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 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
We conducted a double blind study to compare the performance of different methods for the detection of malaria parasite species in the present study was carried out to demonstrate the accuracy and reliability of the proposed methodology. 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 RNA) 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 (χ²) = 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. vivax* and *P. malariae* 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.

<table>
<thead>
<tr>
<th>Plasmodium species</th>
<th>Microscopy</th>
<th>OptiMAL-IT®</th>
<th>Nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. falciparum</td>
<td>53</td>
<td>49</td>
<td>48</td>
</tr>
<tr>
<td>P. malariae</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>P. falciparum/P. vivax</td>
<td>5</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>P. falciparum/P. malariae</td>
<td>-</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>PAN</td>
<td>-</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Negative</td>
<td>18</td>
<td>19</td>
<td>20</td>
</tr>
</tbody>
</table>

PAN – Plasmodium-specific antigen.

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

<table>
<thead>
<tr>
<th>Diagnosis method</th>
<th>M Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>LR+</th>
<th>LR-</th>
<th>FDr</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>OptiMAL-IT®</td>
<td>57</td>
<td>93.1</td>
<td>83.3</td>
<td>94.7</td>
<td>78.9</td>
<td>0.1</td>
<td>5.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>56</td>
<td>96.6</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

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

The performance of OptiMAL-IT® and nested PCR in detecting malaria parasite was evaluated using randomly selected samples (n = 76) from two high 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.

DISCUSSION
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 northeastern 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 frequently 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 in 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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REFERENCES

Dhiman et al.: Nested PCR detection of *Plasmodium malariae*


