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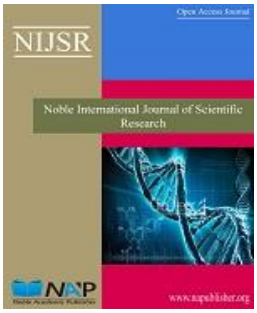

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PREVALENCE OF MALARIA PARASITES USING DIFFERENT DIAGNOSTIC TECHNIQUES AMONG PREGNANT WOMEN ATTENDING A TERTIARY HEALTH INSTITUTION IN BAYELSA STATE, NIGERIA

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ABSTRACT: This study evaluated the prevalence of malaria parasites using different diagnostic techniques among pregnant women attending a tertiary health institution in Bayelsa state, Nigeria. Blood samples were collected from 364 pregnant women attending antenatal clinic at the Niger Delta University Teaching Hospital, Okolobiri, Bayelsa state within the age grade of 18 – 40 years. The blood was analyzed using Microscopy, Polymerase Chain Reaction and Enzyme Linked Immunosorbent Assay. Results showed that 240 cases representing 65.9% were positive using microscopy method, 323 cases representing 88.7% were positive using Polymerase Chain Reaction, and 253 cases representing 69.0% were positive using Enzyme Linked Immunosorbent Assay techniques for malaria parasite detection. Statistically, there was significant difference at $p<0.05$ among the various methods of diagnosis. Based on malaria parasite speciation, the prevalence were in order; Plasmodium falciparum > co-infection (*P. vivax* + *P. falciparum*) > *P. vivax* for each of the techniques. Based on the parasites analysis, co-infection (*P. vivax* + *P. falciparum*) and *P. vivax* showed no significant variations ($p>0.05$) across the various techniques, but significant difference ($p<0.05$) exist across the techniques except for *P. falciparum*. In this study Polymerase Chain Reaction and Enzyme Linked Immunosorbent Assay showed to have higher capacity to detect malaria parasitaemia even at low density when compared to the Microscopy technique. Therefore, there is the need to increase the front burner in the diagnosis of malaria using the current techniques.

Keywords: Diagnosis, Enzyme Linked Immunosorbent Assay, Malaria, Microscopy, Polymerase Chain Reaction.

1. INTRODUCTION

Malaria is a major societal health challenge in many region of the world. [1, 2], Ndiok, et al. [3] reported that malaria is among the major cause of morbidity and mortality in the Sub-Sahara Africa. Authors have reported that 3.28 - 3.4 billion world populations are at risk of malaria infections [1, 4, 5] in 92 countries [6]). World Health Organization [6] reported that 1.1 billion people are at high risk of malaria infection. According to World Malaria report 2018, 203-262 million and 435 000 cases and deaths, respectively were recorded in 2017, which showed a decline of 18% (for malaria cases) and 28% (of death resulting from malaria infection) in 2010 [6]. World Health Organization [7] reported that 212 million and 429,000 malaria cases and deaths, respectively were recorded in 2015. The authors reported that there was a decline of 29% in fatality cases due to prevention and control.

Malaria cases are higher in Africa with prevalence rate of 81 – 93% of total global cases [6-8]. Malaria in children has higher fatality compared to adults. Reports have shown that about half of global malaria death occurs in Nigeria, Democratic Republic of the Congo, Uganda and Ethiopia [8]. The prevalence of malaria in Nigeria differs according to the geopolitical zone. Nigeria Malaria Fact Sheet [8] reported a prevalence of 41 – 50% (North-West, North-Central and South-West), 31 – 40% (North-East and South-South) and 21 – 30% (South-East). In Nigeria, a substantial number of outpatient consultation cases are often treated with malaria. Malaria has been reported among pregnant women, infants and elderly [1]. Nwanosike, et al. [9] reported that 60% and 30% of outpatient visits and hospitalizations, respectively of <5 year children are often treated of malaria.

During pregnancy several physiological changes occurs in women. If not properly managed it could result to other heath concern to both the foetus and mother. Luxemburger, et al. [10] reported that malaria associated with *P. falciparum* and *P. vivax* during pregnancy increases neonatal mortality by lowering birth weight.

Malaria is a parasitic infection caused by a protozoan of the genus *Plasmodium*. Several species of *Plasmodium* exist but five (*P. falciparum, P. vivax, P. ovale, P. knoweski* and *P. malariae*) have been reported to cause malaria [1, 2, 11]. The occurrence of malaria caused by this species of *Plasmodium* differs based on geographical locations [1]. In Nigeria, most cases of malaria cases are caused by *P. falciparum* and *P. vivax*.

Basically, mosquito is iniquitous dipteran fly. Like malaria, several disease conditions (chikungunya, lymphatic filariasis, Japanese encephalitis and dengue fever) has mosquito as their vector [1-3, 12-14]. Malaria parasite is transmitted through an infective bite of the female Anopheles mosquito. Malaria transmission is initiated when the sporozoites of the *Plasmodium* is inoculated by female anopheles' mosquitoes into the human blood stream, sporozoites disappear and invade hepatic cells to establish the liver stage. During the next red blood stage, the gametocytes are taken up by mosquito to ensure the survival of the species.

Malaria is conventionally identified with microscopy approach. With research in science and technology, Enzyme Linked Immunosorbent Assay and polymerase chain reaction were developed for the detection of malaria parasites in human. These methods are applied in several medical laboratories, but their application is still quite low when compare to microscopy method especially in area of malaria endemicity like Nigeria. Therefore, this present study aimed at determining the prevalence of malaria parasites using different diagnostic techniques among pregnant women attending a tertiary health institution in Bayelsa state, Nigeria**.**

2. MATERIALS AND METHODS

2.1. Study Setting

This study was conducted at Niger Delta University Teaching Hospital, Okolobiri, Bayelsa state. The climatic condition of the area is characterized by relative humidity and temperature of 50 – 95% and 28±7°C, respectively all year round. Two predominant seasons occurs in the study area (5 months of dry season station from November to March of the following year) and 7 months of wet season (April to October). In the area, it appears that rainfall pattern is beginning to shift from known conventional period.

2.2. Selection Criteria for Subjects

2.2.1. Inclusion Criteria

Individuals that participated in this study were 364 pregnant women from 18 – 40 years of age that attend antenatal clinic at the Niger Delta University Teaching Hospital Okolobiri for medical attention.

2.2.2. Exclusion criteria

Teenage pregnancy cases (individuals that is less than 18 years of age) and above 40 years of age, and non-pregnant women.

2.3. Blood Collection

A standard venipuncture technique was adopted for sample collection. Approximately 2 mls of blood was collected from each subjects (pregnant women) once per trimester through the three trimesters from the dorsal vein and was dispensed into sterile anti-coagulated EDTA containers.

2.4 Malaria Diagnostic Methods

2.4.1. Microscopic Examination

The malaria parasite test was carried out using the method described by Cheesbrough [15] as applied by Ndiok, et al. [3]. A thick and thin blood films were made in a clean glass slide and stained using Giemsa for 30 minutes. The stained films were allowed to air dry, and then viewed under the microscope using the 100-x objective, which were examined at 100 high power microscope fields. Identification of the malaria parasite was carried out based on the method described by World Health Organization [16], Nkuo, et al. [17].

2.4.2. Determination of Malaria Antibody Titre using Enzyme Linked Immunosorbent Assay

The blood from each of the subjects were centrifuged for 4000 rpm for 10 minutes to extract plasma for anti-malarial parasites antibody using bench top bucket centrifuge (Model: SM 800Bl). Malaria parasites antibody titre in each case was determined using cut off value absorbance of Enzyme Linked Immunosorbent Assay method as described by Kitchen [18]. The Cut off Value was calculated as the mean of three negative controls plus 0.100. Positive cut off value is equal to or greater than 1. Negative cut off value is less than 1. The assay was quality controlled using three negative and two positive controls supplied by the manufacturer of the malaria parasites EIA kit.

2.4.3. DNA Extraction

The extraction of DNA from the pregnant women was determined following the scheme provided by Alberts, et al. [19]. DNA extraction involves three stages; the lysing of the white blood cell to release the DNA, washing off of the DNA contaminants and recovering of the pure DNA. Finally, the pure DNA was Nano dropped for quantification.

2.4.3.1. DNA Testing

The Polymerase Chain Reaction DNA amplification process was used for the determination of *Plasmodium* species, which involves three stages; denaturation of the double stranded DNA, annealing and extension at different temperature. The DNA amplification comprises of two types; primary and secondary or nested amplification. The primary amplification was used to determine the genus *Plasmodium*, while the nested amplification was used to determine *Plasmodium* species. The primers used for the species and genus are presented in Table 1. The template for the primary amplification was the pure extracted DNA while the template used for the nested amplification was the amplified DNA solution of the primary amplification. Two primers (rplu6 and rplu5) were used for primary amplification as both forward and reverse primers. The control samples were 3D7 and Dd2. Each of the four *P.* species (*P. falciparum, P. vivax, P. malariae* and *P. ovale*) has both forward and reverse primers used in the process. In each case, the four *Plasmodium* species were tested for their presence and amplified with two controls (positive and negative) used. *P. falciparum, P. vivax* and *P. malariae* work in the same condition of 30 cycles, while *P. ovale* works in different condition of 45 cycles. The three *P.* species, DNA were denatured first at initial denaturation temperature of 94^{o}C at 3 minutes, while final denaturation temperature 94^{o}C at 1 minute for separation of the double stranded DNA to two single strands, annealing temperature of 50^{o}C at 1.45 minutes for the attachment of the primer to the specific gene of interest on the DNA sequence of the template. The forward primer attach on the template at the 5 prime end while, the reverse primer attach at the 3 prime end. The *P. ovale* has the same denaturation and extension condition as others but with annealing temperature of 57^{o}C at 1.45 minutes. The extension stage which is the process of DNA amplification whereby tag polymerase enzyme activate the addition of Deoxyribose nucleotides at the 3' end of the primer at temperature of 68^{o}C in 1.30minutes and final extension at 7 minutes.

2.4.3.2. Electrophoresis

The nested amplified DNA particles were subjected to Agarose gel electrophoresis at 120v in 20 minutes. Each of the different *Plasmodium* species has its different base pair (bp). Each of the amplified DNA in each case was applied to different well along the negative axis of the Agarose matrix with parallel standard ladder of 100bp arranged in order of the degree of base pair value. Each of the species was determined according to its base pair (bp) lane in line with the standard base pair lane on the molecular ladder. Finally, the various separated bands were identified using ultraviolet (uv) light which was aided by the presence of ethidium bromide in the Agarose gel composition. The direction of separation was from the negative axis to the positive axis through the Agarose matrix.

Table 1. Consensus Primers used for the study

Primer name	Forward primers and Reverse primers Sequence	Size bp
Genus primers		
rPlu6	5'TTAAAATTGTTGCAGTTAAAACG3'	
rPlu5	3'CCTGTTGTTGCCTTAAACTTC5'	
specific primer		

rFAL 1	5'TTAAACTGGTTTGGGAAAACCAAATATATT3'	206
rFAL 2	3'ACACAATGAACTCAATCATCACCCGTC3'	
rMAL1	5'ATAACATAGTTGTACGTTAAGAATAACCGC3'	144
rMAL2	3'AAAATTCCCATGCATAAAAAATTATACAAA5'	
rVIV 1	5'CGCTTCTAGCTTAATCCACATAACTGATAC3'	120
rVIV2	3'ACCTTCCAAGCCGAAGCAAAGAAAGTCCTTA5'	
rOVA1	5'CTGTTCTTTGCATTCCTTATGC3'	375
rOVA2	3'GTATCTGATCGTCTTCACTCCC5'	

Source: Authors

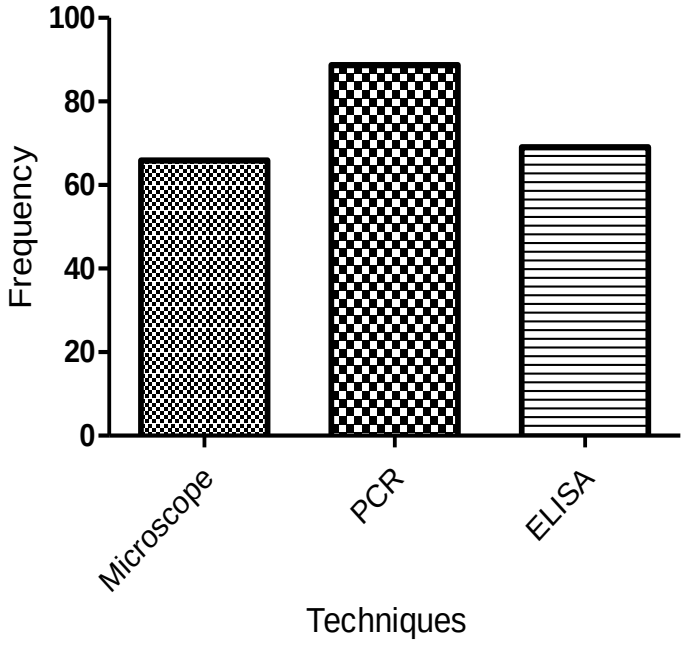
2.5. Statistical Analysis

SPSS was used to carry out the statistical analysis. The data were presented as percentage. Significance was carried using chi-square. The charts were plotted using GraphPad prism 5.

3. RESULTS AND DISCUSSION

Three hundred and sixty four (364) pregnant women were examined for malaria parasites prevalence. Two species of malaria parasites; *P. falciparum* and *P. vivax* were recorded. Co-infection of *P. falciparum* and *P. vivax* also exist. The diagnostic techniques used were Polymerase Chain Reaction, Microscopy and Enzyme Linked Immunosorbent Assay, and their prevalence distribution is presented in Figure 1. Of the 364 number examined 240 representing 65.9% were positive using microscopy method, 323 representing 88.7% were positive using Polymerase Chain Reaction, and 253 representing 69.0% were positive using Enzyme Linked Immunosorbent Assay techniques for malaria parasite detection. There was significant difference at $p<0.05$ among the various methods of diagnosis.

Figure 1. Distribution of different methods in diagnosis of malaria parasites



Source: Authors

The Prevalence of Malaria Parasites by Diagnostic Techniques is presented in Table 2. Polymerase Chain Reaction was more specific for *P. falciparum* and *P. vivax*, with prevalence of 88.7% (with 323 positive cases). Of the 323 positive cases the prevalence were; *P. falciparum* 215 (representing 66.6%), *P. vivax* 49 (representing 15.2%) and co-infection (*P. falciparum* and *P. vivax)* 59 (representing 18.3%). Statistically there was significant different ($p<0.05$) among the prevalence of the different parasites. The Enzyme Linked Immunosorbent Assay has prevalence of 69.0% (with 253 positive cases). Of the 253 positive cases the prevalence were; *P. falciparum* 189 (representing 74.7%), *P. vivax* 26 (representing 10.3%) and co-infection (*P. falciparum* and *P. vivax)* 38 (representing 15.0%). There was significant different ($p<0.05$) among the prevalence of the different parasites. Using microscopy techniques, only *P.*

falciparum were recorded with prevalence rate of 100%. Based on the different methods for each of the parasites group, no significant different at p<0.05 for each of the method of diagnosis for *P. vivax* and co-infection (*P. vivax* + *P. falciparum*). However, significant variation at p<0.05 exist for *P. falciparum.*

Table 2. Prevalence of Malaria Parasites by Diagnostic Techniques

Techniques	Prevalence No. (%)			X^2	P-value
	P.F	P.V	P.F + P.V		
Microscope	240 (100.0)	0 (0)	0 (0)	NA	NA
PCR	215 (66.6)	49 (15.2)	59 (18.1)	51.14	0.000
ELISA	189 (74.7)	26 (10.3)	38 (15.0)	78.50	0.000
X^2	7.347	1.000	0.273	-	-
P-value	0.025	0.317	0.602	-	-

Source: Authors

PCR=Polymerase chain reaction, ELISA=Enzyme linked Immunosorbent assay, P.F=*Plasmodium falciparum*, P.V=*Plasmodium vivax.*

This study has established information for assessing the reliability of the Polymerase chain reaction, Enzyme Linked Immunosorbent Assay and Microscopy techniques for the detection of malaria parasitaemia among pregnant women. This result shows that the detection of malaria using the different methods were in the order; microscopy < Enzyme Linked Immunosorbent Assay < Polymerase chain reaction (Table 2 and Figure 1). The Polymerase chain reaction technique in this study used DNA to amplify *P.* species genes which permits the detection of submicroscopic parasitaemia [20]. *Plasmodium falciparum* predominate malaria parasites species in the area. In addition to underestimating prevalence, it is clear that microscopy really underestimate extent of parasitaemia species existence in the studied area [21, 22].

The Polymerase Chain Reaction method applied in this study is very sensitive and specific for both *P. falciparum* and non-*falciparum* infections, and has many diagnostic advantages over microscopy. In general, Polymerase Chain Reaction assay has specific advantages to microscopy: they are less laborious, they are performed in closed system which minimizes the risk of post-amplification contamination, results can be obtained quite a short period of time, and the analysis design is automated and suitable for multi-purpose studies [23-25]. But microscopy remains the most routine diagnostic technique in use in malaria endemic areas, but its use as a standard may generate misleading results in clinical trials [23, 26-28].

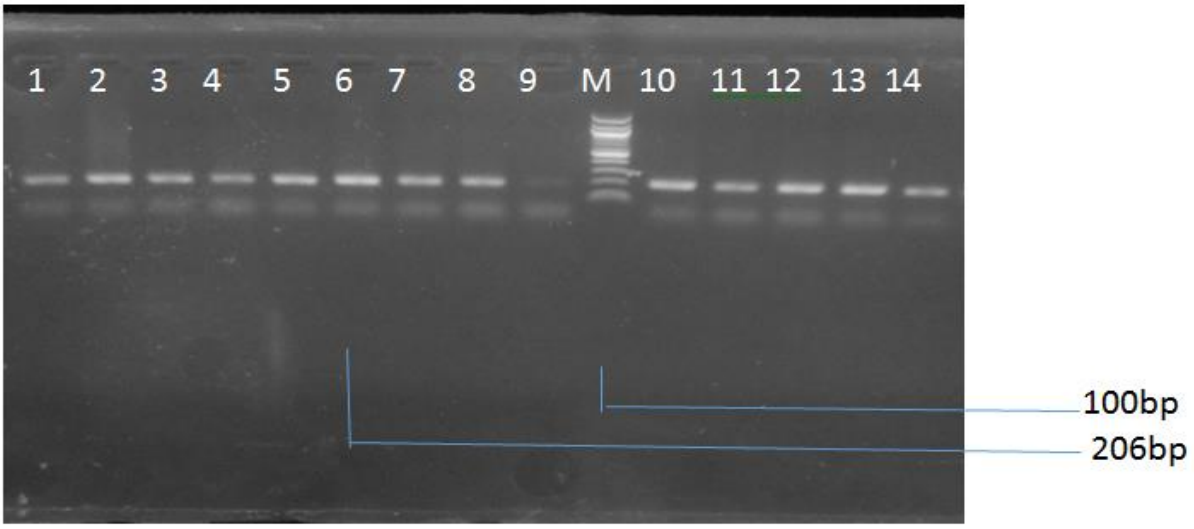
The prevalence rate recorded in this study is higher than previous work in other part of Nigeria. Obianumba [29] reported malaria prevalence rate of 53.9% among pregnant women attending antenatal clinic in Ozubulu, Anambra state. Agomo and Oyibo [30] reported malaria prevalence rate of 7.7 among pregnant women in Lagos. Of these, *P. falciparum*, *P. malariae* and mixed infection of *P. falciparum* and *P. malariae* were 91.6%, 4.8%, and 3.6%, respectively. The authors attributed the low prevalence to scaling-up of malaria interventions, low transmission due to the urban area and high competency in malaria microscopy. Nwonwu, et al. [31] reported prevalence rate of malaria parasitaemia during pregnancy in Abakaliki, Ebonyi state as 29%. Enoch and Ethel [32] reported the prevalence of malaria among pregnant women from two hospital in Port Harcourt, Rivers state as 27.5 – 35.0%. Wogu, et al. [33] reported the prevalence of malaria parasites among some pregnant women attending antenatal clinics in Rivers State, Nigeria as 26%. Inah, et al. [34] reported prevalence of malaria among Pregnant Women in Abi Local Government Area, Cross River State, Nigeria as 40.7%. Okafor, et al. [35] reported prevalence rate of malaria parasites among pregnant women living in Calabar South Local Government Area of Cross River State, Nigeria as 70.1%.

Akinboro, et al. [36] reported prevalence of malaria (associated with Plasmodium *falciparum*) parasitemia as 63.5% among pregnant women in a secondary hospital and a tertiary hospital in Osogbo, South-Western, Nigeria. Adefioye, et al. [37] reported the prevalence of malaria parasites infections among pregnant women in Osogbo was carried out in Ladoke Akintola University of Technology Teaching Hospital as 72%. Omoya and Atobatele [38] reported the prevalence of malaria among Pregnant Women Attending Primary Health Care Centre, Ojo Local Government, Lagos, Nigeria as 65.88% (112 of 170 samples). The authors also reported prevalence of typhoid fever as 67.06% (114 of 170 population) among the same sample subjects. Akinbo, et al. [39] reported prevalence of 24.9% and 18.2% for malaria (associated with *P. falciparum*) and intestinal parasites (*Entamoeba histolytica, Hookworm, Trichuris trichiura, Giardia lamblia and Ascaris lumbricoides*), respectively, and co-infection rate of 43.1%. This is a co-infection of malaria with other disease condition. This trend has been reported

by Bassey and Izah [1]. the difference associated with the malaria prevalence in this study when compared to previous study could be associated to the medical conditions of the individuals, environmental condition and human status (pregnancy, blood group, Rhesus factor, age, gender and educational status) [1] and sensitivity of the technology used for diagnosis.

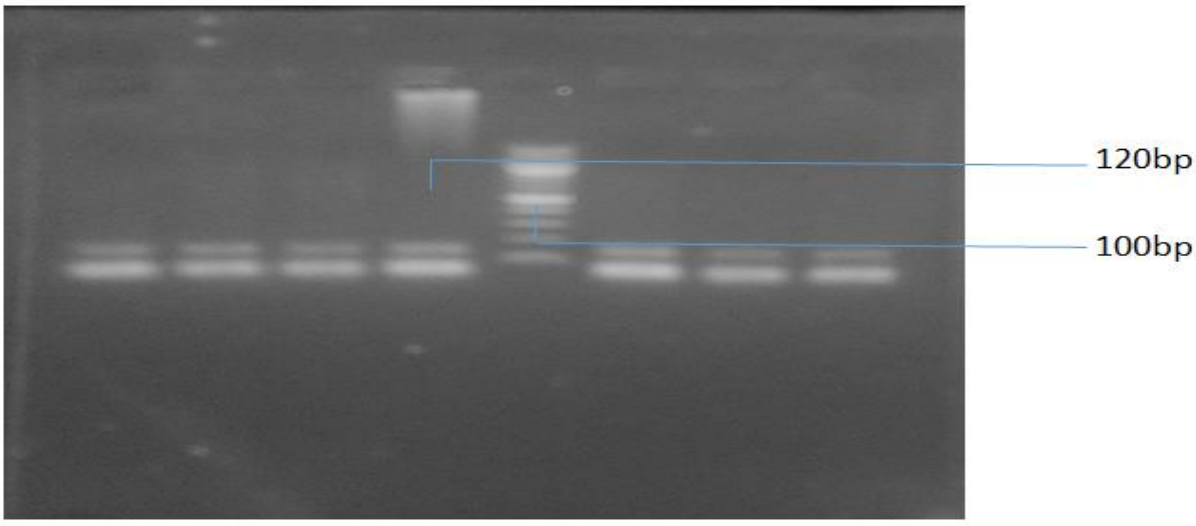
The occurrence of submicroscopic *P. falciparum* and non-falciparum infections were high in this study using polymerase Chain Reaction and Enzyme Linked Immunosorbent Assay analysis targeting the genes detected and malaria parasites antibodies on the four human Plasmodium species (P.falciparum, P. vivax, P.ovale and P.malariae). Microscopy did not detect non-falciparum infections. The detection of submicroscopic infection by polymerase Chain Reaction assay concurred with previous studies [40, 41]. The prevalence of *P. falciparum* detected in this study with peripheral blood sample in pregnancy was similar to a previous study targeting *P. falciparum* DNA [42]. Based on the Agarose gel electrophoresis, Plasmodium falciparum showed at 206bp. Lane 1-12 represent the samples; Lane 13-14 represent control 3D7 & Dd2 while M represent 100bp molecular ladder (Figure 2). While the Agarose gel of Plasmodium vivax showed 120bp. Lane 1-7 represent the samples; while M represent 100bp molecular ladder (Figure 3). Submicroscopy parasitaemia detected by Polymerase Chain Reaction assay have been detected with similar frequency as was in this study by earlier gestational ages in studies in both the Sudan [43] and Mayor, et al. [41]. Submicroscopic infections in the general population are important in serving as a reservoir of parasites for easy transmission [44].

Figure 2. Agarose gel of *Plasmodium falciparum* showing 206bp. Lane 1-12 represent the samples; Lane 13-14 represent control 3D7 & Dd2 while M represent 100bp molecular ladder.



Source: Authors

Figure 3. Agarose gel of *Plasmodium vivax* showed 120bp. Lane 1-7 represent the samples; while M represent 100bp molecular ladder.



Source: Authors

4. CONCLUSION

The prevalence of Malaria Parasites using different Diagnostic Techniques among pregnant women attending a tertiary health institution in Bayelsa state, Nigeria was evaluated. The results found that the prevalence of malaria parasite was high (88.7%). The parasite prevalence was compared using three diagnostic techniques; Microscopy, Enzyme Linked Immunosorbent Assay and Polymerase Chain Reaction. Polymerase Chain Reaction revealed better method for the detection of malaria in pregnancy due to its specificity and sensitivity. Based on the prevalence from the Polymerase Chain, the prevalence of the individual parasites were 66.6% (*P. falciparum*), 15.2% (*P. vivax*) and 18.3% (co-infection viz: *P. falciparum* and *P. vivax*). This an indication malaria is majorly caused by *P. falciparum* in pregnancy women in the study area.

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