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FIRST PHYLOGENETIC EVIDENCE OF *Plasmodium malariae* FROM NORTHEAST REGION OF INDIA

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Abstract- The Northeastern region of India is highly malarious particularly with *Plasmodium (P.) falciparum* along with some amount of *P. vivax* infection. Information about the distribution of *P. malariae* in this region is scanty and only a few reports are available. Tripura, one of the north-eastern states has recently experienced a malaria outbreak in 2014. During this outbreak, the presence of *P. malariae* infection has been suspected and a study has been made to identify the same by molecular tools. A total of thirty one blood samples from suspected malaria patients were obtained from Takarjala community health centre and Khowai hospital of Tripura during this outbreak. Blood samples were examined with Rapid Diagnostic Test (RDT) for the presence of *P. falciparum* infection. All blood samples were also found positive for *P. falciparum* infection in microscopic examination. However, two samples out of these were found to have co-infection suspected to be of *P. malariae.* Presence of *P. malariae* co-infection was confirmed through adopted Nested PCR protocol and further validated by DNA sequencing. Phylogenetic study based on 18S rRNA sequence revealed that these two *P. malariae* isolates have close proximity with isolates from Japan and Uganda. This first molecular evidence of *P. malarie* co-infection from Tripura confirms the sporadic distribution of this parasite in N.E. region. Existence of *P. malariae* infection in this state may have an epidemiological implication for management of this new focus contributing slowly for the malaria case burden in those areas.

Keywords- DNA sequencing, Nested PCR, *Plasmodium malariae*, Phylogenetic study, Tripura

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Introduction

Amongst all mosquito borne diseases, malaria is recognized as a formidable problem in India. The North east region comprising of eight states is malarious having evidence of high incidence of *P. falciparum* infection and also co-infection mostly with *P. vivax*. The main causative protozoan solely responsible for uncomplicated and complicated malaria is *P. falciparum.* The outbreak of malaria due to *P. malariae* in India is not common. However, a malaria outbreak with a *P. malariae* focus in Haryana state was reported thirty five years ago [1]. In last two decades, only few *P. malariae* case reports were reported from some parts of this country [2-8]. In the Northeastern region of India also only two reports of *P. malariae* mono-infection as well as co-infection with *P. falciparum* in Assam and Arunachal Pradesh state of Northeast India [9,10] are available.

A seasonal malaria outbreak that occurred in Tripura, one state in northeast India during the month of June-July, 2014, the presence of *P. malariae* infection was suspected in routine microscopic examination. A study therefore was carried with an objective to determine and document the infection of this *Plasmodium* species by molecular tools and also to analyse the phylogenetic relationship by bioinformatics tools.

Materials and Methods

Collection of Sample

A total of thirty one whole blood samples in K3 EDTA vaccutainer were obtained from one community health centre (Takarjala) and one hospital (Khowai) in Tripura state of northeast during this outbreak. All suspected subjects were tested with bivalent rapid diagnostic test (RDT, SD ALERE Ag KIT) for *Plasmodium* infection.

Microscopic Examination

Blood smears (both thin and thick) were prepared from obtained whole blood samples and stained with Giemsa. Presence of any intra-erythrocytic stages of *Plasmodium* species was examined under 100X magnification in binocular compound microscope (ZEISS, USA).

PCR Amplification of 18S rRNA Gene and Species Detection

Genomic DNA (gDNA) was extracted from 200 µL of whole blood using Qiamp DNA min kit (QIAGEN, Germany) following manufacture's protocol. DNA was eluted in total 100 µL elution buffer from each sample and was stored in -80°C. All DNA samples were subjected to a high sensitive nested PCR protocol for detection of the

four *Plasmodium* species using PCR primers for both genus and species specific amplification and PCR thermal cycle [11] [Table-1]. PCR was performed in a total 20 µL reaction volume for genus specific and 50 µL reaction volumes for species specific amplification. All steps of PCR amplification were carried out using 2X PCR Master Mix (Promega, USA) at 1X concentration, 0.5 µmol of both reverse and forward primer (Intregated DNA Technology, USA) in Veriti 96 well plate thermal cycler (Applied Biosystem). 1µl of extracted DNA was added as template to primary genus specific PCR mixture and 1 µl of amplified genus specific amplicon was added as template to nested species specific PCR mixture. Amplified PCR products specific to species were subjected to 2% agarose gel (prepared in 1X tris-acetate EDTA Buffer, stained with ethidium bromide) electrophoresis (BIO-RAD). Appropriate size of amplified PCR products was detected in a gel documentation system (BIO-RAD) with reference to 100bp DNA ladder (Promega, USA).

Table 1- PCR Primer sequences and thermal cycle for detection of *Plasmodium* genus and species based on 18S rRNA sequence (Snounou, et al [11])

Target Sequence	Primer Name	Primer Sequence	Size	PCR thermal condition
Plasmodium genus	rPLU5	5'-CTTGTTGTTGCCTTAAACTTC-3'	1.2kb	95ºC for 5 min; 24 cycles of 94ºC for 1 min; 58ºC for 2 min; 72ºC for 2 min; then 72ºC for 5 min
	rPLU6	5'-TTAAAATTGTTGCAGTTAAAACG-3'		
P. falciparum	rFAL1	5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3'	205bp	95ºC for 5 min; 30 cycles of 94ºC for 1 min; 58ºC for 2 min; 72ºC for 2 min; then 72ºC for 5 min
	rFAL2	5'-ACACAATGAACTCAATCATGACTACCCGTC-3'		
P. vivax	rVIV1	5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3'	120bp	
	rVIV 2	5'-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3'		
P. malarie	rMAL1	5'-ATAACATAGTTGTACGTTAAGAATAACCGC-3'	144bp	
	rMAL 2	5'-AAAATTCCCATGCATAAAAAATTATACAAA-3'		
P. ovale	rOVA1	5'-ATCTCTTTTGCTATTTTTTAGTATTGGAGA-3'	800bp	
	rOVA2	5'-GGAAAAGGACACATTAATTGTATCCTAGTG-3'		

DNA Sequencing and Phylogenetic Analysis

Amplified products were further processed for DNA sequencing for confirmation of the result and phylogenetic study. Species specific PCR products were purified using QIAquick Gel extraction kit (QIAGEN, Germany) following manufacture's protocol and purified products were outsourced to Bioserve Biotechnologies for DNA sequencing. Obtained forward and reverse sequences from each isolate were edited using BioEdit software and consensus sequence was created. These consensus sequences were searched for similarity by BLAST (Basic Local Alignment Search Tool) [12] with *P. malariae* 18S rRNA sequences from GenBank database and multiple alignment was done by the ClustalW algorithm using MEGA (Molecular Evolutionary Genetics Analysis) software version 6.06. Genetic Distances between the sequences were calculated using the Tamura Nei Model [13,14] and phylogenetic tree was constructed using the Neighbour-Joining Method (bootstrap = 1000). One cluster was considered to be significant on the basis of presence of more than 50% of the permuted trees.

Results

Microscopic examination revealed the presence of *P. falciparum* infection in all 31 collected samples. However two samples out of these were found to have co-infection suspected to be of *P. malariae*.

In PCR assay, 144bp amplified fragment of 18S rRNA sequence specific to *P. malariae* was observed in both the samples (RM04TRIP14 and RM12TRIP14) [Fig-1]. We have further subjected all the samples for PCR using specific primers for the remaining species of *Plasmodium* and found no PCR product specific to *P. vivax* and *P. ovale* in these suspected samples, except the presence of only *P. falciparum* infection in PCR assay.

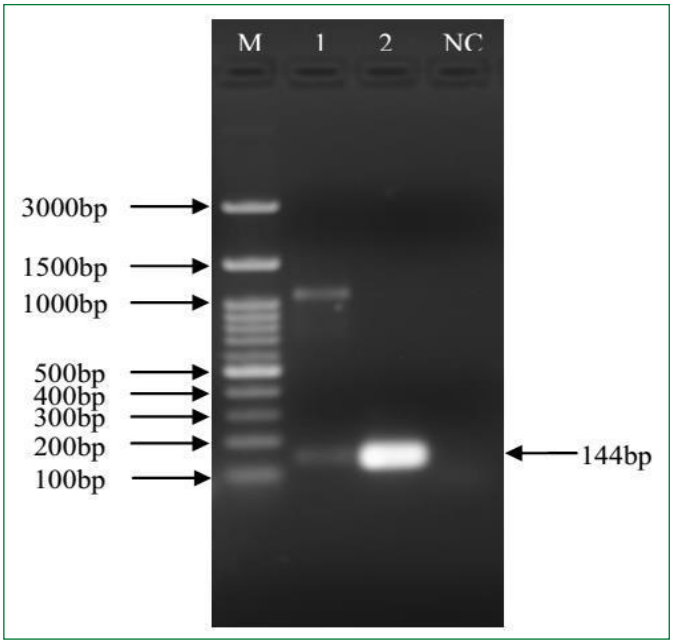

Fig. 1- Gel picture of *P. malariae* species specific amplification in nested PCR. Lane M - 100bp DNA Ladder; Lane 1 and 2 - 144 bp amplified DNA fragments specific to *P. malariae* from RM04TRIP14 and RM12TRIP14 isolate respectively; NC - negative control.

Phylogenetic analysis of *P. malariae* based on 18S rRNA nucleotide sequences revealed that these two isolates (RM04TRIP14 and RM12TRIP14) were found to be sub-clustered with isolate from Japan (AB250682) and Uganda (M54897) having a clue of close proximity of these two Indian isolates with those countries [Fig-2].

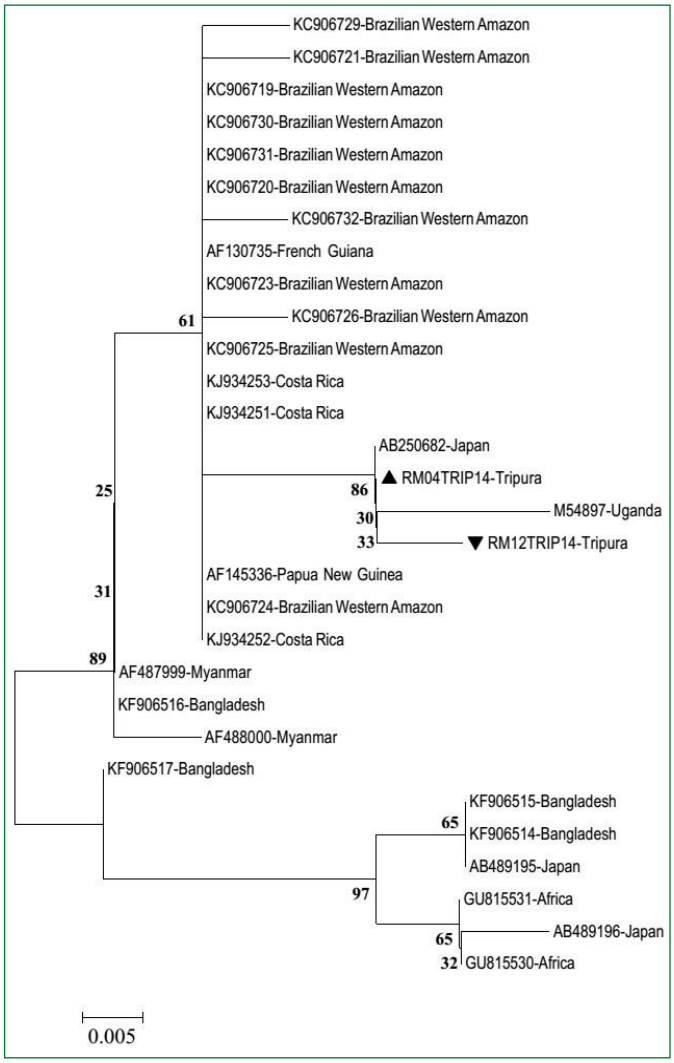

Fig. 2- Phylogenetic tree based on 18S rRNA nucleotide sequences using Neighbour-Joining method (bootstrap = 1000). Tree inferring evolutionary relationship of 18S rRNA sequences of the two *P. malariae* field isolates from Tripura (RM04TRIP14 and RM12TRIP14 coloured in black) with *P. malariae* 18S rRNA sequences from GenBank, GenBank accession number is given against each isolate.

Discussion

This is the first report of *P. malariae* infection from Tripura State of Northeast India. A focus of *P. malariae* infection was detected earlier from Arunachal Pradesh and Assam, another two states of northeast India [9,10]. Though *P. malariae* is believed to be sporadic in this region but its infection in patients may persist as it is not suspected usually and remain undiagnosed or misdiagnosed as *P. vivax* in routine microscopy. *P. malariae* infection was reported from areas where incidence of *P. falciparum* malaria was high [15-17]. The finding of our study is in conformity with this because the northeast region of India is rampant with *P. falciparum* malaria and therefore *P. malariae* mono-infection or co-infection with *P. falciparum* like *P. vivax* may persist. Proper information on epidemiology of *P. malariae* and *P. ovale* infection in malaria patients is still lacking in this part of the country. This parasite, like *P. vivax*, may also spread slowly to other areas during seasonal transmission as *P. malariae* remains for a long time within infected hosts [18]. Pertaining to phylogeny of *P. malariae* in Northeast India, it is the first study of this kind which is also of important concern to elucidate the origin of *P. malariae* isolates and to identify its flow to existing focus.

Conclusions

The presence of *P. malariae* infection in Tripura, Northeast region of India gives the clue of a new focus which may be under selection and at a state of development. The detection of *P. malariae* infection in tribal people of forest fringe and hilly periphery lines of Tripura state, too, may have epidemiological implication for management of this new focus contributing slowly for malaria case burden in those areas.

Abbreviations

RDT: Rapid Diagnostic Test.

gDNA: Genomic DNA.

rRNA: ribosomal RNA.

PCR: Polymerase Chain Reaction.

EDTA: ethylenediamine tetra acetic acid.

BLAST: Basic Local Alignment Search Tool.

MEGA: Molecular Evolutionary Genetic Analysis.

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Author's Contribution: All authors contributed equally in this study. MKD collected the samples of the study interest; MKD and SC performed and assessed all molecular and phylogenetic analysis; PD and MCK finalised the design and type of writing the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of Interest: The authors have declared that they have no competing interest.

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