4(40%)	8(80%)
71-80	8	4	12	6(50%)	8(66.67%)
81-90	2	4	6	6(100%)	6(100%)
Total	151	149	300	130	202

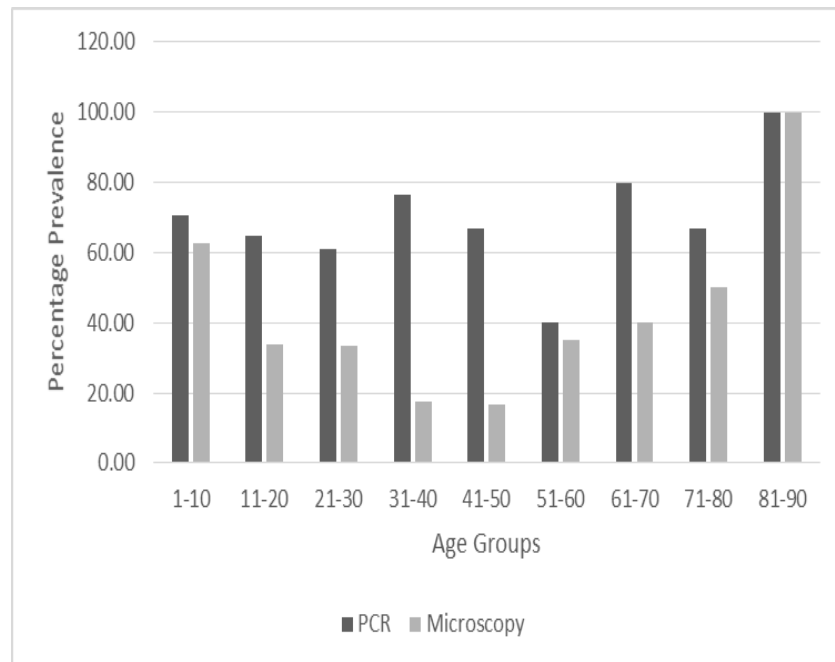


Figure 4.3:- Bar graph showing the number of positives among age groups by nested PCR and microscopy from the samples diagnosed.

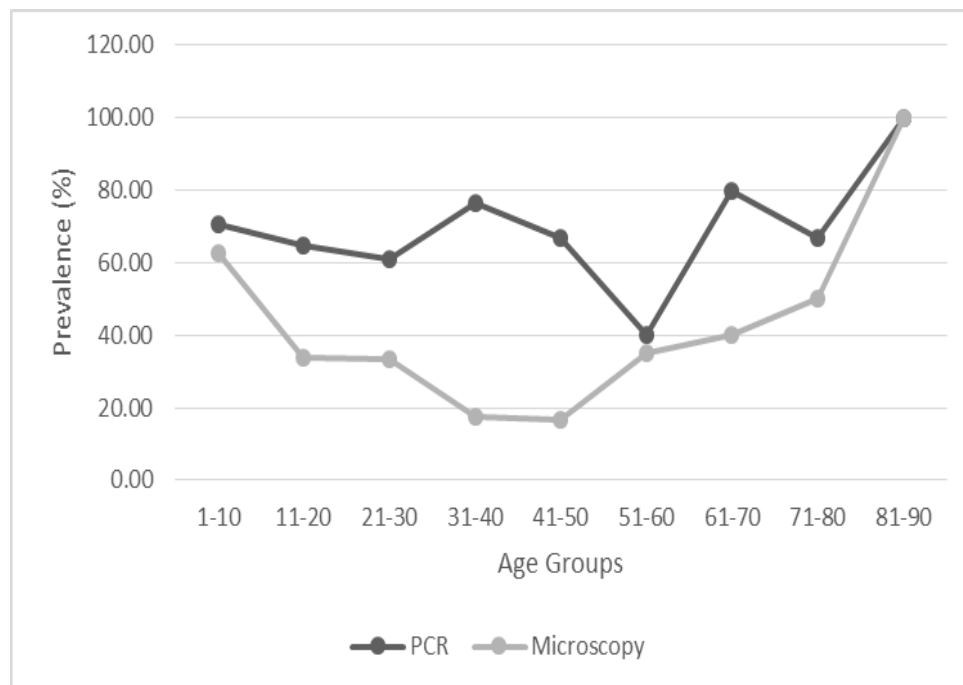


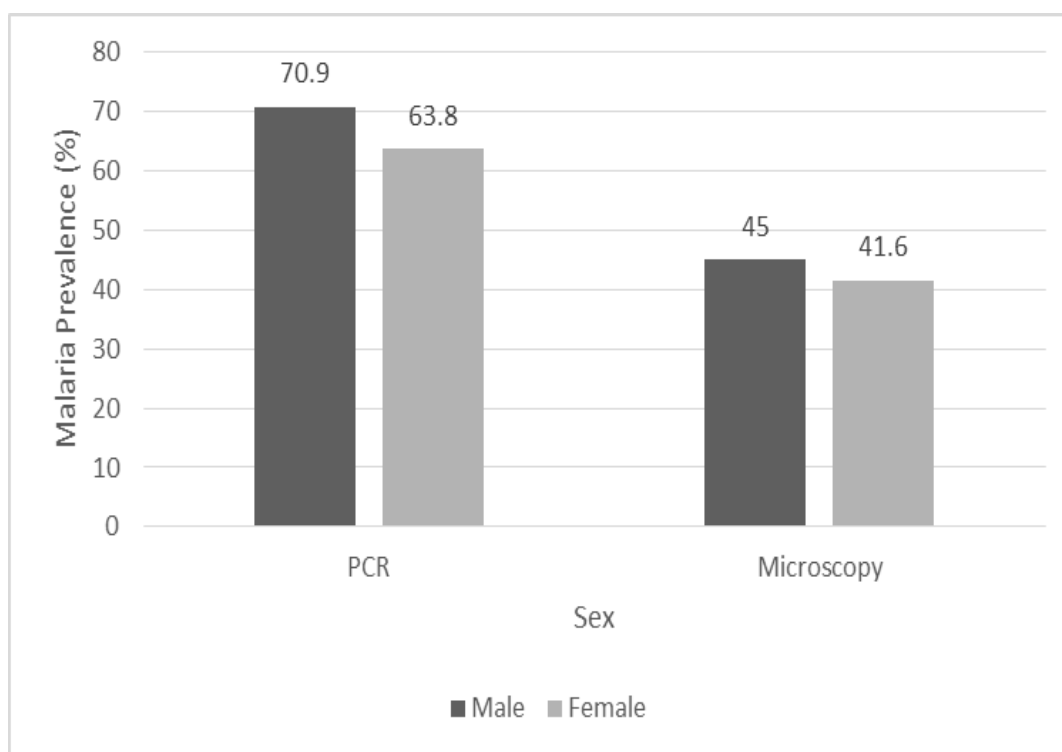
Figure 4.4:- *Plasmodium falciparum* prevalence trend among the different age groups

Sex Distribution

The sex distribution of *Plasmodium falciparum*, showed significant difference at ($P=0.001$, $p<0.05$) (Table 4.3). Table 4.5 showed that males 107/151 i.e. 70.9% were more infected than females 95/149 i.e. 63.8% as detected by PCR, likewise as detected by microscopy 45% and 41.6% in male and female respectively (Figure 4.5).

Table 4.3:- Table showing number of malaria positive samples and prevalence among male and female.

Sex	N	%	PCR	Staining
Male	151	50.3	107(70.9%)	68(45.03%)
Female	149	49.7	95(63.8%)	62(41.6%)
Total	300	100	202	130

**Figure 4.5:-** Bar graph showing sex distribution of *falciparum* malaria prevalence detected by nested PCR and microscopy.**Educational Status**

The level of education of each of the 300 patients involved in the study, were noted.

Considering the educational status of the patients in relation to positive malaria results (Table 4.4), showed no significant difference ($P=0.085$, $p<0.05$) between the groups.

Table 4.4:- Level of education of the patients.

Education	N	%	PCR	Staining
Primary	107	35.7	76(71%)	67(62.6%)
Secondary	64	21.3	46(71%)	25(39.1%)
Tertiary	129	43	80(62%)	38(29.5%)
Total	300	100	202	130

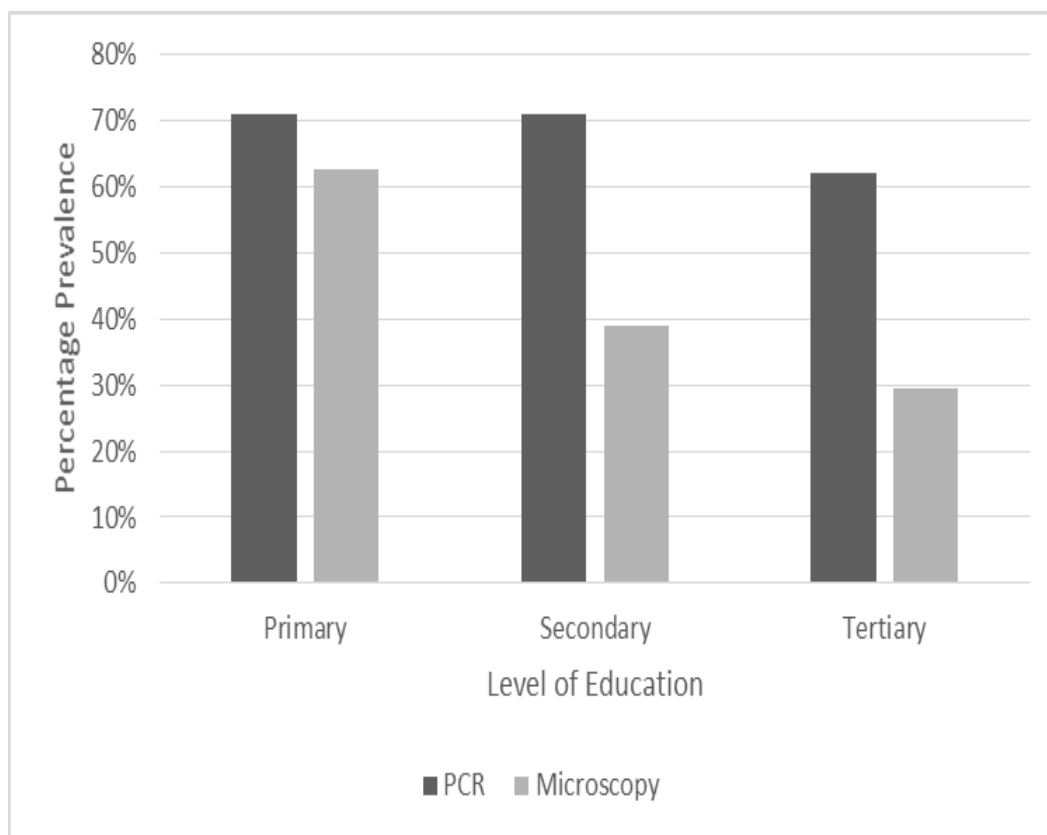


Figure 4.6:- Bar chart showing malaria prevalence with respect to educational status of the patients.

PCR products of specie specific primer

Lane 11-23 represent *Plasmodium falciparum* (206bp) isolates (figure 4.7). Lane 24-25 represent the controls (3d7 and Dd2), while, M represents Quick- Load 100bp molecular ladder.

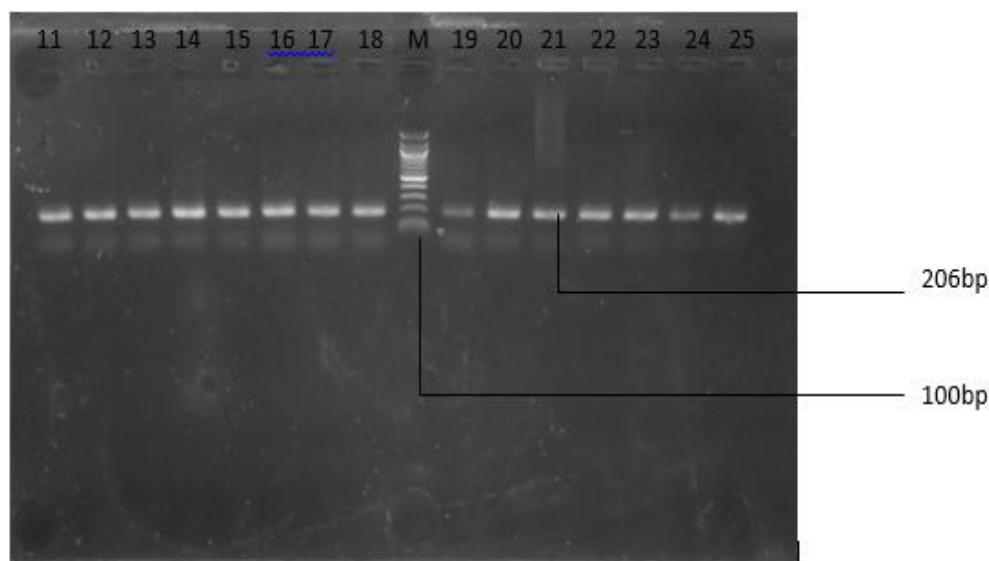


Figure 4.7:- Agarose gel electrophoresis picture showing PCR products obtained using specie-specific primer.

Plasmodium falciparum Identification

Out of the 300 samples that were examined for *Plasmodium falciparum*, 130(43.3%) samples were positive while 170(56.7%) samples were negative using Staining technique (Table 4.5). On the other hand, 202(67.3%) samples were positive and 98(32.7%) samples were negative using the PCR based technique (Table 4.5).

Table 4.5:- Sensitivity and specificity of nested PCR against microscopy in the detection of *Plasmodium falciparum*.

Target <i>Plasmodium</i> spp		
	N	%
<i>Falciparum</i>	300	100
Total	300	100
Diagnostic results from staining technique		
	N	%
Positive	130	43.3
Negative	170	56.7
Total	300	100
Diagnostic result obtained from nPCR technique		
	N	%
Positive	202	67.3
Negative	98	32.7
Total	300	100

Departments where Samples were taken from

There was no statistical significant difference between the means of the compared characters at ($p=0.641$, $p<0.05$). This implies that the results obtained do not depend on the department where the samples were taken from (Table 4.6).

Table 4.6:- Departments where samples were taken from

Department	N	%
OPD	197	65.9
ANC	22	7.4
Pediatrics	20	6.7
MEdLab	60	20.1
Total	299	100

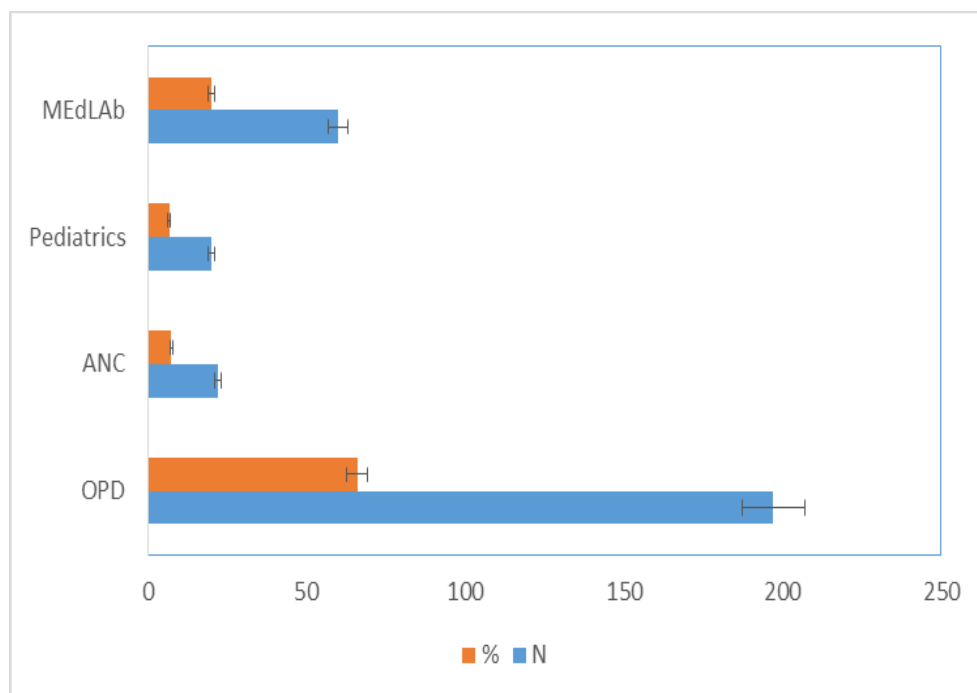


Figure 4.8:- Graph showing departments where samples were taken from.

Awareness for the Existence of falciparum Malaria

300 respondents were selected among the medical profession to determine the awareness of the practitioners to the existence of the infection (Table 4.7). 56(18.7%), 122 (40.7%), 22(7.3%), 82(27.3%) and 18(6%) of the respondents were Doctors, Nurses, Administrative officers, Medical Laboratory Scientists (MLS) and Medical Laboratory Technicians (MLT) respectively (figure 4.9).

Table 4.7:- Sample distribution of interviewed medical personnel.

Personnel	N	%
Doctors	56	18.7
Nurses	122	40.7
Administrative officer	22	7.3
MLS	82	27.3
MLT	18	6
Total	300	100

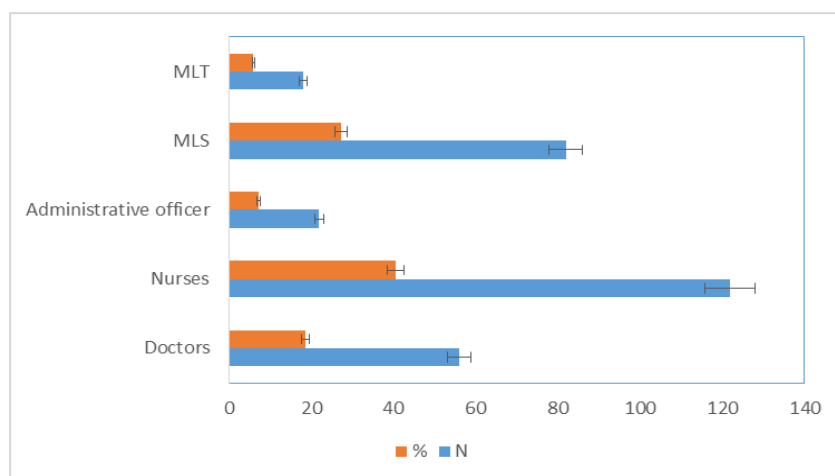


Figure 4.9: Bar graph showing sample distribution of interviewed medical personnel.

Figure 4.10 and Table 4.8 shows the results for the awareness of the occurrence of different species of *Plasmodium* spp. More respondents (100%) were aware of the existence of *Falciparum* species of *Plasmodium* infection than the other species. The differences in their views was not statistically significant at ($p=0.128$, $p<0.05$), meaning that the outcome did not occur by experimental chances.

Table 4.8:- The results for the awareness of the occurrence of different species of *Plasmodium* spp.

Species	Yes	No	Maybe
<i>P. ovale</i>	16	229	55
<i>P.falciparum</i>	300	0	0
<i>P.malariae</i>	0	295	5
<i>P.vivax</i>	1	291	8

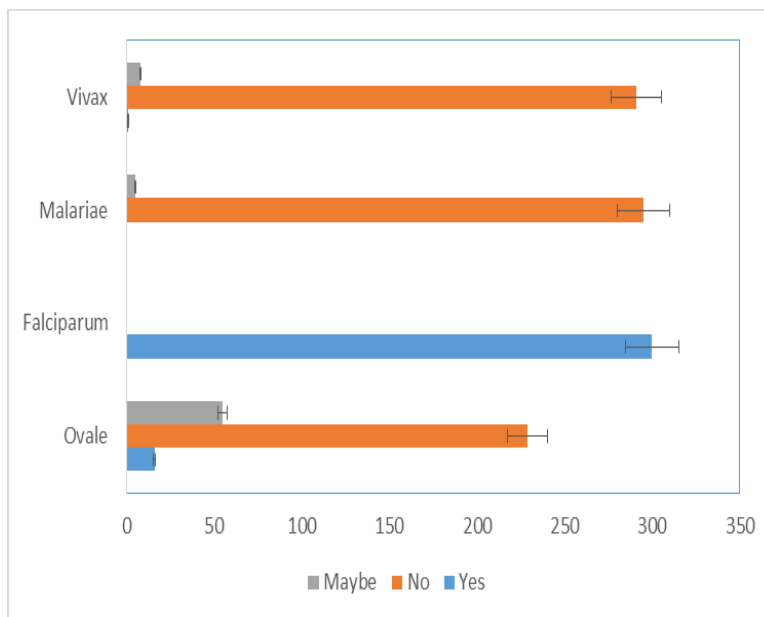


Figure 4.10: Bar graph shows the results for the awareness of the occurrence of different species of *Plasmodium* spp.

Discussion:-

Malaria is a serious disease caused by a parasite belonging to the *Plasmodium* genus and is transmitted by female Anopheles mosquito vector [134]. In Nigeria, the disease is endemic in most areas (case study: Owerri Municipal). In this study, blood samples of patients were collected at three hospitals within the study area. The patients who visited the hospitals reside within the area, hence the samples used in the study were representative of Owerri municipality.

An important aim of the study was to employ molecular method to detect *Plasmodium falciparum* from patients, which is the most common in Africa and is the specie that causes the most severe disease [89]. Nested polymerase chain reaction (nPCR) was used as a diagnostic tool to detect DNA of *Plasmodium falciparum* from blood samples of patients. The blood samples had previously been examined by microscopy, which is one of the main traditional methods. The results from nPCR were evaluated and compared against those of microscopy.

A total of 300 blood samples were collected from patients. The samples were tested for *falciparum* malaria using nPCR and microscopy. Result showed that of the 300 blood samples tested, 202(67.3%) samples were positive and 98(32.7%) samples were negative using the nPCR based technique (Table 4.5). While, 130(43.3%) samples were positive and 170(56.7%) samples were negative using microscopy (Table 4.5). The overall malaria prevalence detected by nPCR at 67.3% in the study area was found to be higher than that detected by microscopy at 43.3%. The nPCR method was however able to detect the parasite DNA in some of the samples that had been categorized as malaria-negative by microscopy, this could be a typical case of low parasitemia and PCR was able to detect the

presence of parasite DNA at levels undetectable by microscopy, as reported by Snounou *et al.*, 1993 [133]. This finding suggests that some samples were misdiagnosed negative by microscopy, which could be said to be false-negative. The prevalence detected by nPCR (67.3%) was a bit lower than that of Ukpai and Ajoku, 2001, who reported a high prevalence of 76% in Owerri municipal among out-patients attending clinics. The prevalence was also close to those of similar studies (though they employed microscopy) reported in other parts of Nigeria, 70.8% in Anambra State (Onyido *et al.*, 2014) and 71.1% in Ogun State (Sam-wobo *et al.*, 2014).

In Table 4.2, comparing the results from microscopy and nPCR among different age groups. It was observed that microscopy showed lesser sensitivity in the groups compared to nPCR, except in the age group 81-90 which had the highest prevalence of 100% of *P. falciparum* infections (table 4.2) compared to the other age groups. Prevalence of malaria in other age groups were also high which was in agreement with Uneke *et al.*, 2005 [150], who recorded higher prevalence among the older age groups in a similar study in Jos, Nigeria. This may be due to the fact that at that age, their immunity to parasitic infections weakens and environmental conditions predisposes them to malaria. The prevalence of parasitic infections among the different age groups in the study was significant ($P < 0.05$) indicating that the occurrences of this infection on these age groups were not the same (table 4.4).

Table 4.3 showed the prevalence of malaria in male and female as detected by nPCR as 70.9% and 63.8% respectively, and also, 45% and 41.6% respectively as detected by microscopy. nPCR showed greater sensitivity compared to microscopy. The prevalence was statistically significant ($p=0.001$, $p<0.05$). Tukey and Fisher's Pairwise Comparison showed that there was significant difference between the number of samples tested, nPCR results and microscopy at $p<0.05$. The study showed that *P. falciparum* infections were more common in the male than in the female subjects (Table 4.3). The sex prevalence supports Ukpai and Ajoku (2001) [151], who reported 78.0% and 72.0% prevalence for male and female respectively. It also agrees with the results obtained in Ogun State [124] and those by Ajero *et al.* 2015 [3], which reported prevalence of 27.5% and 24.3% for male and female respectively. The result conforms to the recorded higher prevalence of *Plasmodium* infection in males than in females in the hospital. However, studies have shown that females have better immunity to parasitic diseases and this was attributed to genetic and hormonal factors [66]. Portilo and Sullivan 1997 [111] suggested that genetic factors could play a role by endowing females with immuno-regulatory potentials to cope better with some disease infections. This may equally be attributed to the fact that males expose themselves to the bites of mosquitoes and other vectors more than females, especially when the weather is hot and during farm work. Exception is found during pregnancy and reproductive ages, when females are more vulnerable to malaria attacks due to immune suppression [9].

Prevalence of malaria was not statistically significant ($P=0.621$, $P<0.05$) among different occupational groups. It showed that people in different occupational groups were equally exposed to malaria attack. Subjecting the data further to Tukey and Fisher's Pairwise Comparison, revealed that prevalence among different occupations was not significant between nPCR and microscopy at $P<0.05$. Though in nPCR, prevalence was highest in unemployed, this could be as a result of inadequate protection greater exposure to mosquito bites due to the nature of their contact with the unclean environment exposing themselves to the risk of mosquitoes (Table 4.1 and figure 4.2). Poor people from low socio-economic status, with inadequate housing facilities and financial constraints are unable to engage in malaria preventive and control measures. They are also unable to purchase effective anti-malaria drugs [2].

The stratification of the prevalence according to educational status gave no significant difference ($p<0.05$) between the means of the compared characters using ANOVA and Tukey Pairwise Comparison. Further subsection of the data to Fisher's Pairwise Comparison showed a significant difference between the number of samples and microscopy result, but there was no significant difference between the means of nPCR result and microscopy. Though high prevalence was found among those with primary and secondary education, in nPCR result there was prevalence of 71% for both status, while microscopy was 62% and 39% respectively. This suggests that, education invariably affects people's perceptions about causes of certain diseases of which malaria was not an exception. Lack of knowledge about the consequences of undue exposure to mosquito bites among primary education may have accounted for high prevalence rate of malaria infection.

Some medical practitioners were selected to determine the awareness of the existence of *Plasmodium falciparum*, *P. malariae* and *P. ovale* in the study area. The number distribution of the practitioners (Doctors, Nurses, Administrative officers, Medical Laboratory Scientists (MLS) and Medical Laboratory Technicians (MLT) were 56(18.7%), 122 (40.7%), 22(7.3%), 82(27.3%) and 18(6%) respectively (Table 4.8). The results recorded showed

that 100% of the medical practitioners were aware of the existence of *falciparum* specie of plasmodial infection than the other species (Table 4.7). The differences in their views were not statistically significant at ($p=0.128$, $p<0.05$), meaning that the outcome did not occur by experimental chance (Table 4.8). According to Markell and Voge, 1992, *falciparum* malaria is almost entirely confined to the tropics and subtropics. The Predominance of *P. falciparum* in the prevalence and intensity of malaria in blood donors in Nnewi, Anambra State, Nigeria was also reported by Umeanaeto *et al.* 2006 [149].

Statistically, there was no significant difference between the means of the compared characters of the departments from which the samples were collected at ($p=0.641$, $p<0.05$). It indicated that the results obtained do not depend on the department where the samples were taken from, but could have depended on the exposure of the patients to the parasite (Table 4.6).

The results of this study indicated that the examination of thick and thin blood smears by microscopy were insufficient for the diagnosis of malaria in this region (Table 4.5). This study supported the idea that sensitivity decreases with microscopical tests as parasitaemia falls below 100 parasites/mL and false negatives are observed [133].

Conclusion:-

This study emphasized that nPCR is an excellent method for obtaining accurate epidemiological data in malaria endemic Owerri municipal (Table 4.5). The diagnosis of *P. falciparum* by nPCR might prevent misdiagnosis, incorrect treatment, false positives, false negatives, the emergence and spread of drug resistance, and the transmission of parasites from a malaria-endemic region to other parts.

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