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Predominance of *Plasmodium malariae-falciparum* Co-Infection by Molecular Speciation in Bangolan, North West Region of Cameroon

Olivia Afa Achonduh, Aristid Herve Ekollo Mbange, Atogho-Tiedeu Barbara, Innocent Ali Mbulli, Daniel Achinko, Palmer Masumbe Netongo and Wilfred Fon Mbacham

The Biotechnology Center, University of Yaounde I, Yaounde, Center Region, Cameroon

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Abstract: Although microscopy still remains the gold standard for the diagnosis of malaria, rapid diagnostic tests (RDTs) and PCR assays have been shown to be sensitive and specific. Very few comparative studies have been reported of the three diagnostic methods on the same samples in vulnerable groups. Microscopy, RDTs and PCR assays were used for detection and speciation of *Plasmodium falciparum* (*Pf*), *Plasmodium malariae* (*Pm*) and *Plasmodium ovale* (*Po*) in patients in a rice culture savanna ecotype. Fifty four children and 16 pregnant women presenting with a fever were recruited. Bloods collected was used for thin and thick smears, perform RDTs and spotted blood on filter paper for DNA extraction and performance of a PCR. Mean parasitaemia was 37,619.06 (± 33,599.04) p/μL and 7,512.5 (± 12,446.11) p/μL for children and pregnant women, respectively. A total of 87.14% were positive by microscopy, 85.71% by RDTs and 90% by PCR. Distribution of *Plasmodium* species as identified by PCR was 72.86% *Pf/Pm*, 11.43% *Pf/Pm/Po* and 5.43% *Pm* while 10% were negative. Cohen's Kappa value for PCR and RDTs was K = 0.75 (CI = 0.28-1.22) while PCR and microscopy was K = 0.64 (CI = 0.18-1.10). Malaria infection in Bangolan was mostly due to mix infection predominantly *P. falciparum*/*P. malariae*.

Key words: *Plasmodium* sp., microscopy, rapid diagnostic tests, PCR, children, pregnant women.

1. Introduction

Malaria affects 300-500 million people annually and accounts for over 1 million deaths, mainly in African children aged less than five years [1]. It has been estimated that the economic burden of malaria is extremely high, accounting for a reduction of 1.3% in the annual economic growth rate of countries in which malaria is endemic, and the consequent long-term impact is a reduction of gross national product (GNP) of more than half [2]. Apart from children less than five, pregnant women are potential reservoir for malaria transmission as most of them are asymptomatic in endemic areas. Clinical consequences of placental malaria comprise maternal anemia, low birth weight (LBW), preterm delivery and consequently increased prenatal mortality (PM). In sub-Saharan Africa, malaria during pregnancy is estimated to account for 3-8% PM, ~35% of LBW [3].

Effective treatment of malaria requires precise laboratory diagnosis and this remains a cornerstone for global malaria control efforts. *P. falciparum*, which can be fatal, must be identified promptly and differentiated from the other *Plasmodium* species that cause malaria. Microscopy still remains the method of choice in the diagnosis of malaria in endemic areas because it is cost effective. However correct identification of *Plasmodium* species by microscopy depends on factors such as; the experience of the microscopists, proper staining of the slides, appropriate maintenance of the

Corresponding author: Wilfred Fon Mbacham, ScD, associate professor, research fields: public health biotechnology and biochemistry. E-mail: wfmbacham@prd-college.eu.

microscope and the time spent reading a slide. The sensitivity of microscopy is approximately 10-30 parasites/μL of blood [4] and this is usually a challenge in malaria endemic areas. These factors may result in incorrect speciation and in ability to detect mix infections and low parasitaemia. On the other hand, rapid diagnostic tests (RDTs) have been reported to have a considerable potential as a tool to improve the diagnosis of malaria [5, 6] and several commercially available tests are sensitive, specific, and stable under operational conditions [7]. However, RDTs have some limitations e.g. HRP-2-based immune- chromatographic tests permit rapid diagnosis of *P. falciparum* malaria, hence their clinical usefulness for the diagnosis of other *Plasmodium* spp. and for monitoring of the therapeutic response is limited. Since HPR-2 is expressed only by *P. falciparum*, these tests will give negative results with samples containing only *P. vivax*, *P. ovale*, *P. malaria* and *P. knowlesi*. Many cases of non-*falciparum* malaria may therefore be misdiagnosed as malaria negative. Polymerase chain reaction (PCR) based techniques have been reported to be more sensitive and specific [8, 9] and have the ability to detect malaria parasites in patients with low levels of parasitemia and identify them to the species level. It is an attractive addition for confirmation of results of other methods.

Microscopy still remains the gold standard for parasitological diagnosis of malaria in Cameroon despite its disadvantages of electrical supply, well trained technicians, and available reagents amongst others. The government is yet to introduce a policy for the use of RDTs for routine diagnose of malaria and PCR is still restricted only to a few research laboratories. The objective of this study was therefore to compare the specificity and sensitivity of microscopy and the RDTs (SD Bioline kits; *Pf*/pan and *Pf* specific kit) and PCR in the detection and speciation of *Plasmodium* species in children and pregnant women in Bangolan village.

2. Materials and Methods

2.1 Study Area

The study was conducted in BBIHC (the Bangolan Baptist Integrated Health Centre) found at latitude 5.50° and 5.55°N, and longitude 10.37° and 10.40°E. Bangolan village is made up of 12,000 inhabitants in 11 neighbourhoods. The village has two raining seasons- a major rainy season from March/April to August/September, and a minor one from October to November. It has an annual rainfall of 1,734.25 mm per annum, a daily temperature range of 27-34 °C. The vegetation is guinea savannah. This village has suffered greatly from the repercussions of the Bamendjin dam constructed in 1975 and it is one of the villages that were not displaced by water. The area is perennially marshy and the people of Bangolan have embarked on rice farming and fishing for their livelihood. The abundance of marshes explains why malaria is transmitted perennially in this area.

2.2 Study Design

Sample collection was done between September and November 2007. A total of 70 patients who sought treatment at the BBIHC were recruited based on clinical signs and symptoms of malaria. The objective of the study was explained to participants or legal guardians who signed the informed assent or consent form prior to sample collection. The enrolled participants consisted of 16 pregnant women aged 180-420 months and 54 children aged 6-168 months. A total of 200 μL of blood collected from each patient by finger prick was used to prepare thick and thin blood smears, perform RDTs and blood spot on filter paper.

2.2.1 Microscopic Examination and Determination of Parasitemia

Thick films stained with 10% Giemsa were used for definitive parasites counts; 200 high power fields were screened before a slide was declared negative. The number of parasites per 200 leucocytes was recorded and converted into parasite density per μL by

assuming an average white blood cell count of 8,000/μL. The mean of 2 slide readings was performed and discrepancies greater than 10% were performed by the microscopy quality assurance expert. Meanwhile the thin film was used for *Plasmodium* species identification.

2.2.2 Rapid Diagnostic Tests

Two types of RDTs were used which are SD Bioline Malaria Ag *Pf*/Pan and SD Bioline Malaria Ag *Pf* test kits (Standard Diagnostic Inc.). The Malaria Ag *Pf*/Pan antigen rapid tests is a one step, qualitative and rapid immunochromatographic test for the differential diagnosis of *P. falciparum* histidine rich protein II (*P.f* HRP-II) and the other *Plasmodium* species (Pan, pLDH). Meanwhile the Malaria Ag *Pf* has one line for control and the other for *P.f* HRP-II. Blood (5 μL) collected from patients was used to perform the tests according to the manufacturer's instructions.

2.2.3 DNA Extraction and Amplification of the 18ss rRNA Genes by Nested PCR

DNA was extracted from dried blood spot on filter papers (Protein Saver Card) by chelex boiling method. All materials and reagents were sterilized using the autoclave (SanoClav). Using a scissors sterilized by flaming, each blood spot on the filter paper was carefully excised and transferred in 1.5 mL microfuge tubes and 1 mL of 0.5% saponin in 1× PBS added. The scissors was flamed in between samples, dipped into distilled water and wiped with a clean tissue paper. The tubes were then inverted several times to completely immerge the filter paper and kept at 4 °C overnight. The following day the solution was discarded and the filter papers washed with 1 mL of 1× PBS and incubated at 4 °C for 15-30 min. During this period, 50 μL of 20% chelex (sterile) were added into 150 μL of nuclease free water in a 1.5 mL microfuge tube and place on a heat block (Bioblock) set at 100 °C. After incubation, the filter papers were transferred into 1.5 mL eppendorf tubes containing hot 20% chelex-100 and nuclease free water. During the incubation period of 10 min each tube was vortex twice, centrifuged at 13,200 rpm for 3 min and the supernatant transferred into a fresh tube. Centrifugation was repeated and the final supernatant (DNA) transferred into a fresh tube and stored at -20ºC for PCR analysis. DNA from previously confirmed positive samples was extracted alongside to serve as positive control. The amplification of the18ss rRNA gene was carried out following the method in Ref. [10] with a T3 thermal cycler (Biometra, Germany). Primers used were obtained from MR4, USA. The first round was carried out in a 20 μL reaction mixture consisting of 12.75 μL, nuclease free water, 2.5 μL of 10× Thermopol buffer (New England Biolabs, USA), 0.5 μL of 10 mM dNTPs, 1 μL of 2.5 μM of each primer (rPLU5/rPLU6), 0.25 μL of 5 U/μL Taq DNA polymerase (New England Biolabs) and 2 μL of template DNA. Amplification conditions were: 95 °C for 15 min, 26 cycles of 58 °C for 2 min, 72 °C for 5 min, and 94 °C for 1 min, followed by 58 °C for 2 min and 72 °C for 5 min. The second round of species-specific amplification was carried out in three separate tubes each containing a single pair of primers of rFAL1/rFAL2, rOVAL1/rOVAL2 and rMAL1/rMAL2. The 20 μL reaction mix contained 13.75 μL nuclease free water, 2.5 μL of 10× Thermopol buffer, 0.5 μL of 10 mM dNTPs, 1 μL of each primer 2.5 μM, 0.25 μL of Taq DNA polymerase (5 U/μL), and 1 μL of the amplicon DNA. The cycling parameters were: 95 °C for 15 min, 30 cycles of 58 °C for 2 min, 72 °C for 5 min, and 94 °C for 1 min, followed by 58 °C for 2 min and 72 °C for 5 min. Electrophoresis of PCR products was carried out on 1.5% agarose gel stained with ethidium bromide alongside molecular weight markers, positive and negative controls. Products were visualized with high performance UV transilluminator, scored and photographed using a digital camera.

2.3 Data Analysis

Sensitivity, specificity, negative predictive value

(NPV), positive predictive value (PPV), percentage agreement (PA), and Cohen's Kappa coefficient (K) with their 95% confidence interval (CI) were determined using Open Epi version 2.2. Kappa was determined according to Altman. [11]. Kappa value express agreement beyond chance and a K value of 0.21-0.60 is moderate, K value of 0.61-0.80 a good and K > 0.80 an almost perfect agreement beyond chance. Other variables measured included true negative (TN), true positive (TP), false positive (FP), and false negative (FN). Sensitivity was calculated as TP/(TP+FN), specificity as TN/(TN+FP), NPV as TN/(TN+FN), PPV as TP/(TP+FP) and PA as (TP+TN)/sample size.

2.4 Ethical Clearance

Ethical clearance for the study was obtained from the Institutional Review Board of Cameroon Baptist Convention Health Board and the National Ethics Committee of the Ministry of Public Health, Cameroon.

3. Results

Among the 70 participants enrolled in this study, 54/70 (77.14%) were children while 16/70 (22.85%) were pregnant women. The mean age and weight for the children was 42 months and 14.17 kg respectively while that for the women were 271 months and 67.63 kg respectively (Table 1).

3.1 Comparison of PCR with Microscopy

Out of the 70 patients tested by microscopy, 61/70 (87.14%) tested positive for malaria parasites. Though microscopy failed to speciate the causative *Plasmodium* species, one case identified as *P. falciparum/P. ovale*/*P. malariae* mixed infections was confirmed by PCR as *P. falciparum*/*P. malariae* mixed infections. Among the microscopically unidentified *Plasmodium* spp. (60/70) PCR identified 48 of the samples as *P. falciparum/malariae* mixed infections, 08 as *P. falciparum*, *P. malariae* and *P. ovale* mixed infections and 03 as *P. malariae* single infections (Table 2). A comparative evaluation of PCR with parasitemia indicated 33.33% detection by PCR when parasitemia was negative (Table 3).

3.2 Comparison of PCR with Rapid Diagnostic Tests

Of the 45 *Pf/Pan* kits tested, 39 were positive, PCR confirmed 30 samples and 7 samples as *Pf/Pm* and *Pf/Pm/Po* mixed infections respectively. Out of the 25 *Pf* specific kit tested, PCR confirmed 18 samples and 1 sample as *Pf/Pm* and *Pf/Pm/Po* mixed infections

Table 1 Baseline characteristics of the study population.

	Children	Pregnant Women
Number of participants	54	16
Mean age (months ± SD)	41.74 (± 38.38)	270.88 (± 48.16)
Mean weight (kg ± SD)	14.17 (±8.97)	67.63 (± 13.37)
Temperature (°C) range	36.0-40.2	35.2-38.2
Geo-mean parasitemia (SD)	37,619.06 (± 33,599.04)	7,512.5 (± 12,446.11)

SD: standard deviation.

Table 2 Comparison of PCR with microscopy for the identification of *Plasmodium* species in Bangolan.

PCR	Microscopy			
	Negative	*Pf/Po/Pm*	*Plasmodium*	Total
Negative	6	0	1	7 (10%)
(*Pf/Po/Pm*)†	0	0	8	8 (11.43%)
Pf/Pm	2	1	48	51 (72.86%)
Pm	1	0	3	4 (5.71%)
Total	9 (12.86%)	1 (1.43%)	60 (85.71%)	70

Pf, *P. falciparum*; *Pm*, *P. malariae*; *Po*, *P. ovale*. †: means microscopy failed to speciate the causative *Plasmodium* species.

Table 3 Comparative evaluation of PCR with parasitemia in children and pregnant women in Bangolan.

(parasites/µL blood)	*Pf/Pm*	*P/Pm/Po*	*Pm*	Negative	Total	Sensitivity, %	Confidence level = 95%
500-1000	3	0	1	0	4	100	51.01 - 100
1001-5000	7	0	0	1	8	87.5	52.91 - 97.76
5001-10000	4	1	1	0	6	100	60.97 – 100
10001-20000	8	0	0	0	8	100	67.56 -100
20001-30000	9	0	1	0	10	100	72.25 -100
30001-40000	4	1	0	0	5	100	56.55 -100
40001-50000	2	0	0	0	2	100	34.24 -100
50001-60000	3	2	0	0	5	100	56.55 -100
60001-70000	2	2	0	0	4	100	51.01 -100
70001-80000	1	1	0	0	2	100	34.24 -100
80001-90000	3	0	0	0	3	100	48.85 -100
90001-100000	2	1	0	0	3	100	48.85 -100
>100000	1	0	0	0	1	100	20.66 -100
negative	2	0	1	6	9	33.33	12.06 - 64.58
Total	51	8	4	7	70	90	80.77 - 95.07

Table 4 Comparison of PCR and RDTs in the speciation of *Plasmodium* species in children and pregnant women in Bangolan.

PCR	RDTs				
	Pf/Pan (3)	*Pf* (2)	*Pf* *	*Pf/Pan**	Total
-VE	1	0	3	3	7 (10%)
Pf/Pm	30	18	0	3	51 (72.86%)
Pf/Pm/Po	7	1	0	0	8 (11.43%)
Pm	1	2	1	0	4 (5.71%)
Total	39 (55.71%)	21 (30%)	4 (5.71%)	6 (8.57%)	70

*Pf** and *Pf/Pan** denotes invalid results; *Pf* (2) denotes presence of two bands (*Plasmodium* spp and control line); *Pf/Pan* (3) denotes the presence of three bands (*Pf*, *pan species* and control line).

Table 5 The sensitivity and specificity of RDTs kits compare to PCR in the diagnosis of malaria in the study population.

RDTs	Nested PCR					
	Sensitivity, %	Specificity, %	PPV*, %	NPV**, %	Accuracy, %	Kappa
Pf kit [a]	95.45 (78.2-99.19)	100 (43,85-100)	100 (84.54-100)	75 (30.06-95.44)	97.44 (80.46-99.29)	0.8344 (0.448-1.22)
Pf/Pan [b] kit	92.68 (80.57-97.48)	75 (30.06-95.44)	97.44 (86.82-99.55)	50 (18.76-81.24)	91.11 (79.27-96.49)	0.552 (0.2675-0.837)

*PPV: positive predictive value; **NPV: negative predictive value; CI: confidence interval 95%;
[a]: the limit of detection of *Pf* kit was 480/µL in children and 520/µL in women; [b]: the limit of detection of *Pf/Pan* kit was 400/µL in children and 2,560/µL in women; 95% confidence intervals are indicated in parentheses.

respectively (Table 4). Three samples diagnosed by the *Pf*/Pan and *Pf* specific kit as *P. falciparum* were identified as *P. malariae* single infection by PCR (Table 4). The detection range for parasitemia was from 400 - 99,600 parasites/µL for *Pf/Pan* kits and from 480-149,600 parasites/µL for *Pf* specific kit in the children population. Meanwhile, for the 16 pregnant women the detection range for parasitemia was 520-38,400 parasites/µL for *Pf* kit and 2560-28,320 parasites/µL with *Pf*/Pan specific kit. Amongst the three methods, PCR was observed to be the most sensitive and specific method (Tables 5 and 6). The concordance between PCR and *Pf* specific kit was higher than PCR and *Pf*/pan kits. The Kappa value between PCR and RDTs was observed to be higher than that between PCR and microscopy in the pregnant women population (Table 6).

Pf/Pan and *Pf* results were invalid tests because in

Table 6 Sensitivity and specificity of nested PCR, microscopy and RDTs in the diagnosis of malaria in the study population.

	Nested PCR					
	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Accuracy, %	Kappa
MIC	100 (64.57-100)	66.67 (35.42-87.94)	70 (39.68-89.22)	100 (60.97-100)	81.2 (56.99-93.41)	0.64 (0.18-1.10)
RDTs	100 (64.57-100)	75 (40.93-92.85)	80 (49.02-94.33)	100 (60.97-100)	87.5 (63.98-96.5)	0.75 (.28-1.22)

MIC: microscopy; RDTs: Rapid diagnostic tests; PPV: positive predictive value; NPV: negative predictive value; 95% confidence intervals are indicated in parentheses.

the course of reaction no control line was observed in the test strip. *Pf* (2): kit specific for *Plasmodium falciparum* and (2) denotes the presence of two bands (one for *Pf* and the other for the control line). *Pf/Pan* (3): kit specific for *P. falciparum* and *pan* species (*P. vivax*, *P. ovale*, *P. malariae*) and (3) denote the presence of three bands (one for *Pf*, one for *pan* species and one for the control line).

3.3 The Sensitivity and Specificity of PCR, RDTs and Microscopy

4. Discussion

Microscopy still remains the method of choice in the diagnosis of malaria in endemic areas because it is an inexpensive, can serve for more than just plasmodium diagnosis and may also provide information species and the state of anaemia. However, this method can sometimes be burdensome, light failures and reagent inadequacies. In addition, the identification of parasite species, especially in the case of low level of parasitemia and a mixed parasite infection can be tricky especially to the untrained eye [12, 13]. In this study the sensitivities and specificity of microscopy, PCR and RDTs in the detection and identification of *Plasmodium* species in children less than 5 years and pregnant women were compared. The nested PCR technique used in this study permitted the identification of *Plasmodium falciparum*, *Plasmodium ovale*, and *Plasmodium malaria* as the three malaria parasites currently found in Bangolan. The parasites as identified by PCR were classified as double (*Pf/Pm*), triple (*Pf/Po/Pm*) and single (*Pm*) infections. The prevalence of mixed infections is often biased by microscopy especially with *P. falciparum* that overshadows the other species by reason of low parasitemia. This study confirms previously reports that PCR is more sensitive and specific in the detection of *Plasmodium* species and can easily differentiate between mix infections. Thin film microscopic differentiation between *Pf* and *Pm* has been reported to be difficult [14] and although *Pm* is a mild infection, it could greatly affect the dynamic of *Pf* manifestation through non-specific and cross-specific immune response [15] that can cause a chronic nephrotic syndrome. Once established, this syndrome does not respond to treatment and carries a high rate of mortality [16]. Moreover, *Pf*/*Pm* are known to co-infect together in endemic areas [17]. In this study, the most prevalent mixed infection was observed to be *Pf/Pm* (77.86%) compared to *Pf/Pm/Po* (11.43%) and no single *P. falciparum* infection was observed. Some of the factors that have been reported to influence microscopic results are the experience of the microscopist, appropriate staining and the time in reading the slides. The inability of microscopy to clearly speciate the *Plasmodium* species in this study might have been influenced by the above factors. Previous studies have reported the sensitivity of PCR in very low parasitemia [11, 18, 19]. A similar trend was observed where 18.75% of aparasitemic pregnant women detected by microcopy were identified by PCR. Hence, though PCR is not a prompt diagnostic method and is not easily affordable, may help in managing treatment of malaria in pregnant women knowing that treatment on the sole basis of microscopy can lead to under-treatment and sometimes over treatment.

Gravidity has been reported to influence the occurrence of *P. falciparum* and parasites densities but not the prevalence of *P. ovale* and *P. malariae* [20]. Thus, this may explain the reason why PCR failed to detect *P. falciparum* in some samples observed to be *P. falciparum* positive by RDTs kits. A study carried out in Burkina Faso by Mockenhaupt et al. who [21] reported that MAKROmed RDT could detect parasites at a submicroscopic level. Similarly in this study two samples that were negative by microcopy were observed to be positive by RDTs and PCR. Moreover, the sensitivity of microscopy, RDTs and PCR were at 87.14%, 85.71% and 90% respectively which agrees with previously reported trend [22]. Pregnant women are the most at risk in this case where *P. falciparum* sequesters in the placenta leading to drastic effects especially in primigravidae. The sensitivity of PCR at low parasitemia was reported in Refs. [11, 19], the authors found that PCR can be effective at 6 parasites/μL or even less. In this study, we obtained 18.75% (3/16) of aparasitemic women by PCR, while Singer et al. [20] reported 46% (164/358), the difference could be due to the small sample. Nevertheless, in this context PCR appears to be limited because it cannot evaluate the parasite density, which is of crucial importance in following the epidemiology of parasites load in a given setting [23]. In the present study, it was also observed that the Cohen's Kappa agreement between PCR and RDTs was higher (K = 0.75, CI = 0.28-1.22) than that between PCR and microscopy (K = 0.64, CI = 0.18-1.10). Moreover, Boonma et al. [24] and Imwong et al. [25] had reported a decrease in the sensitivity of PCR when compared to microscopy as gold standard. The findings from this study therefore indicate that RDTs used could be a good tool for diagnosis after PCR. Although PCR is of great importance in distinguishing malarial species it appears to be limited when sequence variations occur within genomic DNA. In this study, it was observed that primers used to detect *P. malariae* infection were found to cross-react with *P. ovale* genomic DNA in 9/70 of the samples tested. Kawamoto et al. [26] reported a similar observation in their study on spurious amplification of *P. vivax* small subunit RNA gene using the primers for *P. knowlesi*. This situation could probably be due to sequence variations in *P. ovale* 18SS rRNA gene as reported by Basco and Tahar [27]. Although cross-reactivity of *P. malariae* primers with *P. ovale* might be reduced by further optimization of the amplification conditions it is probable that a new set of primers truly specific for *P. malariae* would be preferable.

In summary, although the cost effective microscopy still remains the gold standard for parasitological diagnosis of malaria in field settings, RDTs are becoming popular for routine diagnosis of malaria in vulnerable groups and PCR still remains the most sensitive and specific method. Malaria infection in Bangolan is mostly due to mix infection predominantly *P. falciparum*/*P. malariae* and this could influence treatment response. With the current plans of the National Malaria Program to introduce RDTs for malaria diagnosis at the point of care in all health facilities nationwide, these findings provide useful information for the type of RDT that could be used for effective case management of malaria in this area.

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