Comparative Analysis of the Effectiveness of Polymerase Chain Reaction and Microscopy in Malaria Diagnosis

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Abstract. Malaria is a life-threatening parasitic disease which causes enormous morbidity and mortality in tropical African countries. Successful prevention and treatment of infected individuals heavenly depend on successful diagnosis using recommended techniques. These routine laboratory techniques have different performance indices. Therefore, this study aimed to evaluate the performance of Polymerase Chain Reaction and Microscopy in malaria diagnosis. A total of two hundred consented study subjects were randomly selected and enrolled for the research. Vein puncture technique was use to collect venus blood from the subjects and analysed using microscopy and Polymerase chain Reaction. DNA samples were extracted using Quick-DNA™ Miniprep Plus Kit with catalogue No. D4069. 18SrRNA gene of *Plasmodium falciparum* from chromosome 13 was amplified using the primers F5'AACAGACGGGTAGTCATGATTGAG3' R5'GTATCTGATCGTCTTCACTCCC3'. Malaria prevalence of 167(83.50%) and 105(52.5%) were recorded using microscopy and Polymerase Chain Reaction. Microscopy had a sensitivity, specificity, Positive predictive value and negative predictive value of 84.91, 23.40, 55.53 and 57.89%, respectively, with an overall accuracy value of 0.81. Polymerase Chain Reaction had a sensitivity value of 53.89%, specificity of 54.54%, positive predictive value of 85.79% and Negative predictive value of 18.94% with an overall accuracy of 0.54. Microscopy and Polymerase Chain Reaction demonstrated significant accuracy and relatively good performance indices. Therefore Microscopy and Polymerase Chain Reaction are highly recommended as malaria diagnostic techniques, and further research should be carried out to determine the influence of some biological factors of both the parasite and the host on the outcome of the diagnosis using both Polymerase Chain Reaction and microscopy.

Keywords: Malaria Diagnosis; Polymerase Chain Reaction; Microscopy; Sensitivity; Specificity; Accuracy.

INTRODUCTION

Malaria is an ancient parasitic protozoan disease caused by five parasites (*Plasmodium falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi*) belonging to the genus Plasmodium [1]. *Plasmodium falciparum* is the most predominant specie in sub-Saharan Africa, causing severe disease and death, particularly in young children, usually less than five years of age, pregnant women and immunocompromised individuals [2, 3]. Also,

P. knowlesi causes acute, severe illness but with a low mortality rate [4]. The disease is a significant public health problem in the tropic, especially in Nigeria, where climatic conditions favour the survival of both the parasite (*Plasmodium* specie) and the vector (female Anopheles mosquito) [5, 6]. In 2018, there were 228 million estimated clinical cases of malaria infection and 405,000 malaria-related deaths [7, 8]. Sub-Saharan Africa bears more than 90% of the global malaria cases, with more than 93% of all deaths [9].

Accurate malaria diagnosis is a pillar of malaria control and elimination [10]. Therefore, the only clinical approach is usually discouraged and insufficient for establishing the presence or absence of malaria parasites. This is because such a presumptive diagnosis cannot be entirely relied upon, as the presentation of malaria is highly non-specific and imitates various similar clinical conditions [11]. Therefore, World Health Organisation recommends that all clinically suspected malaria cases be confirmed with parasitological diagnosis, using either a malaria-specific Rapid Diagnostic Test (RDT) or direct visualization of the parasites or their product using microscopy before treatment [12, 13]. Fever, which is usually associated with chills, perspiration, anorexia, headaches, vomiting, high body temperature and malaise, is the most prominent clinical sign and symptom of malaria infection, and quite several people residing in endemic areas of malaria transmission are fully aware of these symptoms, thus led to self-diagnosis and in most cases self-treatment. According to the global malaria policy of universal testing and treatment, detection of malaria cases should be within the first 24 h [14]. Therefore, in the last few decades, there has been a rapid increase and advancement in developing novel malaria diagnostic techniques [15].

Presently, diagnostic and reference laboratories adopt several techniques, which include microscopy, molecular assays (Polymerase Chain Reaction), and serological assays [16]. Still, microscopy remains the mainstay and gold standard technique in most areas where malaria is endemic. Nevertheless, with all this dependence on microscopy, there are some shortcomings attached to it, which include subjective parasite identification and counting by microscopists [17], inherent errors due to sample handling [18], inability to detect mixed species infection especially when it involves *P. ovale* and *P. vivax*, the lower detection limit of between 4–20 parasites/ml even for expert microscopists and lack of steady and inter-rupted power supply in most malaria-endemic countries [19] and in some instances, it may tend to give either false positive or false negative result [20]. Polymerase Chain Reaction (PCR) is a well-known molecular technique that uses the parasite's DNA or RNA (nucleic acid) to diagnose an infectious agent. The method is susceptible and specific as it can detect infectious agents at densities below the threshold level of other conventional techniques. PCR can see, for example, malaria parasites at densities below the threshold level of detection of either microscopy or RDTs [21]. For *Plasmodium falciparum* diagnosis using PCR, 18SrRNA genes are amplified [22]. Unlike other techniques, especially microscopy and RDTs, which are considered less expensive and cumbersome, this Molecular-based technique is relatively expensive and requires a high level of expertise [23]. Malaria diagnostic techniques include Loop-mediated isothermal amplification (LAMP) and flow cytometry. Though PCR is costly, some laboratories and other research institutions adopt it, especially when other conventional diagnostic techniques fail to produce the required and expected result. Therefore this study aimed to compare and evaluate the performance of PCR and microscopy in malaria diagnosis, as there is a lack of data in that regard in the study area.

MATERIAL AND METHODS

Study Area. The study was conducted in Gombe Local Government Area, Gombe State, Nigeria. The Local Government lies between 11°14′07″E and 11°4′42″E, and Latitudes 10°16′48″N and 10°17′24″N with a total land mass of 52 km². According to National Population Commission, the Gombe Local government has a projected population figure of 367,500 people (3.3% annual change). The vegetation of the Local Government is typical of that of Gombe State, which is Sudan savannah and experiences two distinct seasons, the dry season, which usually commences in November-March and the rainy season from April-October, with a mean annual rainfall of 863.2 mm. Agriculture is the primary occupation in the region (mostly Peasant farmers) while some engage in business and few are a civil servant. The local Government being the state capital of the state, both the tertiary (Federal Teaching Hospital) and the secondary (Gombe State Specialist Hospital) health facilities of the state are domiciled in the local Government. This is also in addi-

tion to the primary health care centres strategically located in each local government ward. Also, several private hospitals provide different services, including malaria diagnosis and treatment.

Ethical consideration. The research proposal was submitted to the Gombe State Ministry of Health for approval, after which the consent was communicated via a later MOH/ADM/621/VOL. I/ 222.

Consent of the Subjects. Before collecting a blood sample from the study subjects' verbal and or written consent of the issue was sought after briefing them on the research and the need for them to participate. In a situation whereby the subjects were not mature enough, consent of his/her parents/guardian was sought. All the subjects were assured that all information collected from the study subjects would be strictly used for the research and treated with high confidentiality. In addition, quality control and assurance were assured when handling and treating each sample.

Study Subjects and Inclusion Criteria. A total of two hundred study subjects comprised of males and females of different ages who willingly and voluntarily agreed to participate in the study were used as the subjects for the research. Three recruitment centres were selected: Gombe town maternity (Gidan Magani). Only patients who reported themselves to the selected hospitals (Gombe Town Maternity (Gidan Magani), Sunnah Clinic and Idi Children and Women Hospital Gombe.) with symptoms of malaria (fever) or a history of madness in the last 24 hours and referred by a physician for the screening of malaria infection and in addition they have not used any antimalarial drugs 60 days before the data collection, only subjects with *Plasmodium falciparum* mono-infection were recruited.

Blood sample collection and analysis. The vein puncture technique was used to collect Venus's blood samples with the help of medical personnel. Briefly, a Soft tubing tourniquet was fastened onto the upper arm of the respondents to enable the index finger to feel a suitable vein. The puncture site was then cleaned with methylated spirit (methanol), and venepuncture was made with a needle attached to a 5 ml syringe. When sufficient blood samples had been collected, the tourniquet was removed, and the hand was extracted immediately. The blood was transferred into an EDTA container to the laboratory for analysis.
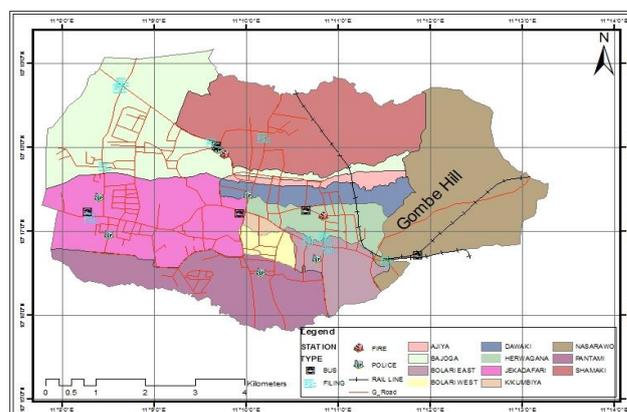

Figure 1 – Map of Gombe Local Government Area

Microscopy. The collected blood samples were analyzed within 1 to 2 hours after collection. Thick and thin films were prepared according to the standard film preparation technique. A drop of blood sample was placed on the centre of grease-free slides. After which, the reverse side of the slides was cleaned with cotton wool, allowed for air-drying, and stained with Giemsa stain for 60 minutes. After which, the slides were washed off gently with clean water. The drops were placed on a rack to air-dry for eventual examination of the drops under the microscope, using oil immersion at 100× magnification to observe for *Plasmodium* parasite. The presence of ring forms and or Trophozoites of *Plasmodium* indicated positive results. In contrast, the absence of either Trophozoites or ring form indicates negative effects after 10 minutes of a thorough examination by a qualified microscopist under a 100 × high power field of the microscope.

Molecular analysis

DNA Extraction. The DNA was extracted using Quick-DNA™ Miniprep Plus Kit with catalogue No. D4069 from Zymo research. Techniques and procedures outlined and recommended by the manufacturers were strictly adhered to. Briefly, 200 µl of biofluid and cell buffer was added to the pieces of the filter paper containing the dried blood sample in the Eppendorf tube. After which 20 µl of proteinase K was added and mixed thoroughly and incubated at 55 °C for 10 minutes to digest the various component of the sample. After which, 200 µl of genomic binding buffer was added. The mixture was then transferred into a zymo spin column in a collecting tube and centrifuge in a refrigerated centrifuge at 1200 rpm for 1 minute. After which, the collecting tube was discarded with the follow-through. 400 µl of DNA pre-wash buffer was added to the column in a

new collection tube and centrifuged for 1 minute. This was followed by adding 700 µl of genomic DNA wash buffer and centrifuge for 1 minute. After which, 200 µl of Genomic buffer was added and centrifuged for 1 minute. The tube was then discarded with the follow-through. Finally, to elute the DNA, 50 µl of DNA elution buffer was added and incubated for 5 minute and then centrifuged for 1 minute.

DNA Confirmation and Purity and Concentration determination. To confirm the presence of DNA in the entire sample extracted, gel electrophoresis was run in 2% agarose stained with 0.5 µl of ethidium bromide and was allowed to run for 1 hour at 100 mA, after which the gel was visualized using Ultraviolent (UV) Trans illuminator. Nanodrop Spectrophotometer was used to determine the concentration and purity of the DNA extracted.

Primers. The primer (F5'AACAGACGGGTAGTCATGATTGAG3' and R5'GTATCTGATCGTCTTCACTCCC3') used were adopted from the work of [24] and validated. All validated Primers were sent to Inqaba biotech Africa's genomic company for synthesis and supply. The primers were Reconstituted/ diluted using the recommended dilution factor (appropriate amount of distilled water) as specified by the manufacturer and stored at -4 °C as stock solution. The actual working solution was obtained by diluting 10 µl of the stock in 90 µl of Nano pure water making (10%).

Amplification of 18Sr RNA gene of Plasmodium falcifarum. The amplification was carried out using a Classic DW-K960 thermal cycler. The reaction was carried in a 25 µl reaction mixture containing 5 µl of the extracted DNA as the template, 1 µl of primer (0.5 µl each of F5'AACAGACGGGTAGTCATGATTGAG3' R5'GTATCTGATCGTCTTCACTCCC3'), 6.5 µl distilled water and 12.5 µl of the PCR Master mix (Containig dNTPs, Mgcl2 and Taq DNA Polymerase). The gene was amplified by setting an initial denaturation at 95 °C for 15 minutes, followed by forty (40) cycles of denaturation at 94 °C for 45 seconds while annealing at 60 °C for 90 seconds and extension at 72 °C for 1 minute. The final extension was carried out at 72 °C for 5 minutes. This band size of 276 bp was used as a control for the confirmation of *Plasmodium falciparum*, while distilled water was used as a negative control for all PCR in the research.
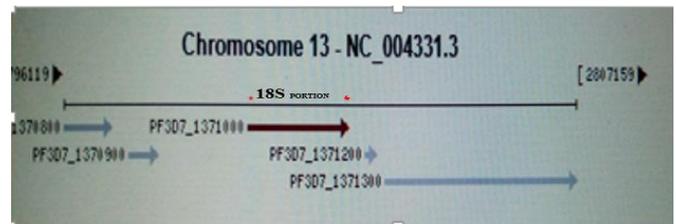

Figure 2 – Chromosome 13 of *Plasmodium falciparum* indicating 18S Portion of Ribosomal RNA gene

Gel Electrophoresis. The amplified genes were subjected to electrophoresis in 2% Agarose stained with Ethidium bromide. The gel was allowed to run for 1 hour at 100 mA, after which the gel was visualized using an Ultraviolent (UV) Trans illuminator.

Determination of performance of Polymerase Chain Reaction and Microscopy. To determine the effectiveness of PCR and Microscopy in malaria diagnosis, its sensitivity, specificity, positive predictive value, negative predictive value and accuracy were calculated using the formulas.

$$Sensitivity = \frac{True\ positive}{True\ Positive\ +\ False\ Negative} \times 100 \text{, (1)}$$

$$Specificity = \frac{True\ Negative}{True\ Negative\ +\ False\ Positive} \times 100 \text{, (1)}$$

$$Positive\ Predictive\ value = \\ = \frac{True\ Positive}{True\ Positive\ +\ False\ Positive} \times 100 \text{, (3)}$$

$$Negative\ Predictive\ value = \\ = \frac{True\ Negative}{True\ Negative\ +\ False\ Negative} \times 100 \text{, (4)}$$

$$Overall\ accuracy = \\ = \frac{True\ positive\ +\ False\ Positive}{True\_P + False\_P + True\_N + False\_N} \times 100 \text{ (5)}$$

RESULTS AND DISCUSSION

Demographic and clinical information of the subjects. Table 1 below shows some essential demographic and clinical characteristics of the study subjects, where the age of the issues ranges from 5-50 years with a mean age of 28.60±10.60. The

mean ambient body temperature of the topics ranges from 33–43 °C with a mean of 37.77±1.92. For the molecular analysis, the concentration of the DNA sample extracted ranges from 100.10–600.2 ng/µl of the sample, and the mean concentration was 300.55±100.03. For purity, the mean value of A260/280 was 1.72±0.55, ranging from 0.7–5.11.

Table 1 – Demographic and Clinical characteristics of the study subject and essential elements of the DNA sample

Characteristics	Mean	Range	Male	Female
Age	28.60 ± 10.60	5–55 Years	114 / 57.0%	86 / 43.0%
Body Temperature	37.77 ± 1.92	33-43 °C		
DNA Concentration	300.57 ± 10.03	100.10-600.2 ng/l		
A260/280	1.72 ± 0.55	0.7–5.11		

Malaria Prevalence using Microscopy and Polymerase Chain Reaction. Of the two hundred blood sample samples analyzed by microscopy, 167(83.5%) was malaria positive. In contrast, a prevalence of 105(52.5%) was recorded when analyzed with Polymerase Chain Reaction, as shown in Figure 2. Statistically, the two techniques show no significant difference (×2= 0.787, df=1, P> 0.05).
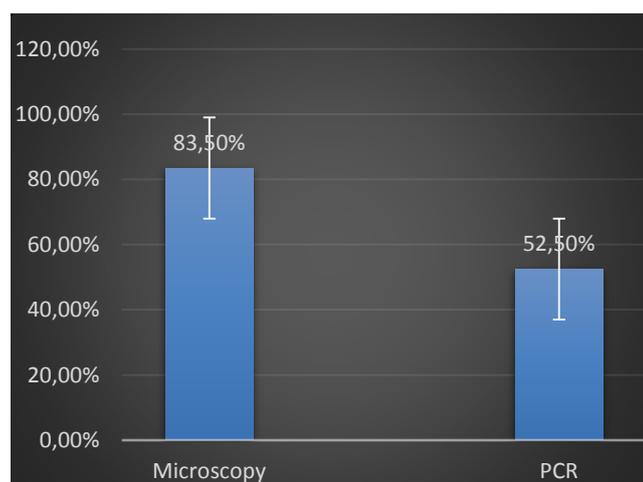

Figure 3 – Malaria prevalence using Microscopy and PCR

Results of Malaria diagnosis using PCR and Microscopy. Table 3 shows the diagnostic results of PCR and Microscopy. Out of the two hundred

samples analyzed by both techniques, 90(45.0%) models were positive by both PCR and microscopy, hence true positive. In contrast, 77(38.5%) were negative with PCR but positive with microscopy. Therefore false negative. 15(7.5%) samples were positive with only PCR. Thus false positives and 18(9.0%) pieces were found to be harmful when tested using PCR and microscopy. Performance of Microscopy revealed that 15(7.5%) samples were found to be falsely negative, and 77(38.5%) and 18(9.0%) models were falsely positive and true negative, respectively.

Table 3 – Polymerase Chain Reaction and Microscopy results

Techniques		Positive	Negative	Total
PCR	Positive	90 (45.0%)	15 (7.5%)	105 (52.5%)
	Negative	77 (38.5%)	18 (9.0%)	95 (47.5%)
Total		167 (83.5%)	33 (16.5%)	200 (100%)
Microscopy	Positive	90 (45.0%)	77 (38.5%)	167 (83.5%)
	Negative	15 (7.5%)	18 (9.0%)	33 (16.5%)
Total		105 (53.0%)	95 (47.0%)	200 (100%)

Performance of PCR and Microscopy in malaria diagnosis. Therefore the overall performance of PCR to microscopy revealed sensitivity, specificity, positive predictive and negative predictive values of 53.89, 54.54, 85.71 and 18.94%, respectively, with an overall accuracy of 0.54. Microscopy revealed sensitivity and specificity values of 85.71 and 18.94%, respectively. In comparison, a positive predictive value of 53.89% and a negative predictive value of 54.54% were recorded, with 0.84 as the overall performance recorded in microscopy, as shown in Table 4.

Table 4 – Performance of PCR and Microscopy in falciparum-malaria diagnosis

Determinant	PCR, %	Microscopy, %
Sensitivity	53.89	85.71
Specificity	54.54	18.94
PPV	85.71	53.89
NPV	18.94	54.54
Accuracy	0.53	0.84.

Notes: PPV – Positive predictive value, NPV – Negative predictive value

One of the strategies to control malaria is the precise laboratory diagnosis to treat positive

cases appropriately [25], and it is one of the primary critical strategies for malaria eradication [26]. In addition, World Health Organisation has realized the importance and impact of novel techniques like PCR on malaria diagnosis regarding reliability and accuracy to overcome various disadvantages and other shortcomings that microscopy faces as the gold standard and other recommended diagnostic procedures [23]. The two diagnostic techniques (PCR and microscopy) used in this study revealed two different results: microscopy reported a very high malaria prevalence of 83.5% and 52.5% by PCR. The difference observed may be attributed to the fact that microscopy produced additional 62 positive samples over the 105 positive samples produced by PCR. However, PCR is more sensitive than microscopy as it can detect infection with parasites as low as 5 parasite/µL of blood sample [27]. The low sensitivity of PCR recorded in the present study might be attributed to other inherent factors of the technique, like the concentration of the parasites' DNA from which the 18srRNA gene was amplified from chromosome 13. This could lead to a decrease in the positive samples by the PCR as such affecting the sensitivity values of the PCR and other performance indices of the technique. In addition, microscopy is one of the most routine laboratory techniques [28] for diagnosing malaria infection in the study area [29]. Therefore, laboratory technologists have a mystery of the technique, which makes it difficult to be affected by either internal or external factors un noticed.

Contrary to the present study's findings, [4] reported almost the exact prevalence of 71.43% and 73.57% by microscopy and Polymerase chain reaction, respectively. This high prevalence reported by the two techniques in the present study is unsurprising. Instead, it only confirms the endemicity of the disease in the study area.

The performance of PCR reported in the present study in terms of sensitivity, specificity and negative predictive values is lower than that of [30], who reported high performance of PCR with 65.3%, 95.6% and 98.8% as sensitivity, specificity and negative predictive values respectively, but with the lower positive predictive value of 33.3% as compared to 85.71% reported in the present status. The sensitivity and specificity documented are also lower than 100% and 79%, respectively, written by [31]. The sensitivity value of 53.89% of PCR registered in this study indicated that PCR could detect 53.89% of the subjects suffering from malaria. The sensitivity of PCR reported in this study is far higher than the 12.63% reported by [32] from southeast Nigeria when Comparing the performance of different Malaria Diagnostic Tools, including PCR among Pregnant Cohorts in Onitsha Christian. At the same time, a 100% specificity value was recorded, which was higher than the 54.54% reported in the present study, which was several individuals free from malaria by PCR. In addition, the present study recorded 85.71% as a positive predictive value, corresponding to the number of subjects that tested positive and had malaria infection. On the other hand, a Negative Predictive Value of 18.94% reported in the present study indicated the number of subjects that tested negative and did not have malaria disease. Though the sensitivity and specificity of PCR recorded in this study are lower than 95% and 90%, respectively, recommended by World Health Organisation (6), the technique (PCR) was able to confirm almost 86% of subjects who have the disease condition (malaria).

Microscopy recorded a sensitivity value of 85.71% in the present study. This is similar to the findings of [33], who reported a sensitivity value of 89.4 but with higher specificity and positive and negative predictive values of 100% each. In addition, the findings from this study also contradict the results of [34], who reported higher sensitivity, specificity and Positive and Negative predictive values of 91.0%, 97.5%, 96.8% and 92.8%, respectively. Microscopy in the present study detected 83.5% of the individuals suffering from malaria infection and 18.94% free individuals. Nevertheless, 53.89% of the individuals tested positive and had malaria. In comparison, 54.54% of study subjects tested negative and did not have malaria infection like in PCR. Sensitivity and specificity values reported in the present study are lower than the recommended 95% and 90% for sensitivity and specificity, respectively.

The accuracy of the two techniques used in this study was 0.53 and 0.84, respectively, for PCR and Microscopy. These findings contradict the finding of [35], who reported a lower accuracy value of 0.42 and 0.4, respectively, for Microscopy and PCR.

CONCLUSIONS

Both PCR and microscopy have demonstrated a high level of sensitivity, and the specificity of PCR

is far higher than that of microscopy. Though the sensitivity and specificity recorded are lower than the World Health organizations' recommended values, the techniques can still provide the minimum required result. Both methods have demonstrated appreciable predictive values with an excellent level of accuracy.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Edem, E. N., Mbong, E. O., & Hussain, S. (2020). Accuracy of Malaria Diagnosis by Microscopy and RDT Methods amongst Pregnant Women Attending Antenatal Clinic in Eket. *Journal Of Infectious Diseases and Diagnosis, 15*(3), 1–3.

2. Adeola, O., Oluyomi, S., Sola, A., Bassey, O., Oluwagbemiga, A., &Olusola, A. (2018). Performance of Microscopy Method and Rapid Diagnostic Tests in Malaria Diagnosis amongst Pregnant Women in Lagos, Southwest Nigeria. *Diversity and Equality in Health and Care, 15*(3), 104–109.

3. Yaya, S., Bishwajit, G., Ekholuenetale, M., Shah, V., Kadio, B., & Udenigwe, O. (2017). Knowledge of prevention, cause, symptom and practices of malaria among women in Burkina Faso. *PLOS ONE, 12*(7), e0180508. doi: 10.1371/journal.pone.0180508

4. Obimakinde, E. T., Simon-Oke I., & Osunyemi, O. S. (2018). The effectiveness of microscopy: Rapid diagnostic test and molecular assay in diagnosing malaria. *Journal of Parasitic Diseases: Diagnosis and Therapy, 3*(1). doi: 10.4066/2591-7846.1000022

5. Musbau, S., Bala, U., Umar, Y. D. (2014). Prevalence of Malaria Parasite Infection among Pregnant Women Attending General Sani Abacha Specialist Hospital Damaturu. *Scholars Journal of Applied Medical Sciences , 2*(6F), 3281–3283.

6. Muhammad, I., Abubakar, B., Mohammed, M. T., Abdullahi, A., & Asiya, M. (2022). Determination of malaria rapid diagnostic test effectiveness compared to microscopy (Gold standard). *DYSONA-Life Science, 3*, 49–56.

7. Sixpence, A., Nkoka, O., Chirwa, G. C., Milanzi, E. B., Mangani, C., Mathanga, D. P., & Ntenda, P. A. M. (2020). Levels of knowledge regarding malaria causes, symptoms, and prevention measures among Malawian women of reproductive age. *Malaria Journal, 19*(1). doi: 10.1186/s12936-020-03294-6

8. Mbanefo, A., & Kumar, N. (2020). Evaluation of Malaria Diagnostic Methods as a Key for Successful Control and Elimination Programs. *Tropical Medicine and Infectious Disease, 5*(2), 102. doi: 10.3390/tropicalmed5020102

9. DePina, A. J., Dia, A. K., de Ascenção Soares Martins, A., Ferreira, M. C., Moreira, A. L., Leal, S. V., Pires, C. M., Moreira, J. M. G., Tavares, M. F., da Moura, A. J. F., Pereira, J. M., Faye, O., Seck, I., & Niang, E. H. A. (2019). Knowledge, attitudes and practices about malaria in Cabo Verde: a country in the pre-elimination context. *BMC Public Health, 19*(1). doi: 10.1186/s12889-019-7130-5

10. Danwang, C., Noubiap, J. J., Souopgui, J., Gaudart, J., Yombi, J. C., & Robert, A. (2021). Accuracy of malaria diagnostic tests performed on non-invasively collected samples: a systematic review and meta-analysis. *BMJ Global Health, 6*(6), e005634. doi: 10.1136/bmjgh-2021-005634

11. Hathiwala, R., Mehta, P. R., Nataraj, G., & Hathiwala, S. (2017). LED fluorescence microscopy: Novel method for malaria diagnosis compared with routine methods. *Journal of Infection and Public Health, 10*(6), 824–828. doi: 10.1016/j.jiph.2017.01.001

12. Fransisca, L., Kusnanto, J. H., Satoto, T. B. T., Sebayang, B., Supriyanto,   , Andriyan, E., & Bangs, M. J. (2015). Comparison of rapid diagnostic test Plasmotec Malaria-3, microscopy, and quantitative real-time PCR for diagnoses of Plasmodium falciparum and Plasmodium vivax infections in Mimika Regency, Papua, Indonesia. *Malaria Journal, 14*(1). doi: 10.1186/s12936-015-0615-5

13. Zaki, W., & Madkhali, A. (2016). Accuracy of immunochromatography diagnostic test versus microscopy in the diagnosis of malaria among clinically suspected patients in Jazan area, KSA. *Parasitologists United Journal, 9*(2), 95. doi: 10.4103/1687-7942.205164

14. Kitutu, F. E., Wamani, H., Selling, K. E., Katabazi, F. A., Kuteesa, R. B., Peterson, S., Kalyango, J. N., & Mårtensson, A. (2018). Can malaria rapid diagnostic tests by drug sellers under field conditions classify children 5 years old or less with or without Plasmodium falciparum malaria? *Comparison with nested PCR analysis. Malaria Journal, 17*(1). doi: 10.1186/s12936-018-2508-x

15. Badiane, A., Thwing, J., Williamson, J., Rogier, E., Diallo, M. A., & Ndiaye, D. (2022). Sensitivity and specificity for malaria classification of febrile persons by rapid diagnostic test, microscopy, parasite DNA, histidine-rich protein 2, and IgG: Dakar, Senegal 2015. *International Journal of Infectious Diseases, 121*, 92–97. doi: 10.1016/j.ijid.2022.04.060

16. Ricciardi, A., & Ndao, M. (2015). Diagnosis of Parasitic Infections: What's Going On? *SLAS Discovery, 20*(1), 6–21. doi: 10.1177/1087057114548065

17. Nijhuis, R. H. T., van Lieshout, L., Verweij, J. J., Claas, E. C. J., & Wessels, E. (2018). Multiplex real-time PCR for diagnosing malaria in a non-endemic setting: a prospective comparison to conventional methods. *European Journal of Clinical Microbiology & Infectious Diseases, 37*(12), 2323–2329. doi: 10.1007/s10096-018-3378-4

18. Gupta, P., Gupta, P., Rao, S., Singh, N., & Kalita, D. (2018). Comparison Between Microscopy And Rapid Diagnostic Tests In Diagnosis Of Malaria At A Tertiary Care Medical Institution In Uttarakhand (A 3-Year Study). *Asian Journal of Pharmaceutical and Clinical Research, 11*(2), 94. doi: 10.22159/ajpcr.2018.v11i2.22141

19. Oboh, M. A., Oriero, E. C., Ndiaye, T., Badiane, A. S., Ndiaye, D., & Amambua-Ngwa, A. (2021). Comparative analysis of four malaria diagnostic tools and implications for malaria treatment in southwestern Nigeria. *International Journal of Infectious Diseases, 108*, 377–381. doi: 10.1016/j.ijid.2021.05.049

20. Richardson, D. C., Ciach, M., Zhong, K. J. Y., Crandall, I., & Kain, K. C. (2002). Evaluation of the Makromed Dipstick Assay versus PCR for Diagnosis of Plasmodium falciparum Malaria in Returned Travelers. *Journal of Clinical Microbiology, 40*(12), 4528–4530. doi: 10.1128/jcm.40.12.4528-4530.2002

21. Nair, C. B., Manjula, J., Subramani, P. A., Nagendrappa, P. B., Manoj, M. N., Malpani, S., Pullela, P. K., Subbarao, P. V., Ramamoorthy, S., & Ghosh, S. K. (2016). Differential Diagnosis of Malaria on Truelab Uno®, a Portable, Real-Time, MicroPCR Device for Point-Of-Care Applications. *PLOS ONE, 11*(1), e0146961. doi: 10.1371/journal.pone.0146961

22. Siwal, N., Singh, U. S., Dash, M., Kar, S., Rani, S., Rawal, C., Singh, R., Anvikar, A. R., Pande, V., & Das, A. (2018). Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by Plasmodium falciparum and P. vivax in India. *PLOS ONE, 13*(3), e0193046. doi: 10.1371/journal.pone.0193046

23. Wardhani, P., Butarbutar, T. V., Adiatmaja, C. O., Betaubun, A. M., Hamidah, N., & Aryati. (2020). Performance Comparison of Two Malaria Rapid Diagnostic Test with Real Time Polymerase Chain Reaction and Gold Standard of Microscopy Detection Method. *Infectious Disease Reports, 12*(11), 8731. doi: 10.4081/idr.2020.8731

24. Londono, B. L., Eisele, T. P., Keating, J., Bennett, A., Chattopadhyay, C., Heyliger, G., Mack, B., Rawson, I., Vely, J.-F., Désinor, O., & Krogstad, D. J. (2009). Chloroquine-Resistant HaplotypePlasmodium falciparumParasites, Haiti. *Emerging Infectious Diseases, 15*(5), 735–740. doi: 10.3201/eid1505.081063

25. Hernandez-Valladares, M., Rihet, P., & Iraqi, F. A. (2014). Host susceptibility to malaria in human and mice: compatible approaches to identify potential resistant genes. *Physiological Genomics, 46*(1), 1–16. doi: 10.1152/physiolgenomics.00044.2013

26. Rifaie-Graham, O., Pollard, J., Raccio, S., Balog, S., Rusch, S., Hernández-Castañeda, M. A., Mantel, P.-Y., Beck, H.-P., & Bruns, N. (2019). Hemozoin-catalyzed precipitation polymerization as an assay for malaria diagnosis. *Nature Communications, 10*(1). doi: 10.1038/s41467-019-09122-z

27. Rodulfo, H., De Donato, M., Mora, R., González, L., & Contreras, C. E. (2007). Comparison of the diagnosis of malaria by microscopy, immunochromatography and PCR in endemic areas of Venezuela. *Brazilian Journal of Medical and Biological Research, 40*(4), 535–543. doi: 10.1590/s0100-879x2007000400012

28. Wilson, M. L. (2013). Laboratory Diagnosis of Malaria: Conventional and Rapid Diagnostic Methods. *Archives of Pathology & Laboratory Medicine, 137*(6), 805–811. doi: 10.5858/arpa.2011-0602-ra

29. Efunshile, M., Amoo, A. O. J., Akintunde, G. B., Ojelekan, O. D., König, W., & König, B. (2011). Use and Effects of Malaria Control Measures in Pregnancy in Lagos, Nigeria. *The Korean Journal of Parasitology, 49*(4), 365. doi: 10.3347/kjp.2011.49.4.365

30. Masanja, I. M., McMorrow, M. L., Maganga, M. B., Sumari, D., Udhayakumar, V., McElroy, P. D., Kachur, S. P., & Lucchi, N. W. (2015). Quality assurance of malaria rapid diagnostic tests used for routine patient care in rural Tanzania: microscopy versus real-time polymerase chain reaction. *Malaria Journal, 14*(1). doi: 10.1186/s12936-015-0597-3

31. Wangai, L., Karau, M., Njiruh, P., Sabah, O., Kimani, F., Magoma, G., & Kiambo, N. (2011). Sensitivity of microscopy compared to molecular diagnosis of P. falciparum: Implications on malaria treatment in epidemic areas in Kenya. *African Journal of Infectious Diseases, 5*(1). doi: 10.4314/ajid.v5i1.66504

32. Uba, C. C., Ikegbunam, M. N., Anagor, I. S., Eleanya, L. C., Ezeumeh, E. N., Ezekwueche, S. N., Okechukwu, E. C., & Esimone, C. O. (2021). Comparative Performance of Different Malaria Diagnostic Tools among Pregnant Cohorts in Onitsha Southeast Nigeria. *Advances in Infectious Diseases, 11*(04), 384–394. doi: 10.4236/aid.2021.114035

33. Khan, S. A., Ahmed, S., Mushahid, N., Anwer, M., Saeed, S., Khan, F. A., Shamshad, G. U., & Joyia, Z. (2013). Comparison of real time polymerase chain reaction with microscopy and antigen detection assay for the diagnosis of malaria. *Journal of the College of Physicians and Surgeons-- Pakistan: JCPSP*, *23*(10), 787–792.

34. Adiatmaja, C. O., Butarbutar, T. V., Betaubun, A. M., & Wardhani. P. (2020). Comparison of Malaria Microscopy and Polymerase Chain Reaction for Identification of Plasmodium in Papua, Indonesia. *Systematic Reviews in Pharmacy, 11*(06). doi: 10.31838/srp.2020.6.16

35. Shankar, H., Singh, M. P., Phookan, S., Singh, K., & Mishra, N. (2021). Diagnostic performance of rapid diagnostic test, light microscopy and polymerase chain reaction during mass survey conducted in low and high malaria-endemic areas from two North-Eastern states of India. *Parasitology Research, 120*(6), 2251–2261. doi: 10.1007/s00436-021-07125-8