Nested PCR detection of *Plasmodium malariae* from microscopy confirmed *P. falciparum* samples in endemic area of NE India

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Abstract: The present study evaluates the performance of OptiMAL-IT® test and nested PCR assay in detection of malaria parasites. A total of 76 randomly selected blood samples collected from two malaria endemic areas were tested for malaria parasites using microscopy and OptiMAL-IT® test in the field. PCR assays were performed in the laboratory using DNA extracted from blood spots of the same samples collected on the FTA™ classic cards. Of the total of 61 field confirmed malaria positive samples, only 58 (95%) were detected positive using microscopy in the laboratory. Sensitivity, specificity, positive predictive value, negative predictive value and false discovery rate of OptiMAL-IT® in comparison to the microscopy were 93%, 83%, 95%, 79% and 5%, respectively. On the other hand, the sensitivity and specificity of PCR assay were 97% and 100 %, respectively, whereas positive predictive value, negative predictive value and false discovery rate were 100%, 90% and 0%, respectively. The overall performance of OptiMAL-IT® and PCR assays for malaria diagnosis was 76% and 97%, respectively. PCR assay enabled the identification of infection with *Plasmodium malariae* Laveran, 1881 in four samples misidentified by microscopy and *Plasmodium*-specific antigen (PAN) identified by the OptiMAL-IT® test. In addition to the standard methods, such PCR assay could be useful to obtain the real incidence of each malaria parasite species for epidemiological perspectives.

Keywords: malaria, diagnostics, blood samples, OptiMAL-IT®, sensitivity, specificity, Assam

Malaria is a complex and evolved infectious human disease that is endemic in north-eastern states of India, where malaria control relies on early active and passive case detection and prompt treatment through malaria camps and village level health workers (Dhiman et al. 2011). Since malaria associated with fever and other symptoms is the most common diagnosis in rural settings, the incorrect diagnosis may have severe public health implications (Anash et al. 2010). A considerable proportion of patients treated for malaria actually do not have malaria (Prasad 2009). Furthermore, missed out true malaria cases may act as epicentres for disease transmission at a local level. Resistance to anti-malarials, decay in health infrastructure, population movement and socio-political unrest are contributing factors to the spread of malaria (Gardner et al. 2002). Accurate diagnosis of malaria cases could be useful in lowering such conjectures and ultimately reducing malaria burden in endemic settings.

Until recently, microscopic examination of blood smears has been a standard method for malaria diagnosis by convenience (Anash et al. 2010). Microscopy, however, requires well trained technical staff and it is also rather

labour intensive and time consuming (Maltha et al. 2010). Hand-held non-microscopic immunochromatographic rapid diagnostic tests (RDT's) are simple, sensitive and specific, and good results have been achieved with them in various endemic regions. Since their introduction during the late 1990's, RDT's have undergone many changes and currently three-band RDT's for detecting both *Plasmodium falciparum* Welch, 1897 specific antigen and *Plasmodium*-specific antigen (PAN-specific) have been developed (Moody 2002).

The performance of the RDT's in the diagnosis of malaria has been validated throughout the malaria endemic regions (Moody 2002, Anash et al. 2010), however, the RDT's were unable to identify mixed infections, owing to the common PAN-specific antibody capture band, when compared to the polymerase chain reaction (PCR) assay. In addition, the RDT's have not been found suitable to diagnose malaria where parasite counts were as low as < 50 parasite/μl (Iqbal et al. 1999).

The pLDH based OptiMAL-IT® has some limitations, but as on today, it has been proved to be one of the best RDT available for on spot detection of malaria parasite

(Moody 2002). In the recent years, PCR assay has been proved to be a sensitive method to diagnose the malaria parasite at species level and in mixed infections, and is expected to detect parasite at very low level (Iqbal et al. 1999, Scopel et al. 2004).

In the north-east India, *P. falciparum* malaria, in particular, emerges with high degree of antimalarial drug resistance and therefore treatment regime differs from that used for the other malaria parasite species. The correct malaria treatment relies on the proper diagnosis of malaria parasite and following the recommended chemotherapy. In the field settings in India, where malaria diagnosis at large is carried out using microscopy, incorrect diagnosis is common and leads to progression of uncomplicated malaria to severe disease (Prasad 2009). Correct treatment would interrupt the indigenous transmission and minimizes the risk of selection and spread of drug resistance to the other areas.

Plasmodium malariae Laveran, 1881 is still uncommon in India and only a few systematic studies have reported it in the country. In the northeastern region, only two studies have reported the presence of *P. malariae* malaria (Dev 2000, Mohapatra et al. 2008), however, only the latter study has evidenced the persistence transmission of *P. malariae* in the region. Molecular methods have the merit of differentiating morphologically similar *Plasmodium* species. The present study was carried out to evaluate the usefulness of PCR assay in malaria diagnosis, detecting the mixed infection and identifying the malaria parasite species, which have been, otherwise, missed out.

MATERIAL AND METHODS

Study area and sample collection

With the help of local medical authority, Defence Research Laboratory organises malaria camps for civil population inhabiting the surrounding areas of defence cantonments. All the malaria positive cases are given antimalarial treatment as prescribed by National Vector Borne Disease Control Programme (NVBDCP). The present samples were collected from patients that reported for malaria testing in the malaria camps during the year 2011 from two different malaria endemic clinical sets, Missamari (92°35'34"E; 26°48'11"N) and Hozai (92°50'47"E; 26°00'14"N), both in Assam, India.

These two areas report *P. falciparum* and *P. vivax* Grassi et Feletti, 1890 cases and no evidence of other human malaria parasite infection are available in these areas. Missamari is a foothill area dominated by various ethnic tribes, whereas Hozai is relatively plain area inhabited by mixed (tribal and non-tribal) population. Bodo and Rabha are main ethnic tribes in Missamari, whereas Karbi, Dimasa and Bodo tribes inhabit Hozai area. We conducted a double blind study to compare the performance of smear reading using microscopy and PCR, while masked to the RDT results in detecting possible malaria parasite species other than the two species encountered frequently.

A total of 61 (37 from Missamari and 24 from Hozai) microscopically confirmed malaria samples and 15 confirmed negative randomly-selected samples were used for the present study.

The entire samples were parallelly checked using OptiMAL-IT® kits (Diamed AG; Lot No. 46110.72.01) in the field and at the same time, 2–3 drops of blood were collected on the FTA™ classic cards (Whatman, Sweden) for PCR assays. These randomly selected 76 samples were re-examined by another senior technician in the laboratory using light microscopy. In case of discrepancies, the results obtained during re-examination using microscopy were recorded as true and used for the present study. The study was approved by the Ethical Review Committee of L.G.B. Regional Institute of Mental Health, Tezpur and informed verbal consents were obtained from the patients.

DNA preparation and nested PCR assay

Parasite DNA was extracted using Qiagen kit and following the standard FTA™ manufacturer procedure. *Plasmodium* species identification was done by nested PCR amplification (Bio-Rad S1000) of the small subunit of the ribosomal ribonucleic acid (18S rRNA) genes using the primers for four human-infecting malaria parasite species excluding *P. knowlesi*, Knowles et Gupta, 1931. PCRs were performed essentially with the primers described elsewhere (Snounou et al. 1993, Johnston et al. 2006). The cycling conditions were similar to Johnston et al. (2006), except for increase in reaction cycles to 35 in the first round and decrease in annealing time to one minute in the nested step for *P. falciparum*, *P. vivax* and *P. malariae*. Genomic DNA isolated from the blood of one of the investigators with no malaria history was used as negative control in the PCR assays. The amplified PCR products were resolved by 2% agarose gel electrophoresis and viewed using UV transilluminator (Syngene G-box).

Data analysis

The sensitivity and specificity of the OptiMAL-IT® and PCR assay for the detection of malaria infection were calculated by using microscopy as reference standard. Positive and negative predictive values (NPV and PPV) referred to the probability that the disease is present and absent when the test is positive and negative, respectively. Positive and negative likelihood ratios (LR) were the probability of a test result with the disease. False discovery rate (FDR) was the proportion of false-positive results out of all positive tests. Overall performance (%) of OptiMAL-IT® and PCR assay in diagnosing malaria was calculated using Youden method (J) (Youden 1959).

RESULTS

Out of a total of 61 microscopy-confirmed malaria positive blood smears in the field, only 58 (95%) could be detected positive using microscopy in the laboratory. However, the difference between the field and laboratory microscopy results was not significant [p-value (p) = 0.69; chi-square (χ^2) = 0.15; relative risk (RR) = 1.128; 95% confidence interval (95% CI) = 0.7463–1.704]. Of these 58 blood smears, 53 (91%) were positive for *Plasmodium falciparum* and 5 (9%) were positive for *P. falciparum* and *P. vivax* mixed infection (Table 1). The OptiMAL-IT® identified *P. falciparum* in 49 (86%) and PAN in 8 (14%) individuals in the field.

Four *P. falciparum* infections confirmed by microscopy were not detected positive by OptiMAL-IT® test. On the other hand, OptiMAL-IT® had detected three *P. falciparum* infections which were not detected by microscopy in the

Table 1. Comparison of OptiMAL-IT®, nested PCR assay and microscopy for detection of *Plasmodium* infection in 76 samples.

<i>Plasmodium</i> species	Microscopy	OptiMAL-IT®	Nested PCR
<i>P. falciparum</i>	53	49	48
<i>P. malariae</i>	-	-	1
<i>P. falciparum/P. vivax</i>	5	-	4
<i>P. falciparum/P. malariae</i>	-	-	3
PAN	-	8	-
Negative	18	19	20

PAN – *Plasmodium*-specific antigen.

Table 2. Overall performance of OptiMAL-IT® and nested PCR in comparison to microscopy.

Diagnosis method	M	Sensitivity	Specificity	PPV	NPV	LR+	LR-	FDR	J
OptiMAL-IT®	57	93.1	83.3	94.7	78.9	5.6	0.1	5.3	0.8
Nested PCR	56	96.6	100	100	90	1	0	0	1

M – diagnosed positive; PPV – positive predictive value; NPV – negative predictive value; LR (+/-) – likelihood ratio (positive/negative); FDR – false discovery rate; J – Youden coefficient.

laboratory. Sensitivity and specificity of OptiMal-IT® as compared to microscopy was 93% and 83%, respectively. PPV, NPV and FDR for OptiMal-IT® were 95%, 79% and 5%, respectively (Table 2). The overall performance of OptiMal-IT® recorded for malaria diagnosis in the present study was 76%.

PCR assay detected *P. falciparum* in 48 (86%) blood samples, *P. falciparum* and *P. vivax* mixed in 4 (7%), *P. falciparum* and *P. malariae* mixed in 3 (5%) and *P. malariae* in one (2%) sample. Four *P. falciparum* and *P. vivax* mixed infections detected by microscopy were detected correctly by PCR. However, in another case, one microscopy detected *P. falciparum* and *P. vivax* mixed infection was identified as *P. falciparum* and *P. malariae* mixed infection by PCR. One *P. falciparum* was identified as *P. malariae* and two *P. falciparum* were identified as *P. falciparum* and *P. malariae* mixed infections by PCR.

Three microscopy-detected negative samples were confirmed to be negative by PCR assay, whereas these samples were detected as positive by OptiMAL-IT®. Two microscopy-positive samples could not be detected positive either by OptiMAL-IT® or PCR. The sensitivity and specificity of PCR assay in detecting malaria parasite was 97% and 100%, respectively, whereas PPV, NPV and FDR were 100%, 90% and 0%, respectively. The overall performance of PCR assays was 97%.

No *P. malariae* infection was diagnosed using microscopy in the present study. However, nested PCR detected three *P. falciparum* and *P. malariae* mixed infections and one *P. malariae* monoinfection. The nested PCR amplicons of *P. malariae* infections were visualised at 144 bp diagnostic band (Fig. 1), whereas *P. falciparum* and

Fig. 1. Detection of *Plasmodium* species amplification in nested PCR. Lanes 1–3: *Plasmodium malariae* (144 bp); lanes 6, 7: *P. vivax* (120 bp); lanes 9–11: *P. falciparum* (205 bp); lanes 4 and 8: standard ladder Sigma (50 bp); lane 5: negative control.

P. vivax were resolved at 205 and 120 bp positions, respectively. *Plasmodium malariae* infections were identified from three males and one female patients of different age groups (11–55 years) belonging to different tribes.

DISCUSSION

The performance of OptiMAL-IT® test kit and nested PCR assay in detecting the malaria parasite was evaluated using randomly selected samples ($n = 76$) from two highly endemic areas of Assam. Out of 61 confirmed samples, 95% were identified malaria positive in the second round microscopy. In the present study, microscopy detected one *P. vivax* and two *P. falciparum* cases, which were later identified correctly as *P. malariae* by PCR.

Microscopic examination has been the diagnostic method of choice in malaria epidemiologic studies and diagnosis. The method is simple, does not require special facilities and enables differentiation among the four well known species of human malaria parasite in most cases (Maltha et al. 2010). Microscopy has limitation that even a skilled person can evaluate only limited number of blood smears per day under field conditions (Gardner et al. 2002). Furthermore, in case of low level of parasitemia and mixed parasite infection, microscopy can sometimes be misleading in identification of malaria parasite species.

The sensitivity and specificity of OptiMAL-IT® was 93% and 83%. OptiMal-IT® detects *P. falciparum* and PAN-specific antigen for *Plasmodium* species. However, four microscopy-confirmed *P. falciparum* cases were detected negative by OptiMAL-IT®. The sensitivity of OptiMAL-IT® has been found low with the lower level of parasitemia or presence of blocking antibodies in the blood. OptiMAL-IT® test also detected three false *P. falciparum* cases, probably due to the presence of pLDH antigen, which has been found to remain in the patient's blood even after initiation of the treatment with antimalarials (Iqbal et al. 1999). The reading beyond the reading time may increase the number of positive PAN-pLDH

lines, apparently explaining for the false-positive cases observed in the tests (Maltha et al. 2010). In the PCR assay, the sensitivity and specificity were 97% and 100%, respectively.

Similar to the present study, the PCR assays have been proved useful in detecting mixed *Plasmodium* infections in various malaria endemic regions (Kimura et al. 1995, Cox-Singh et al. 2008). The PCR assays in the present study were found better than the microscopy in detecting *Plasmodium* species, which were misidentified by microscopy. In mixed infections, most of the time one *Plasmodium* species either dominates or inhibits the other *Plasmodium* species present inside the same red cell population (Kimura et al. 1995).

Two microscopy positive *P. falciparum* samples were detected negative in PCR assay. These two patients were under chloroquine treatment for last 2–3 days and the smears contained only few live parasites. Most of the *P. falciparum* parasites in these smears were either moribund with irregular shape or dead. It has been shown that the DNA extracted from dead parasites did not show positive amplification in PCR assay (Tham et al. 1999). The DNA extracted from these two samples was either unfit for amplification due to poor quality or probably lost during the storage and handling.

In two *P. falciparum* cases, *P. malariae* was identified along with *P. falciparum* as mixed infections, whereas one *P. falciparum* and *P. vivax* mixed infection detected by microscopy was identified as *P. falciparum* and *P. malariae* mixed infection by PCR. Many malaria cases in north-eastern region of India appear with *P. falciparum* and *P. vivax* mixed infection, but only a few cases of *P. falciparum* with *P. malariae* and *P. falciparum* with *P. vivax* mixed infection have been reported (Mohapatra et al. 2008). Mixed infection with two or more *Plasmodium* species is largely underestimated when microscopy is used in malaria detection instead of molecular-based diagnosis (Cox-Singh et al. 2008).

Plasmodium malariae is not very common in this region and has been reported only in two states of north-eastern India (Dev 2000, Mohapatra et al. 2008), and its prevalence in other parts of the region is unknown (Mohapatra et al. 2008). It appears that due to the large number of samples and preconceived notion of diagnosing fre-

quently encountered *P. falciparum* and *P. vivax* malaria parasites, the examiner might have missed the *P. malariae* infection in the present study. In microscopy, *P. malariae* may be confused with other *Plasmodium* species due to the similarity of the growth stages (Scopel et al. 2004). Many studies have revealed that *P. knowlesi* is often misdiagnosed as *P. malariae* in South East Asian countries. Infections with *P. knowlesi* are thought to be potentially more serious and even life-threatening if wrongly treated or remain untreated (Cox-Singh et al. 2008).

Use of antimalarials for malaria treatment in any area largely depends on the type of malaria species present in that particular area. Delayed or wrong diagnosis of malaria parasites increases the risk of complicated disease and relapses, which may be fatal in many cases. Further, due to negative diagnosis, untreated patients may be carriers of malaria parasites and act as epicentres for other non-infected individuals. *Plasmodium malariae* parasite is important from an epidemiological perspective, unlike other malaria parasites, as it can sustain at very low infection rates within the human hosts for many years. This parasite is capable of infecting the mosquito vectors even at very low parasitemia, thus facilitating transmission round the year (Mohapatra et al. 2008).

The current study region falls in malaria zone 2 where, although *falciparum* malaria is dominant, it typically co-circulates and occurs together with the non-*falciparum* malaria (Murray and Bennett 2009). OptiMAL-IT® test in such settings is not much useful in detecting the mixed infections to the species level. Therefore, the nested PCR detection of malaria parasites complemented with the other existing methods is useful to timely obtain the incidence of each malaria parasite species for specific treatment and epidemiological follow up, despite the possibility of rare false positive cases. The present study emphasizes that *P. malariae* may be more prevalent in the region than thought previously. PCR assays employed here may not be used routinely due to various constraints, but can be used at least at primary health centre level for re-evaluation of existing methods.

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