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Usefulness of Polymerase Chain Reaction in the diagnosis of asymptomatic malaria among school age children in Ilorin, Nigeria

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Abstract

Introduction: In Nigeria, microscopy and Rapid Diagnostic Tests (RDTs) are majorly used routinely to diagnose malaria in clinical settings. In recent years, molecular diagnosis has emerged as the most sensitive method for malaria diagnosis. This study determined the usefulness of Polymerase Chain Reaction (PCR) in comparison to microscopy (Giemsa stained thick and thin smears) for the diagnosis of asymptomatic malaria in Ilorin, Nigeria.

Methods: The study enrolled 310 school children aged 4–15 years with no sign or symptoms of malaria. Blood samples were collected for identification of *Plasmodium* species infection using light microscopy and conventional PCR.

Results: The PCR method detected more infection of *P. falciparum* 107 (34.5%) than slide microscopy 81 (26.1%) in the study. Only *P. falciparum* was detected by microscopy while PCR detected mono infection of *P. malariae* (3.2%) and *P. ovale* (0.6%) and mixed infection of *P. falciparum* and *P. malariae* (3.2%). Overall the PCR method detected more malaria parasite compared to microscopy.

Conclusion: The PCR technique, although more laborious and expensive than microscopy, have better diagnostic accuracy and are highly useful for the detection of *P. falciparum* and other malaria species in asymptomatic and low parasitaemia cases.

Keywords: Malaria, Asymptomatic, diagnosis, PCR, Nigeria

1.0 INTRODUCTION

Despite remarkable achievements in reducing malaria mortality, malaria remains the most common cause of morbidity and mortality in sub-Saharan Africa [1,2]. It is a life threatening parasitic disease transmitted by infected female *Anopheles* mosquitoes and a major public health concern worldwide, causing 219 million infections and approximately 90% of the malaria deaths the same year [3]. The disease is endemic in parts of Asia, Africa, Oceania, and Central and South America, with around 90% of the global malaria burden borne by Sub-Saharan Africa [3]. Malaria is endemic throughout Nigeria accounting for 25% of infant mortality and 30% of childhood mortality [4]. About 50% of the population has at least one episode of malaria each year [4]. Nigeria has more reported cases of malaria and deaths due to malaria than any other country in the world [4].

Malaria presents a diagnostic challenge to laboratories in most countries [5]. In tropical countries, malaria may present clinical signs and symptoms like in other diseases thus making the diagnosis of malaria difficult especially in the resource poor regions. Signs and symptoms of malaria include intermittent fever, body aches and pains, malaise, generalized body weakness which may be presented by typhoid disease [5]. Prompt and accurate diagnosis of malaria is essential for effective disease management in any setting; delayed treatment increases the risk of death or serious neurological sequelae [6]. The early symptoms of malaria (fever, chills, headache, body aches, nausea, and vomiting) and physical findings (fever, pallor) are not specific and may be indicative of any number of diseases [5,6]. Common and life threatening infections such as viral and bacterial infections could present same signs and symptoms to malaria making clinical diagnosis quite challenging and unreliable [6]. The signs and symptoms of malaria and other tropical diseases overlap, and this may affect the diagnostic specificity and sensitivity, thereby increasing the wrong use of antimalarial and reduction in the effective management of patients with non-malarial febrile illness especially in the tropical Africa [6]. Therefore precise laboratory diagnosis is required to achieve effective treatment of malaria.

There are several methods in the laboratory for the diagnosis of malaria parasites in the peripheral blood, including microscopy, antigen detection and polymerase chain reaction (PCR). Common tradition is to detect malaria by microscopic examination of blood smears for *Plasmodium* species. However, newer diagnostic methods are being used in both endemic and non-endemic areas to detect malaria parasitemia. Microscopy

has historically been the mainstay of the diagnosis of malaria. The detection of asexual parasites by light microscopy of Giemsa-stained thick and thin films remains the standard laboratory method for the diagnosis of malaria [7,8]. Although easy to apply and cost-effective, this technique assumes that laboratories have certain infrastructure in place with highly skilled professionals and in detecting low level parasitaemia, so the sensitivity may fluctuate depending upon the skill of technician [9-12]. World Health Organization has recognized the urgent need for simple and cost-effective diagnostic tests for malaria to overcome the deficiencies of both light microscopy and clinical diagnosis [13]. To overcome such limitations, several methods have been in use. These include the staining of parasite DNA and RNA with acridine orange, the quantitative buffy coat method (QBC). Rapid Diagnostic Tests (RDTs) methods based on the detection of the enzyme lactate dehydrogenase (pLDH) and circulating *P. falciparum* histidine rich protein-2 (PfHRP-II) [14,15] are now playing an important role in the prompt and accurate diagnosis of malaria.

Polymerase chain reaction (PCR) based assays have been used mainly for the assessment of the sensitivity and specificity of microscopy [16,17]. PCR based tests have shown remarkable capacity to detect malarial parasites in mixed infections and low parasite count and are also sensitive when compared to microscopic examination [18]. PCR can detect malaria infections with parasitemia as low as 0.01–0.2 parasites/ μ L of blood [16,17]. The value of PCR as a means of malaria parasite diagnostic technique lies in its sensitivity, its capability of identifying malaria parasites to the species level, as well as its ability to detect five parasites or less/ μ L of blood [17]. However, it is not appropriate for use in the field, as it is an expensive and complex method.

Many studies have revealed higher sensitivity of PCR in comparison to microscopy mostly for the diagnosis of complicated and uncomplicated *P. falciparum* infection with paucity of information in asymptomatic cases and specie differentiation. The main objective of this study therefore was to evaluate the usefulness of PCR method in the diagnosis of *Plasmodium* species in asymptomatic cases.

2.0 METHODOLOGY

2.1 Study area and population

The study was conducted in Patigia rural community in Kwara State, Nigeria. The town is inhabited by the Nupe people who also exhibit a linguistic repertoire of the Yoruba dialect. The inhabitants are farmers, aquatic sellers/fishers and traders. Pategi is located in Pategi

Local Government Area in Kwara State. The town lies on Latitude Average annual rainfall is about 1270, which falls almost entirely during the wet season. The community falls into stable malaria transmission zone where malaria is present throughout the year with a marked increase during the raining season which normally runs from April to September. The town stands on higher level and the soil can be described as well drained, moderately leached and with moderate humus content. 843'59.988"N and Longitude 545'0.000"E[19]. Average daily temperature is 37°C with minimum mean temperature of 20°C and maximum mean temperature of 39°C.

2.2 Ethical statement.

The study was a cross sectional study. Five primary schools were randomly selected for the study in the LGA capitals. Purposive sampling technique was used to select children for the specimen collection. Blood specimen was taken from 310 primary schools pupils of ages 4-15 years. Inclusion criteria for the study include consent from parent or guardian, no intake of antimalaria during the preceding four weeks and absence of malaria symptoms (vomitting, loss of appetite, fever etc). Sick children and parents who refused to give their consent to participate in the study were excluded.

2.3 Sampling techniques

The study was a cross sectional study. Five primary schools were randomly selected for the study in the LGA capitals. Purposive sampling technique was used to select children for the specimen collection. Blood specimen was taken from 310 primary schools pupils of ages 4-15 years. Inclusion criteria for the study include consent from parent or guardian, no intake of antimalaria during the preceding four weeks and absence of malaria symptoms (vomitting, loss of appetite, fever etc). Sick children and parents who refused to give their consent to participate in the study were excluded.

2.4 Microscopy method

Thick and thin films were prepared from blood collected into EDTA bottle and stained using 10% Giemsa method for 30 minutes. The slides were examined under light microscope (100X oil-immersion objective) by 2 experienced microscopists. Parasitaemia was calculated per 200 WBC assuming 8000 WBC/ μ L of blood [20,21]. The slides were reported only negative upon not detecting any parasites when 200 fields of the slides were examined.

2.5 DNA extraction and molecular detection

Blood samples collected were transported to the

Molecular Biology Laboratory of Ladoke Akintola Univeristy of Technology, Osogbo for PCR diagnosis. Genomic DNA was isolated using QIAgen DNA Mini Kit blood and tissue (QIAGEN, Germany) according to the manufacturers's instructions. The extracted DNA was stored at -20°C until used. Nested PCR assay were carried out as previously described elsewhere [12]. DNA samples were amplified by species-specific primer pairs (Table 1) designed to amplify small subunit ribosomal ribonucleic acid (ssRNA) genes of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* using the PCR technique originally described by Snounou et al. [22]. In brief, both the primary and nested amplifications were carried out in a 20 μ l reaction volume containing 10X buffer, 2.5mM MgCl₂, 200 μ M dNTPs, 200nM primers, and 1U Taq DNA-polymerase with approximately 10ng of DNA template on a PTC-200 Thermal cycler (Prime, UK). The *Plasmodium* genus-specific amplification was followed by *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* species-specific PCR amplification.

Thermal cycling parameters for first round of amplification were: initial denaturation at 94°C for 5 min, followed by 30 cycles of 95°C, 58°C at 30 seconds respectively and 72°C at 1:20min, for annealing temperature. Thermal cycling parameters for second round of amplification were: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C, 58°C, and 72°C for 45 seconds for annealing temperature. In each run negative and positive controls were included.

Genomic DNA from healthy as well as from individuals who have not travelled to malaria-endemic areas were included as negative controls in all PCR diagnostic assays. Amplicons were separated on a 1.2% agarose gel electrophoresis run along with a 1000bp DNA ladder (Invitrogen, Karlsruhe, Germany). The presence or absence of different *Plasmodium* species was confirmed with representative amplicon size that were species-specific. Samples that failed to amplify were subjected to repeated amplification procedures with different PCR additives. Chances of cross contamination was strictly monitored and prevented following stringent quality control procedure.

3.0 RESULTS

The enrollment data of the study group are summarized in Table 1. The mean age was 9.10 (SD $\pm$ 1.937) years. Polymerase chain reaction (PCR) detected more *P. falciparum* 107 (34.5%) parasite than slide microscopy 81(26.1%). Only a single specie of *P. falciparum* was detected by microscopy while *P. malariae* (3.2%) and *P. ovale* (0.6%) and mixed infection of *P. falciparum* and *P. malariae* (3.2%) were detected by PCR.

Table 1: Enrollment data of the participants recruited in the study

Characteristic	Outcome
Number of subjects examined	310
Mean age (years) $\pm$ SD	9.10 $\pm$ 1.937
Sex (male/female)	152/158
Number positive by microscopy (<i>P. falciparum</i>)	81 (26.1%)
Number of subjects positive by PCR	
<i>P. falciparum</i>	105 (33.8%)
<i>P. malariae</i>	10 (3.2%)
<i>P. ovale</i>	(0.6%)
<i>P. falciparum</i> + <i>P. malariae</i>	10 (3.2%)

Figure 1: Agarose gel electrophoresis picture of *P. falciparum* PCR

The PCR and microscopy at different *Plasmodium falciparum* parasite densities is shown in Table 2. All smear positive for *Plasmodium* by microscopy were also positive by PCR at all parasitaemia levels. However, more infection were detected at density below 500 parasites/ μ L.

Table 2: PCR and microscopy at different *Plasmodium falciparum* parasite densities

<i>P. falciparum</i> Parasites/ μ L	No. Detected by microscopy	No. Positive by PCR (%)
1-<99	20	20 (100.0)
100-249	41	41 (100.0)
250-499	10	10 (100.0)
500-999	6	6 (100.0)
1000-4999	4	4 (100.0)

4.0 Discussion

Microscopic examination of Giemsa-stained thick and thin blood smears has been the diagnostic method of choice for *Plasmodium* species identification in epidemiologic studies and medical diagnosis [21]. The method is simple, does not require highly equipped facilities, and in most cases enables differentiation among

the four *Plasmodium* species causing malaria in human when performed by a trained medical laboratory technician. However, microscopy is often time-consuming and laborious, and it is estimated that even a skilled person can evaluate only 60 to 80 specimens per day under field conditions [23,24]. This method can sometimes be misleading in identifying parasite species, especially in the case of low level of parasitemia and a mixed parasite infection [24] or modification by drug treatment. PCR based methods have been consistently shown to be powerful tool for malaria diagnosis [21]. Studies else where have shown that nested PCR, is more sensitive diagnostic method for malaria than microscopy, particularly in cases of low parasitemia and mixed infections [18]. The PCR analysis for *Plasmodium* species done in this study showed a sensitivity of 80.4%. In the present study, slide microscopically failed to detect parasite in some positive samples, giving some false negative results. False negatives are a big public health problem because the patient would miss correct diagnosis and treatment, thus not complying with the rule “fast and correct diagnosis, and treatment with confirmed presence of the parasite” [25]. This could have grave implications in health, transmission, and possibly mortality. Accurate diagnostic methods are the basis for adequate disease control and avoiding resistance to antimalarial drugs or the spread of resistance.

In the present study, 12 cases (10 cases for *P. malariae* and 2 cases for *P. ovale*) which were not detected by microscopy were detected by PCR. This might be due to the presence of low level of parasitemia. Other studies have also reported the ability of PCR to detect low level of parasitemia undetected by conventional microscopy [26-28]. Nested PCR could detect parasite DNA even in very low parasite count where microscopy could not detect, and PCR could also distinguish the species which was misdiagnosed by microscopy [16,17]. In practice, PCR-based assays may not replace microscopy for routine diagnosis in developing country Nigeria. However, nested PCR would be useful in discriminating the low parasitic infection cases strongly suspected by the clinicians but not supported by microscopy. Though it was observed that slide microscopically failed to detect parasite in many positive samples.

However PCR-positive results were obtained for same samples with coinfection of other species of *Plasmodium* detected which were not detected by microscopy. This observation is in line with other studies elsewhere [29-32]. The low sensitivity by microscopy can be due to several factors. It could be as a result of suboptimal staining of the smear which may result into poor staining

of the malaria parasites. Giemsa staining method is commonly employed by most clinical laboratories in the tropical countries to stain malaria smears. In Nigeria, the stain is readily available over the counter in most medical equipment stores and patent medicine stores with questionable quality. Even with the good quality stain and staining method, poor quality and inadequately maintained microscope could fail to detect parasites at low parasitaemia. An experienced person in a reference laboratory would not be expected to detect parasitaemia lower than 50 parasites/ μ l by microscopy [33]. The microscopist who is not adequately trained and fatigued by high workload could miss out low parasite density. However, even when working under best conditions microscopy based malaria slide examination cannot detect the very low parasitaemia that PCR-based method can achieve. The PCR could detect a low sensitivity of 0.5 parasites/ μ l [31,32,34], which would practically be impossible for an experienced person achieve with light microscope. Furthermore, inability to detect low parasitaemia by microscopically may probably be as a result of submicroscopic malaria infections which might be related to the immunity status. The present study was among asymptomatic infected school age children. In malaria endemic countries, acquired immunity is associated with the submicroscopic infections that may not be seen under microscope easily by the clinical technician. Submicroscopic infections have been reported in high transmission regions in sub-Saharan African countries such as Ghana and Uganda among children [35,36]. Previous studies have shown that malaria parasite densities is associated with the stage of the infection [37,38], level of acquired immunity [25,39] and the genetic diversity of circulating parasite clones [40]. In addition to the argument that malaria parasitaemia can be influenced by immunity, there could be false PCR positivity as it can be explained by other mechanisms. PCR may remain positive for several week after systemic clearance of malaria from an effective drug treatment, as a result of residual asexual parasites and presence of gametocytaemia [26]. This may be eliminated by employing a mitochondrial PCR. This is sensitive in detecting gametocytes because gametocytes stage harbour up to eight mitochondria organelles, compared to the ring stage which harbours only one organelle [30]. Failure to observe good laboratory practices in a PCR laboratory can result in false positivity due to contamination. Contamination can occur in all steps of the procedure giving false amplification products [30].

In this study the sensitivity and specificity for PCR were 80.4% and 100.0%, respectively. Other studies obtained

in Nigeria had obtained different sensitivity and specificity. Ogbolu and others obtained a sensitivity and specificity of 29.4% and 100% in a study in Ile-Ife [27] in south western Nigeria, Kanyi and others 53.8% and 100% in Lagos [28] and Waggoner *et al.* obtained 97.1% and 93.5% also in Lagos [39]. It has been estimated that a diagnostic test with 95% sensitivity and 95% specificity requiring minimal infrastructure would avert more than 100,000 deaths and about 400 million unnecessary treatments [40].

Our results highlight the importance and usefulness of PCR in the diagnosis of asymptomatic malaria in among children. Although microscopy is cheap, readily assessible and can be used in field epidemiological study, however, PCR would give the correct prevalence of the running parasite in the community because the submicroscopic parasites would eventually be detected. For prevalence studies it is better to continue using microscopy as a reference technique and PCR to confirm negative results from microscopy.

Conflict of interest

The authors declare that there is no conflict of interest

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