

Original Research Article

Comparative Analysis in Utilizing Different Diagnostic Methods for *Plasmodium Falciparum* Detection within Owerri Municipal

ABSTRACT

Malaria is one of the major public health issue in Africa as its affects human beings at different level. However, any wrong diagnosis of malaria infection can lead to severe ill health, death of the infected individual. This study was carried out to compare different diagnostic methods of malarial detection within owerri municipal in Imo State Nigeria. Blood samples were collected from patients and diagnosed via microscopy, antigen assay technique and polymerase chain reaction. About 200 blood samples collected obtained utilizing the ethylene diamine tetra acetic (EDTA) bottles and examined. In 200 blood samples, microscopy had 75 total number of positive cases and Antigen assay had 83 while, PCR had 94. However, percentage prevalence of about 37% were obtained from microscopy though, about 41.5% were observed in Antigen Assay and 47% for Polymerase chain reaction. There was high malaria prevalence recorded in relation to age within the age range of 31 to 40. The Sensitivity and specificity of the three diagnostic methods shows that PCR had the highest for both while, microscopy had the least sensitivity followed by RDT. Moreover, positive predictive value and negative predictive value assessment were analyzed. The results showed that Microscopy has a 0.2% chance of missing a true positive and antigen assay has 0.1% while, PCR have 0%. Therefore, more emphasis should be given to PCR as the possibility of an error during diagnosis is less.

1.0 INTRODUCTION

The malaria parasite, *Plasmodium falciparum* is the highly widespread, pathogenic and deadliest of all five *Plasmodium* species and so can cause severe and lethal malaria infection in humans, if treatment is delayed [1]. Hence, the global focus on malaria as a public health problem remains a trans-generational trend especially in malaria endemic regions such as Africa and some parts of Asia. This is clearly in an attempt to eradicate malaria and curb its afflictive impacts on the health, economic and social lives of the inhabitants of these regions. Despite the fact that a notable decline in malaria mortality was achieved globally between 2010 and 2018, the estimated rate of the pandemic in Nigeria still amounts to 27% of the disease burden in Africa [2 & 3]. Moreover, the COVID-19 outbreak during the last quarter of 2019 stirred a possible negligence in malaria elimination plan worldwide, thereby causing the malaria mortality rate to increase by 2% between 2019 and 2021 [1]. Consequently, the World Health Organization (WHO) approved the T3 (Test, Treat and Track) strategy as a therapeutic intervention needed for precise diagnosis of every suspected case and proper treatment with antimalarial drugs [4]. One of the hurdles yet to be surmounted in malaria control and eradication process, is incorrect diagnosis due to different degrees of sensitivity of the malaria diagnostic tools. It is therefore crucial to decipher an accurate, quick, specie-specific and highly sensitive test technique required for an effectual malaria surveillance and treatment. Microscopy has been generally accepted as the gold standard in laboratory diagnosis of malaria [5 & 6]. Its positive attributes include being cost effective and relatively sensitive when properly handled by a good microscopist [3 & 5]. However, it has some undeniable limitations like high demand of expertise and power supply [3], reduced reliability at low density parasitaemia [4], inability to differentiate mixed species infection (*P. ovale* from *P. vivax*) and being time-consuming [3]. Malaria

antigen assay is a rapid diagnostic test (RDT) aimed at detecting histidine rich protein 2 (pHRP 2) of *Plasmodium falciparum* in human whole blood sample. The malaria RDT kits are quite handy, economical, time saving and highly recommended for use both in the laboratories and the field; as such, they can easily be transported and operated by unskilled personnel in the absence of electricity supply [4 & 6]. False positive results are commoner with malaria antigen assay, due to its tendency to detect pHRP-2 in the blood up till a month after the treatment of an active infection [7] and this disqualifies it as a follow-up method [8]. Furthermore, several studies [3,4,5,6, 7 & 8] have confirmed polymerase chain reaction (PCR) as a quick, very sensitive, specific and more reliable diagnostic method that detects parasite DNA, even as low as 0.5–5 parasites per microliter (μL) of blood. The PCR method makes better diagnosis in asymptomatic and sub-microscopic patients compared to microscopy and RDT [7]. Notwithstanding, the PCR machine is usually found in regions that have sophisticated and well-equipped medical centres, with readily available power supply and well trained technicians needed to operate it [8]. This makes it very expensive to maintain, difficult to use and unfit for field work. In Nigeria, the use of microscopy and PCR techniques in malaria diagnosis is often handicapped by low power supply, mostly at the rural parts of the country where high transmission rates are recorded. Therefore, the study is to ascertain the sensitivity and specificity of the three diagnostic methods and determine the frequencies of cases missed by traditional methods that are detected by PCR. Understanding proper diagnostic method is imperative in eliminating the mistake of clinical presumption and drug pressure which leads to resistance. Thus most treatment failure is caused by improper diagnosis.

Keywords: Malaria, diagnosis, Plasmodium falciparum, PCR.

2.0 STUDY METHODS

2.1 Study Area

The study was conducted within Owerri municipal, Imo state. Owerri is a diverse city positioned in the tropical rain forest zone of Southeast Nigeria. The population of people in the location is approximately 3.9 million people (Census, 2006). The mean daily temperature is about 26.4°C.

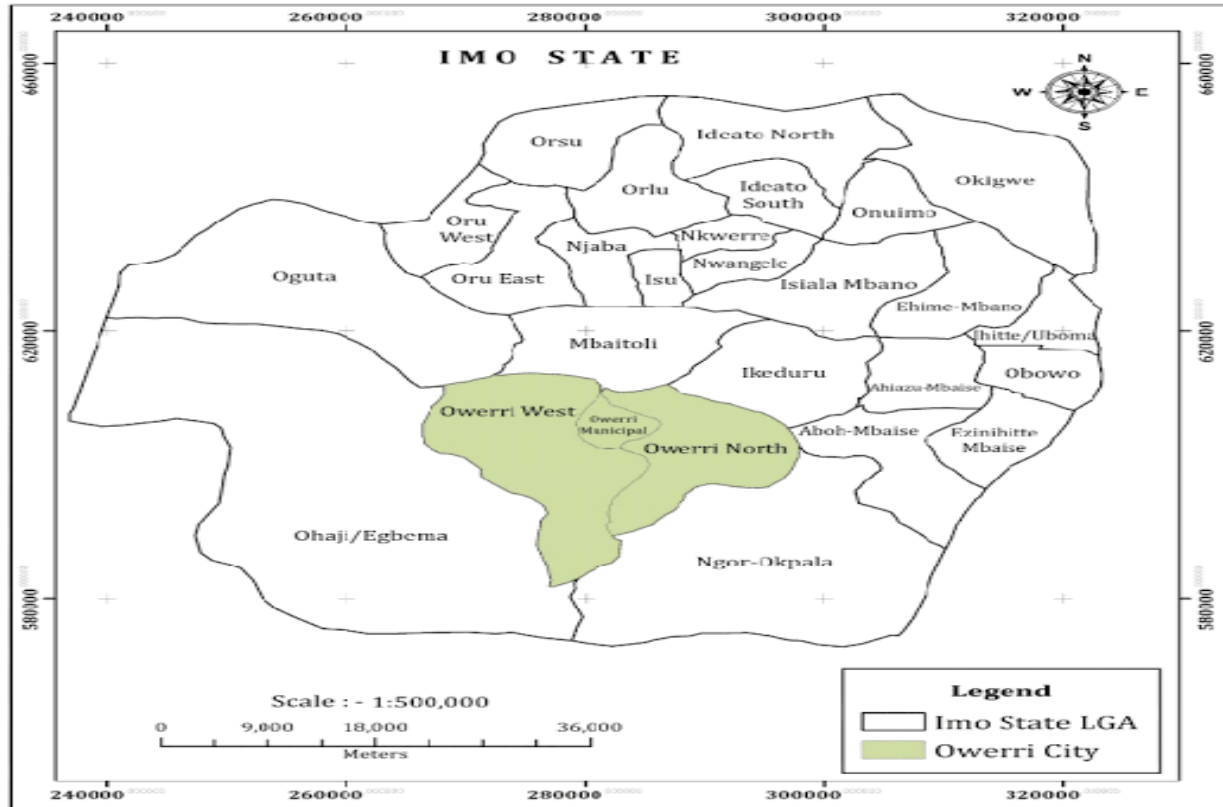


Fig 1. Map of owerri municipal (Onwuadiochiet *al.*, 2021).

2.2 Study Population

The research was conducted utilizing both gender of about 21- 60 age bracket who came for malaria diagnosis at Everight Diagnostic Laboratory Owerri. About 200 patients were nominated for the study due to cost, lack of funding and time factor, exclusion criteria include those below 21 years of age due to the challenge of informed consent and those who have use anti-malaria medication 2 weeks earlier.

2.3 Sample collection

Patient data was collected with the use of questionnaire with references to age, sex, marital status and whether pregnant, nursing mother or not pregnant. With the use of needles and syringe, blood specimen were taken intravenously from each patient and transferred into an Ethylene Diamine Tetra-Acetic (EDTA) bottle to avoid coagulation and covered tightly. Specimen was labeled correctly with the following data: M for male and F for female, with the following age range; 21-30, 31-40, 41-50 and 51-60. Of the total 200 patients 111 were females and 89 were males. Of the 111 females 28 were pregnant, 12 were nursing mothers while 71 were single females [14].

2.4 Microscopic testing for *Plasmodium falciparum* parasite

A slide was taken and labeled in respect to the label on the EDTA bottle. A drop of blood was taken from the bottle and placed on the microscopic slides. A smear was made by sliding forward the drop of blood using a cover slip. It is confirmed to be useable if it has a head, a tail and thin enough. The film was allowed to air dry for about 5-10 min and soaked in methanol briefly. The film was allowed to air dry for

another 2-5min before soaking in safranine red for 2mins. The fixed slides was washed off using running water tap and fixed again in methylene blue for another 2 min. Afterwards the fixed blood films were washed off and allowed to air dry [14].

2.4.1 Microscopic Examination of *Plasmodium falciparum* parasites

A drop of glycerin was mounted on the fixed blood films to avoid drying of the specimen, and help in the refractive index of the microscope. It was then covered with a cover slip and mounted on a (Hisense) microscope with a magnification of 100x. The slides were scanned systematically both vertically and horizontally through the field. Presence of *Plasmodium* trophozoite ring form if present in the blood film signified a positive result and were counted. According to standard laboratory report. Presence of 5-10 trophozoites or schizonts was assigned +, 10-20 was assigned ++, 20 and above was assigned +++. This system is used to report the level of parasitemia [17].

2.5 Antigen based testing of *Plasmodium falciparum* parasites (RDT kit).

SD Bioline Malaria Ag Pf./ Pv diagnostic Test Kit (product code: 05FK50 By Standard Diagnostics) was used for the detection of histidin-rich protein 11 (HRP11) antigen of *Plasmodium falciparum* in human whole blood. The test exploits the principle of lateral immune chromatography whereas, the specimen flows through the membrane after the addition of the assay diluents. The RDTs devices were labeled accordingly. 20µl of venous whole blood was obtained from the sample with micropipette and dispensed into the round specimen well according to the manufacturers instruction, 3 drops of diluents was added into the device well according to the manufactures protocol.[5]

2.5.1 Antigen Examination of *Plasmodium falciparum* parasites

Sample was allowed to move for 15 to 20 minutes and results were read. Presence of color band in the control line (C) is a prerequisite for the test to be valid. Presence of “C” and “P.f” colour bands indicates plasmodium positive test result. The absence of the tinted band in the test region indicates a negative result [18].

2.6 PCR testing for *Plasmodium falciparum* parasites.

The DNA extraction method followed the mini-prep spin column technique (Machererey Germany). Blood DNA extraction kit used were supplied by Qiagen (Qiagen Valencia, CA, USA), following the manufacturers instruction, 25µl of protinase K solution was pipette into a 1.5ml lysis tube. 200µl of whole blood was pipetted into the lysis tube. 200 µl buffer B3 was pipetted into the lysis tube and the solution was mixed properly by pulse vortexing for 10s. The lysis tube was incubated at 70°C for 10min and mixed by pulse-vortexing. The lysis tube was centrifuged briefly at 2000 rpm for 1s to remove drops from the lid. 210µl chilled ethanol was added to the sample (96-100%) and mixed by pulse vortexing for 5s. The entire lysate was loaded to the nucleospin Dx blood column placed in a collection tube and the lid was closed. It was centrifuged for 11min at 11,000rpm. The collection tube was discarded along with flow-through and a new collection tube was replaced. The necleospin was opened and 500µl buffer BW was added to the column and was centrifuged at 11,000 rpm for 1min the flow-through was discarded and replaced with a new collection tube. The nucleospin was opened and 600µl Buffer B5 was added. The nucleospin was centrifuged at 11,000rpm for 1min. The collection tube with flow-through was discarded

and a new one was replaced. It was centrifuged for 1min at 11,000rpm, the nucleospin was placed in a clean elution tube. The nucleospin Dx Blood column was opened and 200µl Buffer BE was added directly to the center of the membrane. It was further centrifuged at 11,000rpm for 1min to elute the DNA from the column. The pure DNA collected in collection tube was transferred to the PCR room for evaluation [15].

2.6.1 PCR confirmation of *P. falciparum* DNA

20µl of PCR master mix was prepared in a microtube on iceblock and distributed equally to the fresh sample microtubes placed on the clock block. 2.5ul H₂O RNAs free water, 10ul 2x master mix, 0.5 µl MgSO₄, 1µl forward primer, 1 µl reverse primer, 5 µl Taq DNA Polymerase and 5µl of DNA was added to the mastermix to make the total reaction volume of 25µl. This is in consonance with the method used by Pedro *et al.*, 2018. The primer sequence is based on the 18S rRNA mitochondria DNA of *Plasmodium falciparum* described by Pedro *et al.*, 2018. The Qiagen Rotor Gene Q was used to perform the amplification process for the polymerase chain reaction process to detect *Plasmodium falciparum* from the isolated plasmid DNA as given: 45 cycles with denaturation temperature of 95°C for 25s, annealing temperature of 60°C for 35s and extension at 72°C for 20s. Results were observed through Real Time PCR (RT-PCR) [3].

2.7 Sensitivity: Positive Sensitivity describes the capacity of the test to properly categorize an individual as infected. Calculation of sensitivity is true positive divided by true positive with false negative. This indicates the probability of positive sensitivity when disease is present while, negative sensitivity illustrates the test capacity to appropriately sort an individual as being infection free. Specificity is true negative divided by true negative with false negative. This demonstrates the Probability of the test being negative when disease absent. [9]

2.7.2 Positive Predictive Value (PPV): This predictive value informs about the percentage of patients that responded positive to the test. This shows positive infected patients that are really infected and if this percentage is near to 100, it suggests efficiency as Polymerase chain reaction as is the Gold standard. PPV Positive predictive value is true positive divided by true positive with false positive. This shows Probability of the patient having disease when test is positive. Negative Predictive Value. This informs about the percentage of patients that responded negative to the test however, negative predictive value shows negative uninfected patients that are actually infection free and if this percentage is near to 100, it suggests efficiency as microscopy which is the Gold standard. The negative predictive value is true negative divided by false negative with true negative. The Probability of patient not having disease when test is negative. Other calculations such as positive likelihood, negative likelihood and diagnostic ratio were also obtained [5].

2.8 Ethical permit: Patients gave full informed consent before the research was performed. Ethical authorization was issued by the Ministry of Health, Public Health Department, Owerri Imo State.

2.9 Statistical Analysis.

After the experimental research, statistical calculations were made following established protocol to obtain the percentage prevalence, sensitivity, specificity, test accuracy and positive and negative predictive value. Obtained data were analyzed using R software to obtain the significant difference via ANOVA and associations utilizing Chi-square for the three diagnostic methods in relation to age and sex.

Significant differences, associations and relationships were analyzed with a p value of 0.05. P <0.05 was considered statistically significant.

3.0 RESULTS AND DISCUSSIONS

3.1 Results

Table 1, 2 and 3 displays the results of malaria prevalence regarding the different diagnostic methods utilized, age and gender. The results in table 1 shows that out of 200 blood specimen collected, microscopy had 75 total number of positive cases, 83 by Antigen Assay while 94 by PCR. With percentage prevalence of 37% for microscopy, 41.5% for Antigen Assay and 47% for PCR. However, there was no significant difference between the test results and the diagnostic methods utilized ($X^2=3.7356$, P-value=0.1545). This indicates that the outcome of the test was not influenced by the method applied.

Table 1. Prevalence of malaria in relation to the three diagnostic methods.

Diagnostic method	Microscopy	Antigen Assay	Polymerase chain Reaction(PCR)
Total number tested	200	200	200
+ cases	75	83	94
- cases	125	117	106
% + prevalence	37	41.5	47
% - prevalence	63	58.5	53

Legend :+(positive), - (negative), %(percentage), $X^2=3.7356$, P-value=0.1545

The malariaprevalence in relation to age showed higher values of 27(42%) for microscopy, 27(42%) for Antigen Assay and 24(37%) within the age range of 31-40 compared to other age range as was shown in table 2. There is no significant difference between the positive cases and different diagnostic method as the P-value (0.5414) was obtained as against the test of significance at (P>0.05). However, chi-square showed there was no significant association between the positive cases and the age group as the p-value is 0.05923. This suggest that the aforementioned age group are not statistically significant to effect the outcome of the positive cases.

Table 2: Prevalence of malaria in relation to various age group.

Age range	Tested persons	+%Microscopy	+% Antigen Assay	+% Polymerase chain Reaction(PCR)
21-30	50	23(46%)	25(50%)	20(40%)
31-40	64	27(42%)	27(42%)	24(37%)
41-50	47	10(21%)	10(21%)	27(57%)
51-60	39	15(51%)	21(53%)	23(58%)
Mean age	200	75	83	94

Legend :+(positive), %(percentage). $X^2=12.125$, P= 0.05923

Table 3 presents the results of the malaria prevalence in relation to sex through the three diagnostic methods, however out of 111 females who visited the center, 28 were pregnant, and 12 were nursing mothers while 71 was single. For males, Microscopy detected 40 samples as positive, antigen assay detected 38 as positive while PCR confirmed 43 as positive. There is no significant difference between the positive cases and the gender considering that the p-value is 0.5085. Conversely, there is no

significant association between the positive test results and gender in view of the p-value of 0.5462 which is greater than $P > 0.05$. This demonstrates that the positive diagnostic results are not altered by gender.

Table 3: Malaria prevalence in relation to gender.

Gender	Tested persons	+%Microscopy	+% Antigen Assay	+% Polymerase chain Reaction(PCR)
Male	89	40(45%)	38(42.6%)	43(48%)
Female	111	35(31%)	45(40%)	51(46%)
Total	200	75	83	94

Legend :+(positive), %(percentage), $\chi^2 = 1.2096$, $P=0.5462$

The table in 4 (a, b, c) shows the two by two comparisons of the different diagnostic methods utilized in the analysis. This table indicates the level of false positive and true negative of the diagnosis. In table 4a, Microscopy had 75 true positive results, 0 false positive, 19 false negative and 106 true negative as confirmed by PCR when utilized as a reference standard. A false negative of 19 signified those results detected negative by microscopy but positive by PCR.

Table 4a: Two by two comparative table between PCR and Microscopy.

		PCR RESULTS	
		POSITIVE	NEGATIVE
MICROSCOPY	POSITIVE	75	0
	NEGATIVE	19	106

Table 4b shows that Antigen Assay had 81 true positive result, 02 false positive, 13 false negative and 104 true negative as confirmed by PCR exploited as reference standard while, A false negative of 13 signified those results detected negative by Antigen Assay but positive by PCR.

Table 4b: Two by two comparative table between PCR and Antigen Assay.

		PCR RESULTS	
		POSITIVE	NEGATIVE
ANTIGEN	POSITIVE	81	02

	NEGATIVE	13	104
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In table 4c, there was impediment of detection by microscopy maybe because of low level of parasitemia as was shown in the false negative of 8. However, it was detected by antigen assay. There was a true negative of 117 and 0 false positive.

Table 4c. Two by two comparative table between Antigen Assay and microscopy.

		ANTIGEN ASSAY	
		POSITIVE	NEGATIVE
MICROSCOPY	POSITIVE	75	0
	NEGATIVE	08	117

The performance characteristics of the three methods of diagnosis was calculated as shown in figure 2 and 3. Figure 2 and 3 below shows the various degrees of variation in the parameters measured. The sensitivity is the probability that the people tested positive are truly positive while the specificity is the probability that the people tested negative are truly negative. The result in fig 2 indicates that microscopy and RDT had the highest specificity while, PCR had the least specificity, however, PCR had the highest sensitivity while, microscopy had the least sensitivity. This suggests that PCR is more reliable during diagnosis as the sensitivity and specificity are nearly 100%. This also, includes the test accuracy.

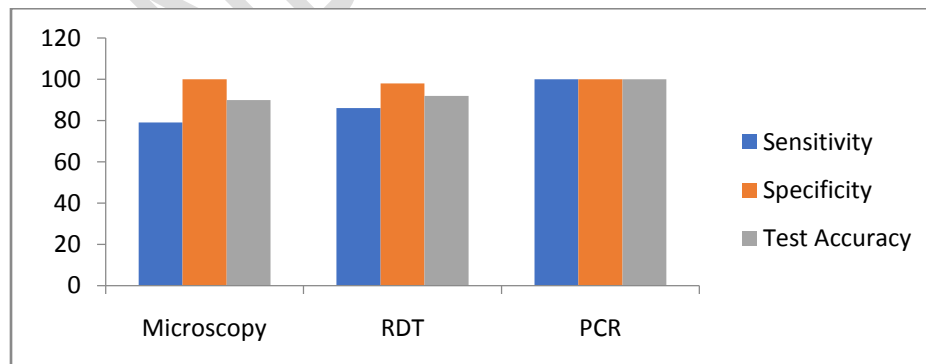


Fig 2: Performance degree of the three diagnostic methods.

The analysis in figure2 shows that the sensitivities of the methods are 79.6% for microscopy, 86 % for Antigen assay and 100% for PCR while the specificity is 100% for microscopy, 98% for Antigen Assay and 100% for PCR. Other value to consider is the test accuracy which is 100% in PCR, higher than other methods.

Table 5a: Predictive value of the three diagnostic methods

Test	Positive predictive value	Negative predictive value	False positive rate	False negative rate
Microscopy	100	84	0.01	0.2
RTD	92	88	0.1	0.1
PCR	100	100	0.0	0.0

The false positive is the probability that a positive result will be issued when the true value is negative. Table 5a shows that microscopy appears to have 0.01% probability while, antigen test (RTD) method has 0.1 and PCR has 0% probability of false positive. The false negative on the other hand is the probability that a true positive will be missed by the test. Microscopy has a 0.2% chance of missing a true positive while antigen assay has 0.1%, PCR have 0%.

The diagnostic likelihood was displayed in table 5b while, figure 2 display the graphical representation. Diagnostic likelihood ratio is more clinically expedient than the exclusive utilization of sensitivity in estimating the possibility of disease in a patient.

Table 5b. Showing positive and negative likelihoods as well as the diagnostic odd ratio.

Diagnostic method	Positive likelihood	Negative likelihood	Diagnostic odd
Microscopy	39.8	0.20	1
Antigen assay	43	0.14	1
PCR	0	0	1

The chances of positive response in patience with disease relative to the chances in patience without disease is diagnostic ratio. A diagnostic odd ratio of 1 means a positive outcome, a diagnostic odd ratio of less than 1 means the test is inappropriate, greater than 1 means a better performance. The proportion of anticipated test result in patients with the disease to the patients without the disease is diagnostic likelihood. This explains the possibility of a patient being infected with a disease or condition. The higher the ratio, the more likely there is an infection of the disease or condition. The lower the ratio, the less likely there is no infection of the disease or condition. However values closer to 0 have a higher decrease in probability of disease.

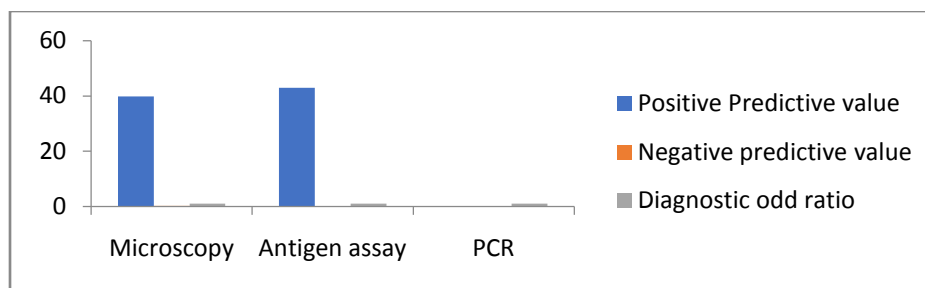


Fig3: The probability variations of diagnostic odd ratio.

3.2 Discussion

The prevalence of malaria in relation to diagnostic method, age and gender indicate the quality of malaria diagnosis within the study area. However, with the rate of false positives and negatives have consequences in malaria control and intervention programs, the impact of the diagnosis can be ascertained. The high positive prevalence recorded with PCR diagnosis reveals the loopholes obtainable with microscopy and antigen assay diagnosis, this does not imply that the microscopy and antigen assay diagnostic procedure are inept. Besides, the antigen assay similarly attained a moderate positive malaria prevalence. Though, considering the less practice of preventive methods of transmission within the study area might have contributed to the observed prevalence [9]. Moreover, the prevalence in relation to age and gender demonstrates PCR lead in the positive prevalence cases. Nonetheless, the variation observed in the percentage of prevalence within the age group can be attributed to sluggish attainment of effective immunity to malaria while, there is no specific reason for the observed variation within the gender [10]. The utilization of PCR as a gold standard illustrates the possibility of having low level of parasitemia in the peripheral blood which may be challenging when exploiting microscopy for detection. Further, the 02 false positive recorded elucidate the samples detected positive by Antigen Assay but confirmed negative by PCR, those additional infections might not have been because of *Plasmodium falciparum* but, could be as a consequence of gene mutations. Frequently, the remnant of antigen in the blood after treatment of malaria contributes to the inefficiency of characterizing individuals with the infection therefore, leading to issuance of false positive results [3]. This could advance the concerns of drug resistance pertaining to upsurge in drug usage. Implication of a high number of false positive in any test will lead to uninfected individuals being treated with antimalarial drugs. This negates the very essence of malaria rapid diagnostic test utilization in evidence based treatment of malaria [19]. There is an implication of cost increment of malaria treatment owing to more drug sales to unaffected population. The reluctance of parasitemia to circulate albeit producing the HRP2 in the blood stream will ensure that microscopy test will be negative, as the segregated parasite does not circulate in the peripheral blood [20]. In this case, true positive cases will be misclassified as false positive, thus increasing their number in a sample and situation of this nature could not be ruled out in a hospital setting. Conversely, rapid diagnostic test will be positive assuming the matured sequestered forms of the parasites remained intact. It has been shown that serum rheumatoid factor, cross reaction and other parasitic diseases may also raise the number of false positive during tests with HRP2 malaria RDTs [21]. The positive predictive value of PCR and Microscopy is 100% which indicates the efficiency of the diagnosis while the negative predictive value of 100% and 88% expresses the reliability of the diagnostic methods [12]. Moreover, the Gold standard had efficient sensitivity and specificity while microscopy had a high specificity with 90% test accuracy and

100% test accuracy for PCR. The PCR deployed as a gold standard has proven to be more reliable in line with its sensitivity and specificity.

4.0 Conclusion

Our findings further confirmed PCR as the most sensitive, reliable, specific and accurate diagnostic method for detecting *Plasmodium falciparum*, amongst the three diagnostic methods compared in this study. However, considering the high cost of PCR usage or maintenance in Nigeria and the positive predictive values obtained for PCR, antigen assay (RDT kit) and microscopy; we also recommend microscopy and antigen-based methods as suitable, accurate and sensitive diagnostic tools in malaria testing.

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