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Molecular investigation of sub-microscopic and mixed *Plasmodium* species infection in North-Central Nigeria

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ABSTRACT

Objective: To assess the level of sub-microscopic and mixed *Plasmodium* species infection in children in North-Central Nigeria.
Methods: Blood sample was obtained from 960 apparently healthy children aged 2–18 years. Plasmodial parasites were identified by Giemsa-stained light microscopy and by DNA amplification of the 18S rRNA gene.
Results: A total of 126 out of 960 samples (13.1%) were positive for plasmodial parasites by microscopy while 284 of the 960 samples (29.6%) were positive by the nested PCR assay. The prevalence of sub-microscopic infection was 16.5% (158/960). The proportion of microscopic asymptomatic infections was found to be significantly higher in younger children than in older children ($\chi^2 = 16.86$; $df = 2$; $P = 0.014$), while sub-microscopic infections were more frequent in older children than in younger ones. Mono-infections of *Plasmodium falciparum*, *Plasmodium malariae*, and *Plasmodium ovale* were 96.1%, 1.1%, and 0.7%, respectively while 2.1% of the samples had mixed infections.
Conclusions: Our results showed that sub-microscopic infections were more prevalent in the study region and this has consequences for sustaining malaria transmission in the area. The inability of microscopy to correctly identify non-*falciparum* species and mixed *Plasmodium* species infection in this study clearly shows the importance of molecular screening tools for active field surveillance.

1. Introduction

Malaria remains a major public health problem in tropical and sub-tropical regions of the world, with an estimated 3.2 billion people in about 97 countries and territories of the world still at risk of being infected and developing the disease[1,2]. Although significant progress has been achieved in recent years at reducing malaria, latest estimates showed that about 198 million cases of malaria still occurred globally in 2013, resulting in about 584000 deaths, most of which were in sub-Saharan Africa and a substantial proportion of this was from Nigeria[2].

Human malaria is primarily known to be caused by four species of plasmodial parasites: *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*) and *Plasmodium malariae* (*P. malariae*). However, *Plasmodium knowlesi* which traditionally infects monkeys, is now known to infect human populations in South-East Asia[3,4] and was acknowledged to be a fifth species of human malaria parasites[5,6]. Of the human malaria parasites, *P. falciparum* predominates in Africa[7] although there are areas within this region that are co-endemic for other species of human malaria parasites.

A phenomenon which is widely acknowledged is the fact that individuals in malaria endemic regions harbour malaria parasites without presenting with clinical symptoms of the disease; they are said to have sub-clinical level of infections[8,9]. Some of these infections are detectable by microscopy and are referred to as patent infections or asymptomatic infections while others, consisting basically of very low-density parasitaemia, and below the detection threshold of light microscopy, are referred to as sub-patent or sub-microscopic infections[8].

Studies have shown that sub-microscopic infections were usually missed by light microscopy[10-14]. This is because light microscopy, although generally acknowledged as the conventional method for parasitological diagnosis of malaria, has several limitations: it has limited sensitivity and specificity especially when parasite density is very low and it relies on the expertise of the microscopist as well

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as on the quality of reagents and microscopes[15]. Furthermore, in areas that are co-endemic for multiple species of human plasmodial parasites, microscopy may not be able to make a distinction between species in mixed infection; consequently, non-*falciparum* species as well as mixed species infections may be underestimated or entirely missed[10,16,17], thereby making it difficult to assess the exact disease burden of malaria in a particular area or region. This therefore, makes the deployment of more sensitive tools imperative for epidemiological surveys, especially for the detection of sub-microscopic parasitaemia as well as low density minority species in mixed species infection.

For more than two decades, molecular techniques have been developed and used for malaria parasite identification and one of the most commonly used molecular test is the polymerase chain reaction (PCR)-based amplification of the DNA sequence of the 18S ribosomal RNA (rRNA) gene[18-22]. PCR-based assay has been shown to be more sensitive and specific in the detection and identification of *Plasmodium* species, and they more readily detect mixed infections[23-27]. They are therefore, useful for epidemiological studies in addition to being an assay of choice in critical situations where confirmatory diagnosis is required.

In Nigeria, it is known that the predominant species of parasite causing malaria in humans is *P. falciparum*, which has been implicated in over 90% of malaria cases[2]. However, the level of sub-microscopic infection due to *P. falciparum* or other *Plasmodium* species has not been sufficiently studied. Similarly, there are scant data on the impacts of minority species, especially *P. malariae* and *P. ovale*, on the overall burden of malaria in Nigeria[2]. Few studies that have tried to assess the level of mixed infections essentially in clinically healthy or asymptomatic individuals were conducted in South-West Nigeria[28,29]. This study therefore, was aimed at determining the level of sub-microscopic and mixed *Plasmodium* species infection by PCR-based amplification of the 18S rRNA gene, in apparently healthy children resident in Lafia, North-Central Nigeria.

2. Materials and methods

2.1. Study population

This study was conducted in Lafia, a city located in North-Central Nigeria which is located 8.49 latitude and 8.52 longitude (8°29'10.4" N, 8°31'14.8" E), and lies within the Nigeria's Guinea savannah ecological zone as described previously[30].

We enrolled children and teenagers from 2 to 18 years of age into the study in a community-based, cross-sectional survey. The criteria for enrolment were: (i) the child must be apparently healthy, with no symptoms compatible with malaria or any history of fever within the preceding 48 h or pyrexia (axillary temperature 37.5 °C) at the time of sampling; (ii) the child must have been residing in the community for at least one year. A child who had antimalarial drug administration within 28 days preceding enrolment was excluded from the study.

The study protocol was reviewed and approval by the Ethics Review Committee of the Dalhatu Araf Specialist Hospital and the Nasarawa State Ministry of Health, Lafia, Nasarawa State, Nigeria. The study participation was voluntary. Prior to being included in the study, informed consent was obtained from parent or guardian of each child (except those who were 18 years) after the study protocol has been explained to them. At the point of collection, all samples were de-identified with respect to confidentiality of participants.

2.2. Sampling and parasite identification

Blood sample of approximately 0.5 mL was collected by venepuncture from each child and from it, three drops were spotted on labelled Whatman 3 MM filter paper, air dried and individually sealed in plastic bags until use. Thick and thin blood smears were prepared for microscopic examination. Slides were stained with freshly prepared 5% Giemsa stain for 20 min at room temperature. Thick and thin blood films were then examined for malaria parasites. Examination of the blood films were performed by trained malaria microscopists. Parasitaemia were quantified relative to 250 white blood cells (WBC) on thick films and estimated as parasites per μL assuming a mean WBC of 8 000 per μL of blood as described[30]. Blood films were scanned on high-power field (HPF) at 1 000 magnification for approximately 5 min before they were labelled negative if no parasites were seen.

Parasite DNA was extracted from the dried blood spots on filter paper using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, as described previously[31]. We genotyped all samples for four human *Plasmodium* species (*P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*) using the nested PCR technique based on oligonucleotide primers that target the 18S rRNA gene described previously[32]. The primary reaction was performed using the rPLU5 and rPLU6 primer pairs for the genus. This was then followed by four sets of nested reactions using the specific primer pairs rFAL1, rFAL2; rMAL1, rMAL2; rOVA1, rOVA2, and rVIV1, rVIV2 for *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* detection, respectively. Primer sequences were synthesized by Operon Biotechnologies GmbH, Cologne, Germany. The PCR programme used was: denaturation at 95 °C for 5 min followed by 25 cycles (30 cycles in nested) of 1 min at 94 °C, 2 min at 60 °C and 2 min at 72 °C and a final extension period of 5 min at 72 °C. All PCR assays were performed using a BIOMETRA TB1 thermal cycler (Biotron, Göttingen, Germany). Electrophoresis of the PCR products was done on 1.5% agarose gel, and visualized by trans-illumination with ultraviolet light after staining with SYBR® Green. Fragment sizes were calculated relative to a standard size 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany).

2.3. Statistical analysis

Data entry was done in Microsoft® Excel, 2007 (Microsoft Corporation) while the SPSS version 11 (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis. Normally distributed variables were compared using the student's *t*-test. Numerical data not conforming to normal distribution were log-transformed. The *Chi*-squared test was used to compare proportions or categorical variables. Prevalence of *Plasmodium* species was measured by both microscopy and by PCR in each child. The results of microscopy were used to determine asymptomatic infections; while the results of the PCR assay, in combination with microscopy results, were used to determine sub-microscopic infections. The sensitivity and specificity of microscopy was determined taking PCR as the 'reference'. Cohen's kappa (κ) was used to measure the degree of agreement

between microscopy and PCR-based test with their 95% confidence intervals (*CI*). *P*-values less than 0.05 were considered significant.

3. Results

A total of 960 children were enrolled into this study after satisfying the inclusion criteria. Of these participants, the prevalence of microscopic asymptomatic infection (*i.e.* presence of parasitic infection detectable by microscopy in apparently healthy individuals) was 13.1% (126/960), while the prevalence of sub-microscopic infections (*i.e.* presence of parasitic infection undetectable by microscopy but detectable by PCR) was 16.5% (158/960). Overall, PCR was able to detect sub-clinical infections in 284 (29.6%) participants.

On average, microscopy detected 44.4% of all PCR-detected infections in this study. The sensitivity of microscopy for the detection of *Plasmodium* species compared to PCR assay was 44.37% (95% *CI* = 38.50%–50.35%) and measure of the degree of agreement by Cohen's kappa (κ) was 0.520 (95% *CI* = 0.461–0.580), assuming PCR as a 'gold standard'.

Of the total *Plasmodium* isolates detected by the PCR-based assay, 96.1% were *P. falciparum*, 1.1% were *P. malariae*, and 0.7% were *P. ovale* mono-infections while 2.1% (6/284) were mixed infections of *P. falciparum* with either *P. malariae* or *P. ovale* (Table 1). All slide-positive results by microscopy were also PCR-positive. Microscopy however, was unable to identify cases of mixed infections, but misdiagnosed four of the six cases of mixed *Plasmodium* species infections identified by PCR assay as mono-infection of *P. falciparum* (Table 1). Three of the misdiagnosed cases were mixed infections of *P. falciparum* with *P. malariae* while one was mixed infections of *P. falciparum* with *P. ovale*. Of the isolates positive by microscopy, 3.2% (4/126) had both sexual (gametocytes) and asexual stages of the parasites present. The presence of sub-microscopic gametocytes by PCR assay were not investigated in this study. Overall, the positive predictive value and specificity of microscopy for detection of *P. falciparum* species compared to the PCR assay was 96.92% (95% *CI* = 92.31%–99.16%), but was zero for other species of human plasmodial parasites ($P < 0.001$).

Stratifying by age group, there was a trend for infections to be sub-microscopic in older children than in younger children (Figure 1), although this association did not reach a statistically significant level ($P > 0.05$). On the other hand, microscopic asymptomatic infections were found to be significantly more prevalent in younger children than in older children ($\chi^2 = 16.86$; $df = 2$; $P = 0.004$). In addition, the proportion of sub-microscopic infections was higher among male participants (53.8%) than among female participants (46.2%) in the study population.

Table 1
Distribution of *Plasmodium* species infections in the study population ($n = 960$).

Infection category	Microscopy ($n = 126$)	PCR ($n = 284$)
(Single) Mono-species infection		
P. falciparum	122	273
P. malariae	0	3
P. ovale	0	2
P. vivax	0	0
(Mixed) Multi-species infection		
P. falciparum + *P. malariae*	0	4
P. falciparum + *P. ovale*	0	2
P. falciparum misdiagnosis	4	0

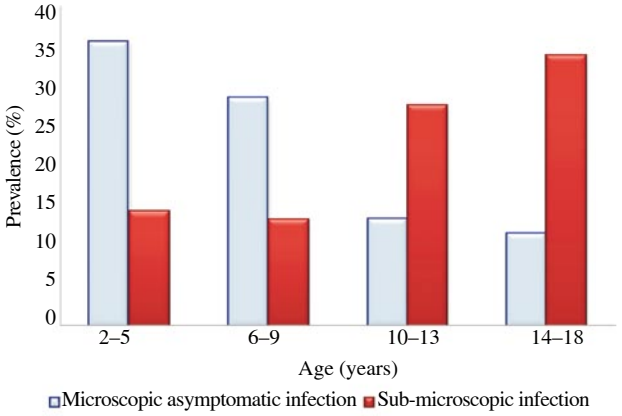

Figure 1. Age-specific prevalence of asymptomatic and sub-microscopic infections.

4. Discussion

Nigeria is top on the list of malaria-endemic countries with high number of infections in sub-Saharan Africa[2]. Although considerable progress has been made at reducing malaria burden in the country over the years, through interventions such as indoor residual spraying (IRS), insecticide-treated bed nets (ITNs), intermittent preventive treatment in pregnancy (IPTP) and the use of artemisinin-based combination therapy (ACT), more needs to be done in order to sustain the gains achieved and more importantly, to move towards actualization of transition from malaria control to pre-elimination as Nigeria is not yet listed as a malaria-eliminating country[33,34]. In order to achieve this goal, an important first step would be to evaluate the overall disease burden in the country and this will require the deployment of appropriate detection tools for proper assessment.

In this study, the levels of sub-microscopic and mixed *Plasmodium* species infection in apparently healthy children were determined by PCR assay of the 18S rRNA gene. We found a higher prevalence of sub-microscopic infections (16.5%) compared with microscopic asymptomatic infections (13.1%). Our results showed that the prevalence of *Plasmodium* species infection detected by PCR was twice that detected by microscopy [29.6% (284/960) versus 13.1% (126/960)], even though the specificity of microscopy relative to PCR, for the detection of *P. falciparum* was very high. In a previous study by May *et al.*[28], in South-West Nigeria, a typical rain forest zone with intense and stable malaria transmission, a slightly higher prevalence of sub-microscopic *P. falciparum* parasitaemia (19.6%) was found.

Expectedly, our data showed that majority of the parasite isolates were mono-species infection of *P. falciparum* (96.1%). This is consistent with previous report discussed elsewhere, where about 96% of infections were also found to be due to *P. falciparum*[35]. It is however, in contrast with the data discussed by May *et al.*[28]; where 68.3% of infections were found to be *P. falciparum*. The prevalence of mixed *Plasmodium* species infection in our study was 2.1%; and in all cases, *P. falciparum* was found to be present. We did not find in this study, mixed infection with more than two different *Plasmodium* species. This is in contrast with the data reported elsewhere[28], that found cases of mixed infections with up to three different *Plasmodium* species.

Furthermore, our data showed a higher proportion of sub-

microscopic infections in older children than in younger children. On the contrary, we found that the prevalence of microscopic asymptomatic infection decreases significantly with age, as more infections tend to be within the detection threshold of microscopy in younger children than in older children. This result is consistent with previous findings in Cambodia[36], Uganda[37], and Ethiopia[11] where older ages were generally associated with increased sub-microscopic carriages; as well as in North-Eastern Tanzania where individuals older than 15 years were three times more likely to have sub-microscopic infections than younger individuals[38]. In a systematic review, Okell *et al*.[18] also found that older age groups were generally associated with increased sub-microscopic carriages.

Our results are in tandem agreement with previous studies showing that molecular detection techniques have greater sensitivity and specificity than either microscopy for the identification of single and mixed species infections[23,39-42], as well as sub-microscopic infections[11,43]. The detection of *Plasmodium* parasites at very low density parasitaemia is difficult by microscopy and thus requires a molecular approach especially for surveillance or epidemiological studies[44]. Current methods which rely on PCR-based assays of ribosomal RNA genes to detect circulating parasites have been suggested to represent the overall gold standard of malaria diagnostics[45], and is increasingly used in epidemiological studies.

Although sub-microscopic infections are rarely associated with clinical disease, they have been shown to be associated with the sustenance of transmission especially in areas of low transmission intensity, where slide prevalence is low[46,47]. As countries with high transmission intensity, including Nigeria, transit to the elimination phase of the Global Malaria Eradication Program (GMEP), it is envisaged that the intensity of transmission will decline across the different ecological zones in these countries. This will no doubt lead to significant reduction in disease incidence and the level of low-density sub-microscopic carriers will presumably rise. When this stage is reached, the import of sub-microscopic infections as key reservoirs of human-to-mosquito transmission, as well as the decision as to whether sub-microscopic carriers should be considered priority for intervention[48], will be critically significant.

In conclusion, we found that sub-microscopic infections are prevalent in the study region and that parasite prevalence in the region generally may be much higher than estimates by local authorities who rely on standard light microscopy for diagnosis. This has consequences for sustaining malaria transmission in the area. Furthermore, the inability of microscopy to correctly identify non-*falciparum* species and mixed *Plasmodium* species infection in this study clearly shows the unquestionable importance of molecular tools for epidemiological surveys and as suitable complement to microscopy especially for reference laboratories.

Conflict of interest statement

We declare that we have no conflict of interest.

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