Human malaria is a protozoan infection caused by five *Plasmodium* species including *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and more recently *P. knowlesi*¹, differing greatly from each other in their clinical manifestation, responses to antimalarial drugs, transmission potential and the nature of immunity they elicit in human hosts². In India, 2.0 to 3.0 million cases of human malaria are reported annually by the surveillance mechanism of the National Vector Borne Diseases Control Programme (NVBDCP)³. *P. vivax*, accounting for about 65 per cent cases, is the most prevalent species of human malaria parasite in India followed by *P. falciparum* which contributes nearly 35 per cent cases⁴. Since sixties, the north-eastern region of India comprising of Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Nagaland, Tripura and recently included Sikkim State has been accounting for 8-12 per cent of all reported malaria cases in India. *P.
P. falciparum and P. vivax are the two common species of malaria parasite in this region with the predominance (60 - 80%) of the former. The sporadic presence of P. malariae has been reported from time to time in Orissa, West Bengal, Madhya Pradesh, Karnataka, Tamil Nadu, Kerala and Assam. P. ovale is the rarest infection with only four cases reported in India, one each from Kolkata, Orissa, Delhi and Assam. The P. malariae reported in earlier studies was based on morphology under the microscope, the technique employed routinely for identification of malaria parasite in India under NVBDCP. Under microscope, however, differentiating P. malariae from P. vivax at early ring stage is difficult. Molecular identification methods such as polymerase chain reaction (PCR) score over the conventional microscope for the accurate identification of malaria parasite species as evident by the report from Malaysia where microscopically identified P. malariae cases were actually found to be of P. knowlesi in PCR identification. Among various PCR methods available for identification of human malaria parasite the nested PCR is considered as one of the most sensitive methods.

During the conduct of our malaria epidemiological studies in Lohit district of Arunachal Pradesh we came across atypical malaria parasites resembling closely to P. vivax which, however, could not labelled as P. vivax because of the host cell morphology. However, in a few cases the parasite looked like P. malariae raising the suspicion of the presence of P. malariae, though there has not been any past record of P. malariae infection in that area. In order to confirm this possibility we carried out a survey employing both microscopic as well as nested PCR techniques for parasite species identification in Lohit district of Arunachal Pradesh.

**Material & Methods**

A cross-sectional study, duly approved by the Institutional Ethical Committee of Regional Medical Research Centre, Dibrugarh, was carried out covering all the 22 villages under Chongkham Community Health Centre (CHC) in Lohit district of Arunachal Pradesh bordering Myanmar. This malaria endemic CHC covered a population around 25,000, mostly of Khambi (an indigenous tribe) and Chakma (migrants from Chitagong Hill tracks of Bangladesh during 1971). The area is mostly plain with evergreen forest criss-crossed by numerous rivulets.

A house-to-house fever survey was carried out in the villages during May-August 2005. Giemsa stained thick and thin blood smears from all the fever cases were examined microscopically (Olympus Binocular compound microscope, CX 41, Philippines) and malaria positive cases were treated as per the guidelines of NVBDCP. All malaria positive cases were administered chloroquine (10 mg/kg of body weight) on days 0 and 1 and 5 mg/kg body weight on day 2. P. falciparum cases showing signs of therapeutic failure to chloroquine were treated with artesunate and sulphadoxine/pyrimethamine combination drugs. All the malaria positives, except P. falciparum were administered primaquine at the rate of 0.25 mg/kg body weight daily for 5 days whereas P. falciparum cases were administered 0.75 mg/kg body weight of primaquine as a single dose. About 1 ml venous blood from all malaria positive cases, irrespective of the species, was collected, after obtaining informed written consent from all adults and in case of minor from guardians in acid citrate dextrose (ACD) coated tubes for molecular identification and stored at 4°C till further processing. All malaria positive cases were evaluated clinically and repeat blood smears were collected from the treated patients on days 2, 3, 7, 14, 21 and 28 to evaluate the antimalarial drug response.

**Nested polymerase chain reaction:** Parasite DNA was extracted from the whole blood using QIAamp® DNA mini kit (Qiagen, Germany) as per the manufacturer’s instructions. The nested PCR method based on the sequence of 18S small subunit ribosomal RNA (ssr RNA) gene, was employed with the following modifications: (i) 0.625 U of Taq polymerase (Promega, USA) and 0.8 µM of each primer (Bangalore Genei, India) were used. (ii) Annealing temperature of 54°C for 2 min was used in the first amplification reaction (species specific) using thermocycler (BIO-RAD, USA, My cycler).

The product of the second amplification reaction (genus specific) was resolved on 2.5 per cent agarose (Promega, USA) gel and documented in gel documentation system (BIO-RAD, Italy) using the software Quantity One version 4.0 to determine the band size.

**Results**

Screening of 1,995 fever cases from the 22 villages revealed malaria infection in 675 (slide positive rate 33.8%) subjects through microscopy (Table I). The P. falciparum was the predominant infection (85.5%). Malaria parasites in 9 smears from 2 villages named Block-1 and Block-2 were identified as P. malariae.
based on the following morphological features—nearly normal size of the parasitized red blood cell (PRBC), stretched trophozoite across PRBC showing typical band form with coarse stippling (Fig. 1), slightly amoeboid parasite with large dark brown pigment, number of merozoites per schizont 7-10, occupying almost the entire PRBC (Fig. 2). One of these smears (CKM-1627) also had *P. falciparum* parasite along with *P. malariae*. All the nine cases of *P. malariae* were from Chakma tribe.

Eight of the nine *P. malariae* cases were males and one female with median age of 40 yr (range 6-52yr). All patients having *P. malariae* infection were febrile at the time of survey with average axillary temperature of 37.9°C (37.6 - 38.9°C) and intermittent fever history of 3-8 days with headache. However, the quarten nature of fever could not be ascertained. Spleen with mild tenderness was palpable in only four cases. All these cases were successfully treated with chloroquine 25 mg/kg of body wt in 3 divided dosages with no evidence of parasite in any repeat blood smears on subsequent days in 8 cases. However, in the mixed infection (*P. malariae* with *P. falciparum*), though *P. falciparum* persisted up to day 3, *P. malariae* was absent from day 2 onwards. The clinical symptoms in all the 9 cases resolved after day 1.

Resolution of second amplicon of the nested PCR in 7 samples produced a *P. malariae* diagnostic band of 144bp (Fig.3). In one sample (CKM-1325), 2 bands of 144 and 120 bp were obtained, indicating mixed infection of *P. malariae* and *P. vivax*. In microscopic examination this smear was identified having mono infection of *P. malariae* (Table II). Another sample (CKM 1627) amplified two bands at 144 and 205 bp positions, indicating mixed infection of *P. malariae* and *P. falciparum* similar to the result obtained in microscopy.

### Discussion

Though *P. malariae* is not very common in most parts of India, its presence has been reported from several parts of the country. Ours is probably the first report of detection of *P. malariae* from the north-eastern State of Arunachal Pradesh which is also confirmed through PCR technique. It is worth noticing that as many as 8 cases of *P. malariae* were found in two villages suggesting that the prevalence of *P. malariae* is much higher in the north-eastern region of India than the recorded.

<table>
<thead>
<tr>
<th>Village name</th>
<th>BSE</th>
<th>Pos</th>
<th>Pf</th>
<th>Pv</th>
<th>Pm</th>
<th>Mixed(Pv+Pf)</th>
<th>Mixed(Pf+Pm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block-1</td>
<td>296</td>
<td>152</td>
<td>134</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Block-2</td>
<td>100</td>
<td>44</td>
<td>41</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total (2 villages)</td>
<td>396</td>
<td>196</td>
<td>175</td>
<td>11</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Grand total(22 villages)</td>
<td>1995</td>
<td>675</td>
<td>577</td>
<td>78</td>
<td>8</td>
<td>11</td>
<td>1</td>
</tr>
</tbody>
</table>

BSE, Blood slide examined; Pos, Malaria positives; Pf, *P. falciparum*; Pv, *P. vivax*; Pm, *P. malariae*; Mixed, more than one *Plasmodium* species

**Fig.1.** *P. malariae* band form trophozoite (inset, marked with arrow) in peripheral thin blood smear.

**Fig.2.** *P. malariae* schizont in peripheral thin blood smear.
P. malariae is likely to be confounded with P. vivax during microscopic examination of thick blood smear. The ring forms of the two species are so similar that Garnham described the ring forms of P. malariae in blood films as “rather like those of P. vivax although less amoeboid and with a more dense ring of cytoplasm”. We suspect that many of the P. malariae cases in areas of its prevalence in India may be misdiagnosed as P. vivax in routine microscopic examination, as has occurred in Suriname. The prevalent practice is examination of thick and thin smear stained with Jaswant Singh and Bhattacharyya (JSB) stain for routine malaria microscopy under NVBDCP (then NMEP). Though the method is sensitive and parasite detection is adequate, many a times it fails to diagnose the species of atypical parasites, possibly due to the morphological changes induced by haemolysis hampering the identification of infecting species or inadequate knowledge and experience of the microscopists to identify malaria parasites other than P. falciparum and P. vivax. Moreover, P. malariae and P. ovale species are difficult to identify if the typical morphology of the infected host cell is damaged. Whatever may be the reason it appears that the present estimate of P. malariae in India through routine microscopy is an underestimate. PCR based identification method due to its sensitivity and specificity even at low level (submicroscopic) of parasitaemia is a better diagnostic tool. However, factors like high cost of equipment and trained technical manpower may act as a major constraint and limit the use of PCR as a routine tool. We detected one mixed infection of P. malariae and P. falciparum in microscopy whereas the PCR method identified 2 mixed infections of Pm with Pf and with Pv. The quantum of mixed infections is generally underestimated when optical microscopy is used for parasite identification as compared to molecular based tools.

P. malariae is sustained at very low infection rates among the sparse and mobile human population. This is because, unlike other human malaria parasites, P. malariae can remain for decades within a human host and in a state potentially infectious to mosquitoes thus, facilitating the transmission of this species. All P. malariae cases detected in this study were from Chakma population who had migrated from Chitagong Hill track region of Bangladesh decades earlier to Arunachal Pradesh. The link between their migration and detection of P. malariae in them needs further

### Table II. Conventional microscopy vs nested PCR results of P. malariae isolates

<table>
<thead>
<tr>
<th>ID No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Conventional microscopic result</th>
<th>Nested PCR result</th>
<th>Final diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKM-638</td>
<td>25</td>
<td>M</td>
<td>Pm</td>
<td>+</td>
<td>Pm</td>
</tr>
<tr>
<td>CKM-727</td>
<td>40</td>
<td>M</td>
<td>Pm</td>
<td>+</td>
<td>Pm</td>
</tr>
<tr>
<td>CKM-1017</td>
<td>45</td>
<td>M</td>
<td>Pm</td>
<td>+</td>
<td>Pm</td>
</tr>
<tr>
<td>CKM-1176</td>
<td>52</td>
<td>M</td>
<td>Pm</td>
<td>+</td>
<td>Pm</td>
</tr>
<tr>
<td>CKM-1325</td>
<td>40</td>
<td>M</td>
<td>Pm</td>
<td>+ +</td>
<td>Pm+Pv</td>
</tr>
<tr>
<td>CKM-1926</td>
<td>49</td>
<td>M</td>
<td>Pm</td>
<td>+</td>
<td>Pm</td>
</tr>
<tr>
<td>CKM-1567</td>
<td>30</td>
<td>M</td>
<td>Pm</td>
<td>+</td>
<td>Pm</td>
</tr>
<tr>
<td>CKM-1627</td>
<td>18</td>
<td>M</td>
<td>Pm + Pf</td>
<td>+ +</td>
<td>Pm+Pf</td>
</tr>
<tr>
<td>CKM-1840</td>
<td>6</td>
<td>F</td>
<td>Pm</td>
<td>+</td>
<td>Pm</td>
</tr>
</tbody>
</table>

Pm, P. malariae; Pv, P. vivax; Pf, P. falciparum; Po, P. ovale; +, Positive; -, Negative
investigation. *P. malariae* has been implicated causing renal damage and nephritic syndrome\(^\text{23}\). Therefore, estimating the actual case load is necessary. Though we detected *P. malariae* infection only in one geographical location, its presence in other similar areas of north-eastern region cannot be ruled out suggesting the need for further studies using molecular tools to find out the exact prevalence of *P. malariae* in this part of the country.

**Acknowledgment**

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**References**