7. Plasmodium Species Diagnosis for Transmission Interruption
7.1 Introduction

Malaria is a complex and well evolved infectious disease of human importance and 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., 2010b; 2011). Since fever with other symptoms is the most common diagnosis for malaria in rural settings, the incorrect diagnosis may have severe public health implications (Ansah et al., 2010). Many people are asymptomatic for malaria and therefore do not get treatment. On the other hand a considerable proportion of patients who are treated for malaria actually do not have malaria (Prasad, 2009). Further, missed out true malaria cases may act as epicentres for the disease transmission at 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 lessening such conjectures and ultimately reducing the malaria burden in endemic settings.

Until recently, microscopic examination of blood smears has been a standard method for malaria diagnosis by convenience (Ansah et al., 2010). Microscopic examination of blood smears involves smearing of the blood obtained by finger pricks and has been the most economic and preferred diagnosis of malaria that rely on the identification of each malaria parasite species on the basis of some distinguishing characteristics. Using the blood smear, an experienced microscopist can detect parasite levels as few as 5 parasites/μL of blood; however, the diagnosis of species can be
difficult because the early trophozoites stages of all four major malaria parasite species look identical and it is never possible to diagnose species on the basis of a single ring form and therefore the species identification is always based on several trophozoites. Despite that some of the *Plasmodium* species look very similar under microscope in some of the stages in life cycle but may have different pathogenicity and one may cause greater severity than the other, so it is important to identify and treat infections immediately. 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 in various endemic regions (Msellem *et al*, 2009). These are commercially available tests that allow the rapid diagnosis of malaria even by unskilled people and in the settings where traditional laboratory based techniques for diagnosing malaria are not available. The RDT’s have an important role in the peripheral of health services capability because majority of the rural clinics and health staff do not have the ability to diagnose malaria in the field due to lack of infrastructures and trained technicians to examine the blood smears. Further, in the non endemic regions, the health workers may have very limited experience in diagnosing and identifying malaria parasites upto the species level. The RDT tests are regarded as complements to conventional microscopy in the field areas because the tests are simple and the procedure can be performed on the spot in field conditions. These tests use finger-prick blood and the complete test takes 15 to 20 minutes only. Since introduction during the late nineties, RDT’s have undergone many changes and currently three-band RDT’s for detecting both *Plasmodium falciparum* specific antigen and *Plasmodium* specific antigen of the four species (PAN-
specific) have been developed (Moody, 2002). Although, the performance of the RDT’s in the diagnosis of malaria has been validated throughout the malaria endemic regions (Ansah et al, 2010; Moody, 2002; Msellem et al, 2009), RDT’s were unable to identify the mixed infections due to common PAN-specific antibody capture band as compared to the PCR assay. Further, RDT’s have not been found suitable to diagnose malaria, where the parasite count was as low as <50 parasite/μl (Iqbal et al, 1999). The pLDH based OptiMAL-IT® has some limitations, but as of today it has been proved to be one of the best RDT available for on the spot detection of malaria parasites (Moody, 2002; Jain et al, 2014). In the recent years PCR assay has been proved to be a sensitive method to diagnose the malaria parasite at species level and mixed infection and can be expected to detect parasite at very low level (Bourgeois et al, 2010; Iqbal et al, 1999; Joveen-Neoh et al, 2011; Rougemont et al, 2004; Scopel et al, 2004; Zakeri et al, 2002, 2010).

In the North-East India, *P. falciparum* malaria in particular emerges with a high degree of antimalarial drug resistance and therefore a treatment regime differs as compared to the other malaria parasite species (Dua et al., 2003; NVBDCP, 2010). Correct treatment would interrupt the indigenous transmission and minimizes the risk of selection and spread of drug resistant into the other areas (Black et al, 1994; Prasad, 2009). *Plasmodium malariae* is still uncommon in India and only a few systematic studies have reported it in the country (Dev, 2000; Dhangadamajhi et al, 2009; Mohapatra et al, 2008). In North-Eastern region only two studies could report the presence of *P. malariae* malaria (Dev, 2000; Mohapatra et al, 2008); however, only one study has evidenced the persistence transmission of *P. malariae* in the region (Mohapatra et al, 2008). The molecular methods have merit in differentiating
morphologically similar *Plasmodium* species. The present study has been carried out to evaluate the usefulness of PCR assay as compared to conventional microscopy and RDT test in malaria diagnosis and detecting the mixed infection, if any, in an endemic rural setting of North-East India for correct identification of the malaria parasite species which could have been missed out, otherwise.

7.2 Methodology

7.2.1 Study area and sample collection

Samples were collected from patients that reported for malaria testing in the malaria camps from two different malaria endemic areas, Missamari (26° 48' N to 92° 35' E) and Hozai (26° 01' N to 92° 50' E) of Assam. These two areas have reported *P. falciparum* and *P. vivax* cases but 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 a plain area inhabited by mixed (tribal and non-tribal) population. Bodo and Rabha are main ethnic tribes in Missamari, while Karbi, Dimasa and Bodo tribes inhabit Hozai area. Double blind study was conducted to compare the performance of smear reading using microscopy, RDT and PCR. A total 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. All samples were parallely checked using OptiMAL-IT® kits (Diamed AG; Lot No. 46110.72.01; Fig. 7.2.1.1) in the field itself following manufactures standard procedure (Fig. 7.2.1.2). Further at the same time 2-3 drops of blood were collected on the FTA™ classic cards (Whatman, Sweden) for PCR assays.
All randomly selected samples were re-examined by another senior technician in the laboratory using light microscopy. In discrepancies, the results obtained during re-examination in laboratory using microscopy were recorded as true and used for the present study.
7.2.2 DNA preparation and nested PCR assay

Parasite DNA was extracted using the Qiagen kit and following the standard FTA™ manufacturer procedure. *Plasmodium* species identification was done by nested PCR amplification (Bio-Rad S1000) of the small sub-unit ribosomal ribonucleic acid (18s-rRNA) genes using the primers for all four human malaria parasite species. PCR was performed with the primers described elsewhere (Johnston *et al*, 2006; Snounou *et al*, 1993). First round amplification was performed under following conditions: for *P. falciparum*, *P. vivax* and *P. malariae* - Initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 60°C for 2 min, extension at 72°C for 2 min (35 cycles), final extension at 72°C for 5 min and for *P. ovale* - Initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, extension at 72°C for 1 min 30 sec (30 cycles), final extension at 72°C for 5 min. The nested PCR was performed under the following conditions: for *P. falciparum*, *P. vivax* and *P. malariae* - Initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min (30 cycles), final extension at 72°C for 5 min and for *P. ovale* - Initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, extension at 72°C for 1 min 30 sec (45 cycles), final extension at 72°C for 5 min. Genomic DNA prepared from the blood of one of the investigators with no malaria history, was used as a negative control in the PCR assays. The amplified PCR products were resolved by 2% agarose gel electrophoresis and viewed using a UV transilluminator (Syngene G-box).

The sensitivity and specificity of the OptiMAL-IT® and PCR assay for the detection of malaria parasite 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 (Altman and Bland, 1994). 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 which is used for comparing two data sets obtained either from two different diagnostic tests conducted at a place or one diagnostic test conducted at two different places (Youden, 1959).

7.3 Results

Out of 61 field confirmed malaria positive blood smears using microscopy, only 58 (95.1%) could be detected positive using microscopy in the laboratory (Table 7.3.1). However, the difference between the field and laboratory microscopy results was not significant ($p = 0.7; \chi^2 = 0.2; RR = 1.1; 95\% CI = 0.75-1.70$). Of these 58 blood smears, 53 (91.4%) were found positive for *P. falciparum* and 5 (8.6%) for *P. falciparum* and *P. vivax* mixed infection (Table 7.3.1). The OptiMal-IT® identified *P. falciparum* in 49 (85.9%) and PAN-specific in 8 (14.0%) individuals. There was discrepancy in 7 *P. falciparum* cases, 4 of which were confirmed positive by microscopy but negative with OptiMal-IT® test. On the other hand, OptiMal-IT® detected 3 *P. falciparum* infections which were not detected by microscopy. Sensitivity and specificity of OptiMal-IT® as compared to microscopy was 93.1% and 83.3% respectively (Table 7.3.2), whereas PPV, NPV and FDR for OptiMal-IT® were 94.7%, 78.9% and 5.3% respectively. The overall performance of OptiMal-IT® recorded for malaria diagnosis in the present study was 76.4%. PCR assay detected *P. falciparum* in 48 (85.7%) blood samples, *P. falciparum* and *P. vivax* mixed in 4 (7.1%), *P. falciparum* and *P. malariae* mixed in 3
On the other hand, two samples which were detected positive by microscopy could not be detected positive either by OptiMAL-IT® or PCR. The sensitivity and specificity of PCR assay in detecting malaria parasite were 96.6% and 100% respectively, while PPV, NPV and FDR were 100%, 90% and 0% respectively. The overall performance of PCR assays was 96.6%.

No *P. malariae* infection could be diagnosed in the field and laboratory using microscopy. However, nested PCR identified three *P. falciparum* and *P. malariae* mixed infection and one *P. malariae* mono infection. The nested PCR amplicons of *P. malariae* infections were visualised at 144 bp diagnostic band, whereas *P. falciparum* and *P. vivax* were resolved 205 and 120 bp positions respectively (Fig. 7.3.1). The *P. malariae* infections were identified from three males and one female patients of different age groups (11-55 years) and different tribes. In the microscopy two *P. falciparum* and *P. malariae* mixed infection were diagnosed as *P. falciparum*, one *P. falciparum* and *P. malariae* mixed infection as *P. falciparum* and *P. vivax* mixed infection and one *P. malariae* as *P. falciparum* mono infection.

![Fig. 7.3.1: Plasmodium species amplification in nested PCR. Lane 1-3: Plasmodium malariae (144bp); Lane 6-7: P. vivax (120bp); Lane 9-11: P. falciparum (205bp); Lane 4 and 8: standard ladder Sigma (50 bp), Lane 5: negative control.](image-url)
7.4 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, 95.1% were identified malaria positive in the second round microscopy. In the present study, microscopy detected two *P. vivax* and one *P. falciparum* cases which were later resolved to *P. malariae* by PCR. Microscopic examination has been the diagnostic method of choice in malaria epidemiologic studies and diagnosis. The method is simple and does not require special facility and in most cases enables differentiation among the four species of human malaria parasites (Maltha *et al*, 2010). However, it has limitation that even a skilled person can evaluate only a limited number of blood smears per day under field conditions. Further, in the case of low level of parasitemia and mixed parasite infection, microscopy can sometime be misleading in correct identification of malaria parasite species.

In present study the sensitivity and specificity of OptiMAL-IT® was found to be 93.1% and 83.3% respectively. OptiMal-IT® detects *P. falciparum* and PAN specific antigen for *Plasmodium* species; however, four cases confirmed for *P. falciparum* by microscopy were detected negative by OptiMAL-IT®. The sensitivity of OptiMAL-IT® has been found to decrease with the lower level of parasitemia or presence of blocking antibodies in the blood (Iqbal *et al*, 1999). OptiMAL-IT® test also detected three false *P. falciparum* cases and could be due to presence of pLDH antigens which have been reported to remain in the patient’s blood even after initiation of the treatment with antimalarials (Iqbal *et al*, 1999). The test reading observed beyond the reading time may increase the number of positive PAN-pLDH lines (Maltha *et al*, 2010). In the PCR
assay the sensitivity and specificity was found to be 96.6% 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 (Zakeri *et al*, 2002). The PCR assays in the present study were found better compared to microscopy in detecting the *Plasmodium* species which were misidentified by microscopy. In the mixed infections mostly one *Plasmodium* species either dominates or inhibits the other *Plasmodium* species present inside the same red cell population (Kimura *et al*, 1995). Two samples found positive for *P. falciparum* were detected negative in PCR assay. Loss of DNA extracted from two samples during storage and handling can not be ruled out at present stage.

In two *P. falciparum* cases, *P. malariae* was identified along with *P. falciparum* mixed infections, whereas one *P. falciparum* and *P. vivax* mixed infection detected by microscopy was identified as *P. falciparum* and *P. malariae* mixed infection in PCR. Many malaria cases in North-Eastern region appear with *P. falciparum* and *P. vivax* mixed infection; however, only a few cases of *P. falciparum* with *P. malariae* and *P. falciparum* with *P. vivax* mixed infection have been reported (Dhiman *et al*, 2011; Mohapatra *et al*, 2008; Rabha *et al*, 2012). The mixed infection cases are largely underestimated while using microscopy in malaria detection instead of molecular based diagnosis (Black *et al*, 1994; Carter and Mendis, 2002). *P. malariae* is not very common in this region and has been reported only from two states of North-Eastern India (Dev, 2000; Mohapatra *et al*, 2008), however its prevalence in other parts of the region is unknown (Mohapatra *et al*, 2008). Further in microscopy, the *P. malariae* is confused with *P. vivax* due to similarity at the growth stages (Scopel *et al*, 2004). Use of antimalarials for malaria treatment 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 (Black et al, 1994). Further, due to negative diagnosis the untreated patients may be carriers of malaria parasites and act as epicentres for other non-infected individuals. *P. 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 even at very low parasitemia is capable of infecting the mosquito vectors thus facilitating transmission round the year (Carter and Mendis, 2002; Mohapatra et al, 2008).

Present study area falls in malaria zone-2, where although, *falciparum* malaria is dominant but it typically co-circulate and occur together with the non-*falciparum* malaria (Murray and Bennett, 2009). OptiMAL-IT® test in such settings in not much useful as it is not able to detect the mixed infection. Therefore, despite possible rare false positive cases, the nested PCR detection of malaria parasites complemented to the other existing methods will be useful to timely obtain the incidence due to each malaria parasite species for specific treatment and epidemiological follow up.

The present study also concludes that *P. malariae* may be more prevalent in the region than thought previously. *P. malariae* cases were locals and not visited other areas in recent time. Therefore results strongly evidenced that the local transmission of *P. malariae* was maintained. PCR assays employed in the present study may not be used routinely due to various constraints but can be used in between atleast at a primary health centre level for re-evaluation of existing methods.