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Comparison of immuno-fluorescence microscopy and optical microscopy after Giemsa staining in the diagnosis of malaria during pregnancy in Buea, Cameroon

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ABSTRACT

Malaria in pregnancy remains a significant threat in sub-Saharan Africa as it is associated with sub-optimal pregnancy outcomes. Due to the fact that the current standard diagnostic method is cumbersome, we assessed the diagnostic performance of an alternate method-fluorescent microscopy, compared to optical microscopy after Giemsa staining- the current standard.Venous blood samples were collected from 407 consenting pregnant women and tested by optical microscopy (OM) after Giemsa staining and by fluorescent microscopy (FM) using the Partec-Cyscope (Partec GmbH, Munster, Germany). All participants were asymptomatic at the time of enrollment. Of the 407 samples tested by OM, 255 (62.5%) were plasmodium-positive. Of the 255 OM positive samples, 207 were also plasmodium-positive by FM, thus a sensitivity of 81.1% (95% confidence interval (CI): 75.8, 85.8%). Of the 152 samples negative by OM, 75 were also negative by FM, thus a specificity of 49.3% (95%CI: 41.1, 57.6%). The positive and negative predictive values of FM were respectively 72.9% (95%CI: 67.3, 78.0%) and 61.0% (95%CI: 51.8, 69.6%). The percentage agreement between both methods was 69.3% (Kappa=0.32, p-value<0.01). There was moderate agreement between FM and OM results. The low specificity and negative predictive value of FM suggest a high likelihood of false negative results if FM is used in place of OM. OM after Giemsa staining remains the gold standard.

Keywords: Diagnostic performance, sensitivity, specificity, positive predictive value, negative predictive value, Cameroon.

INTRODUCTION

Malaria is a major health problem in sub-Saharan Africa and the symptoms of uncomplicated malaria are non-specific and similar to many other disease syndromes (CDC-Malaria, 2014). According to the latest

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WHO data published in April 2011, Malaria deaths in Cameroon reached 23,179 or 9.77% of total deaths. The age adjusted Death Rate is 72.99 per 100,000 of the population placing Cameroon at the number 13th position in the world (WHO, 2011). People living in areas where malaria is endemic are often familiar with symptoms of uncomplicated malaria such as elevated temperatures, perspiration, and weakness. They frequently diagnose themselves leading to the problem of malaria over-diagnosis (Pell et al., 2014; Lenz et al., 2011) and presumptive treatment (Bardaji et al., 2008; Ughasoro et al., 2013). *Plasmodium falciparum* infections during pregnancy in Africa rarely result in fever and therefore remain undetected and untreated (Hassan et al., 2011). Increased risk of maternal and foetal complications which include maternal anaemia and low birth weight are some of the complications associated with malaria infection in pregnancy (Sutherland and Hallett, 2009; Krafts et al., 2011). Such severe symptoms call for improved, rapid and accurate diagnostic methods. The accuracy of malaria diagnosis is the key to malaria control (Nicastri et al., 2009; Batwala et al., 2010).

Thick and thin blood films stained with Giemsa and observed using optical microscopy is the current gold standard for malaria diagnosis (Elhassan et al., 2011). This method however is time consuming and requires trained microscopists to identify the parasites. In practise, freshly prepared stain is to be used daily for precise results and this is not always done. This leads to inaccurate diagnosis and therefore presumptive treatment. Due to decreasing incidence and prevalence as well as lower parasitemia but rapid emergence of resistance and expensive drugs (Hassan et al., 2010), alternate rapid diagnostics tests need to be evaluated. This is particularly important in pregnancy when changes in the immune response could potentially affect the performance of rapid tests based on antibody detection (van Eijk et al., 2012).

An alternative technique for use is the FM. This is a DNA specific staining kit, delivered as ready-prepared and ready-to-use test slides carrying the dry/lyophilized reagents already on the slide surface. In this study, the diagnostic performance of FM is compared to that of the gold standard-OM to assess the performance of FM in the rapid diagnosis of malaria during pregnancy and this paper on the diagnosis of malaria is part of a study titled 'HIV in Pregnancy: co-morbidity with malaria and Hepatitis B virus (HBsAg) in Buea, South West Region of Cameroon.

MATERIALS AND METHODS

Study area, design, inclusion/exclusion criteria

The study area was antenatal clinics (ANCs) in the Buea Health District of Cameroon. Two clinics, the Buea Road Health Centre (BRHC) and the Provincial Hospital Annex Buea (PHAB) were used. The data was collected from July to September, 2010. The study population comprised of 407 pregnant women attending the above mentioned ANCs. These women consented to the study and were eligible.

Study procedures

All eligible and consenting women were interviewed using a standardized questionnaire. After this, 10 ml of whole blood was collected from each participant into EDTA tubes. This quantity of blood was taken from participants because this sudy was part of a larger study involving pregnant women.

Samples were transported from the collection sites to the Faculty of Health Sciences Laboratory, University of Buea, in an ice box containing ice packs.

In the laboratory, thin and thick blood films were made on clean dried glass slides. After the smears dried, they were stained with 10% Giemsa stain for 10 minutes. Slides were then viewed under the light microscope using 40X and 100X (oil immersion) when they got dry.The presence of the parasite was confirmed as positive and the absence of the parasite as negative after examination of 100 high power (100X objective) microscope fields (Cheesbrough, 2010).

Ten micro litres (10 µl) of whole blood from the EDTA tubes were placed onto the dried reagent spot on the test slides for Partec fluorescent microscopy (GmbH, Munster, Germany). Slides prepared in duplicate were tilted in all directions in order to distribute the drop of blood. A cover slip was put on them and they were incubated for 5 minutes at room temperature. Slides were then placed under the Cyscope, the white light was switched on and the red blood cells were observed. UV light was then switched on and the presence or absence of the parasites confirmed. Both slides were read by experienced microscopists and evaluated qualitatively as being positive or negative for plasmodia.

Data analysis

Statistical analysis was conducted using STATA version 9 (STATA Corp., College Station, Texas, USA). The evaluation of the performance of FM compared to OM (as a gold standard) was done by computing an overall sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Considering that OM is not a perfect gold standard, the overall percentage agreement between FM and OM was computed using a Kappa interrater agreement statistic. The above performance characteristics were finally compared within subgroups of participants defined by age, gestational age (trimester), gravidity, use of bed-nets and prior use of anti-malarials. The proportions were all compared using Chi-square tests, with the statistical significance level being set at $p<0.05$.

Ethical considerations

Ethical clearance for this study was obtained from the Cameroon National Ethics Committee. Participation in this study was voluntary. A signed consent form was obtained from all participants. All procedures were standard and only involved minimal risk to the participants.

RESULTS

A total of 407 women consented to the study. The age of participants ranged from 15 to 45 years, with a mean (+/- standard deviation) of 26.4 years (+/- 5.0). Most of the participants (97.3%) were in their 2nd and 3rd trimester and 27% of the participants were primiparous. Gravidity ranged from 1-8 and the mean gravidity was 2.4 while the median was 2. Table 1 summarizes the participants' characteristics.

Of the 407 samples tested by OM, 255 (62.5%) were malaria-positive. Of these 255 positive by OM, 207 (81.18%) were also malaria-positive by FM, thus a sensitivity of 81.1% (95%CI: 75.8, 85.8%). Of the 151 samples that were negative by OM, 75 were also negative by FM, thus a specificity of 49.3% (95% CI: 41.1, 57.6%). The positive and negative predictive values of FM were respectively 72.9% (95% CI: 67.3, 78.0%) and 61.0% (95% CI: 51.8, 69.6%) (Table 2).

The percentage agreement between both methods was 69.3% (Kappa=0.32, p-value <0.01).

The performance of FM compared to OM within sub-groups of women is summarised in Table 3. FM was significantly more specific in younger women than in older women (59.7% versus 42.2%, p=0.03). The performance characteristics were similar among all other subgroups compared.

Table 1: Summary of participants' characteristics.

Characteristics	Number	Percentage (%)
Period of gestation		
First Trimester	11	2.7
Second Trimester	219	53.9
Third Trimester	176	43.4
Gravidity		
1	142	35.2
2	101	25.0
3+	161	39.8
Anti-malarial use		
No	149	37.1
Yes	253	62.9

Table 2: Overall Performance of Fluorescent Microscopy (FM) compared to Giemsa Optical Microscopy (OM) in pregnant women in Buea.

Fluorescent Microscopy	Giemsa stain Positive	Giemsa stain Negative	Total
Positive	207 (81.18%)	76 (50.33%)	283 (69.70%)
Negative	48 (18.82%)	75 (49.67)	123 (30.30%)
Total	255	151	406

Table 3: Performance of FM compared to OM by characteristics of 407 pregnant women in the Buea Health District.

PARTICIPANTS' CHARACTERISTICS		SENSITIVITY %	PV	SPECIFICITY %	PV	PPV %	PV	NPV %	PV	AGREEMENT %	PV
Age	< 26	82.5		59.7	0.03	78.6	0.08	65.6	0.30	74.3	0.06
	>26	80.0	0.61	42.2		69.2		56.5		65.6	
Trimester	1st & 2nd	84.9	0.09	48.4	0.69	71.5	0.46	61.7	0.11	70.4	0.63
	3rd	76.7		51.7		75.4		53.5		68.2	
Gravidity	1	75.0		58.0		76.1		55.8		69.0	
	2	80.0	0.07	61.3	0.06	82.4	0.05	57.6	0.31	74.3	0.51
	3+	88.2		39.7		66.7		71.1		67.7	
Bed net use	NO	81.3	0.91	48.5	0.44	72.9	0.88	60.4	0.52	69.2	0.66
	YES	82.1		57.9		74.2		68.6		72.3	
Anti-malarial use	NO	86.3	0.09	53.7	0.39	76.6	0.28	69.1	0.15	74.5	0.08
	YES	77.8		46.3		70.7		55.1		66.0	

PV=Predictive value.

DISCUSSION

From the findings of this study, the sensitivity of FM was high (81.1%) while the specificity was somewhat low (49.3%). The low specificity and negative predictive value of FM suggest a high likelihood of false negative results if FM is used. Kimbi et al. (2013) also used the FM in Buea, Cameroon to identify malaria in pupils, though they did not compare it to the OM and reported a prevalence rate of 33.4%. In their study, the positive and negative predictive values of the cyscope were 94.1% (95% CI: 87.4% - 97.8%) and 95.3% (95% CI: 85.4% - 99.2%) respectively. Hassan et al. (2011) carried out a similar study in pregnant women in Sudan and found the sensitivity of the FM cyscope fluorescence microscopy to be 97.6% (95% CI: 92.2% - 99.6%) with a specificity, higher than in this study, 89.1% (95% CI: 77.5%-95.9%). Another study by Nkruhmah et al. (2011) carried out among Ghanaian children, reported a high sensitivity (100%) and specificity (97.4%) of the FM and in Uganda, in a study with women of child bearing age and children from peri-urban and rural areas, a high sensitivity (92.1% in adults and 86.7% in children) was recorded for FM (Sousa-Figueiredo et al., 2010). Compared to this study in which we recorded a low specificity, the Ugandan study recorded a lower specificity (38.8% in adults and 28.6 % in children), for FM (Sousa-Figueiredo et al., 2010). The presence of other inclusion bodies which appear blue under FM may be responsible for the low specificity.

This study did not count parasite numbers both for the OM and FM since participants were already on malaria prophylaxis and were not febrile. As a whole, the accepted level of sensitivity for rapid diagnostic test (RDT) in diagnosing malaria is a sensitivity of 95% at a parasite density of 100 parasites/µl (Cheesbrough, 2010). The detection threshold in Giemsa-stained thick blood film has been estimated to be 4–20 parasites/µl. Under field conditions, a threshold of about 50–100 parasites/µl blood is more realistic. In rural settings with less skilled microscopists and poor equipment, a still higher threshold is likely (Sousa-Figueiredo et al., 2010). However, it was reported recently in the another study that parasite counts obtained from FM were

significantly lower than those obtained from Giemsa stained light microscopy (Nkruhmah et al., 2011). When the performance of FM was compared to that of OM by participants' characteristics within subgroups (Table 3), the sensitivity by age was greater (82.5%) for those less than 26 years of age while those greater than 26 years of age was 80.0% with a predictive value of 0.61. This finding is consistent with other reports where age group of < 24 years was reported to be at a high risk (Wongsrichanalab et al., 2007).

Conclusion

There was moderate agreement between FM and OM. The low specificity and negative predictive values of FM suggest a high likelihood of false negative results if FM is used in place of OM.

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