Polymerase chain reaction detection of human host preference and *Plasmodium* parasite infections in field collected potential malaria vectors

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This study was carried out to determine the human host preference and presence of *Plasmodium* parasite in field collected *Anopheles* mosquitoes among four villages around a military cantonment located in malaria endemic Sonitpur district of Assam, India. Encountered malaria vector mosquitoes were identified and tested for host preference and *Plasmodium* presence using PCR method. Human host preference was detected using simple PCR, whereas vectorial status for *Plasmodium* parasite was confirmed using first round PCR with genus specific primers and thereafter nested PCR with three *Plasmodium* species specific primers. Out of 1874 blood fed vector mosquitoes collected, 187 (10%) were processed for PCR, which revealed that 40.6% had fed on human blood; 9.2% of human blood fed mosquito were harbouring *Plasmodium* parasites, 71.4% of which were confirmed to *Plasmodium falciparum*. In addition to *An. minimus*, *An. annularis* and *An. culicifacies* were also found positive for malaria parasites. The present study exhibits the human feeding tendency of *Anopheles* vectors highlighting their malaria parasite transmission potential. The present study may serve as a model for understanding the human host preference of malaria vectors and detection of malaria parasite inside the anopheline vector mosquitoes in order to update their vectorial status for estimating the possible role of these mosquitoes in malaria transmission. The study has used PCR method and suggests that PCR-based method should be used in this entire malarious region to correctly report the vectorial position of different malaria vectors.

Keywords: *Anopheles*, *Plasmodium*, Host preference, PCR

Introduction

Human-vector contact is an important component in disease transmission and used to evaluate the risk of vector-borne disease.¹ In addition to prevalence of *Anopheles* mosquitoes, its host preference and parasite transmission rate provide insight into the malaria epidemiology in an area of interest. Proper knowledge of blood meal preference of *Anopheles* vector mosquitoes, particularly from the human host, provides information on anthropophilic behaviour and is useful to demonstrate the changing patterns of host preference.² Northeastern states of India are highly endemic for *Plasmodium falciparum* and *P. vivax* malaria incidence as compared to *P. malariae* malaria, of which only few cases have also been reported.³⁻⁶ Malaria transmission in the region is uninterrupted and supported by *An. minimus*, *An. fluviatilis*, and *An. dirus*

mosquitoes.^{4,6-8} In addition, *An. annularis*, *An. culicifacies*, and *An. philippinensis* have also been incriminated as vectors of secondary importance.^{6,9-12} Most of the *Anopheles* vectors in Southeast Asia exist in cryptic forms that are difficult to separate using morphological characters.^{13,14} *An. philippinensis* and *An. nivipes* are difficult to differentiate based on morphological characters even at species complex level; hence, they have been denoted as *An. philippinensis/nivipes*.¹¹ The host preference and incrimination data of these vectors is scanty in the region and only a few systematic studies have been carried out earlier.⁵ Various methods such as salivary gland dissection,¹⁵ ELISA,¹⁶ and gel diffusion¹⁷ are available to detect the blood meal in mosquito; however, the PCR-based methods are more reliable and sensitive.^{2,5,15,18} In addition to recognizing the blood meal ingested by mosquito, PCR-based methods are specific to detect the *Plasmodium* species present within the vector mosquito.² The present study was designed to understand the human host preference of malaria

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vector mosquitoes and to identify the anopheline species which could have role as malaria vectors.

Material and Methods

A 5-month long study was carried out in four different villages (GPS position: 26°40'44.9"N to 92°47'42.5"E, 26°41'09.4"N to 92°46'46.6"E, 26°41'56.9"N to 92°48'09.9"E, 26°42'01.2"N to 92°46'50.1"E) surrounding the Solmara cantonment in Sonitpur district of Assam, India, during April–August 2010. Mosquitoes were collected using light traps (John Hock, Gainesville, FL, USA) from the human dwelling sites, which had integrated cattle sheds, so that mosquitoes could get equal chance to bite human as well as animal host. The traps were placed near unscreened windows in rooms with sleeping human and operated during 1800–0600 hours. In this way, collections were made from four selected sites of each village on weekly basis. In the study area, domestic animals such as cow, goat, cat, dog, pig, fowl, and duck were also available as possible hosts for mosquitoes. A total of 1874 blood fed *Anopheles* vector mosquitoes comprising of *An. minimus*, *An. annularis*, *An. culicifacies*, and *An. philippinensis* were identified. Mosquitoes have been identified to the species complex level following morphological characters and no molecular assays were performed for identification purpose. Out of total 1874 mosquito specimens, 187 (sum total of 10% of each vector species) were selected randomly and processed for DNA extraction using QIAamp DNA mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. We have tested only 10% of the collected mosquitoes due to resource limitation. PCR

assay for human host preference was performed using primers as described earlier,² whereas PCR for *Plasmodium* detection and nested PCR for *P. falciparum*, *P. vivax*, and *P. malariae* detection was carried out with primers described elsewhere¹⁹ (Table 1) with slight modifications in cycling conditions (Table 2). Immediately after identification, the selected mosquitoes were processed for PCR assay. Laboratory reared *Culex* mosquitoes fed on human and mouse blood were used as positive and negative controls for human host preference PCR, while known malaria-positive and -negative blood samples obtained on FTA classic cards (Whatman, Piscataway, NJ, USA) were used as controls for *Plasmodium* detection PCR. Each sample was tested twice, using two different thermal cycler machines (Bio-Rad, Hercules, CA, USA and Eppendorf, Hamburg, Germany) on two different days. Only those samples which showed positive *Plasmodium* specific results were used in nested PCR. PCR products obtained were visualized in 1.8% agarose gel electrophoresis in a gel documentation system (Syngene, Iselin, NJ, USA). Vector mosquito densities were compared using ANOVA followed by Tukey–Kramer test of multiple comparison, whereas the anthropophilic index was compared using Chi-square test. The statistical analyses were carried out using GraphPad InStat software.

Results

Human host preference

Seventy-six (40.6%) of 187 vector mosquito specimens tested were found fed on the human host. *An. philippinensis*/*nivipes* was the dominant species

Table 1 Primers used for human host preference and nested PCR for detection of malaria parasite in the mosquito

Species	Primer	Sequence (5'–3')	Bases	Product size (bp)
<i>Homo sapiens</i>	HUM 1	CGA GAG TTC TCT GGA AGA ATT GA	23	519
	HUM 2	TGA TAG CCT GGA AGT GAC AAA AT	23	
<i>Plasmodium</i> specific	PLU 5	CCT GTT GTT GCC TTA AAC TTC	21	1100
	PLU 6	TTA AAA TTG TTG CAG TTA AAA CG	23	
<i>P. falciparum</i>	FAL 1	TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT	30	205
	FAL 2	ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC	30	
<i>P. vivax</i>	VIV 1	CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC	30	120
	VIV 2	ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA	30	
<i>P. malariae</i>	MAL 1	ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC	30	144
	MAL 2	AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA	30	

Table 2 PCR reaction conditions for human host preference and *Plasmodium* parasite detection

Primer pairs	Cycling conditions
HUM 1/HUM 2	94°C–4 minutes 35 cycles of: 94°C–30 seconds, 55°C–30 seconds, 72°C–1 minutes 72°C–8 minutes
PLU 5/PLU 6	94°C–5 minutes 32 cycles of: 94°C–1 minutes, 60°C–1 minutes, 72°C–2 minutes 72°C–4 minutes
Nested primers	
FAL 1/FAL 2	94°C–4 minutes
VIV 1/VIV 2	30 cycles of: 94°C–1 minutes, 55°C–1 minutes, 72°C–2 minutes
MAL 1/MAL 2	72°C–4 minutes

recorded in large number, whereas *An. minimus* was least abundant and recorded in less number ($F=34.9$; $P<0.0001$). The overall anthropophilic index (AI) of *An. minimus*, *An. culicifacies*, *An. annularis*, and *An. philippinensis/nivipes* was 90%, 37.3%, 26.9%, and 45.9%, respectively (Table 3). *An. minimus*, although scanty in distribution, had the highest AI as compared to the other vectors (Chi-square=15.3; $P=0.0016$).

Detection of *Plasmodium* parasite

Only seven mosquitoes, two each of *An. culicifacies* and *An. annularis* and three of *An. minimus*, were positive for the presence of human malaria parasite (Table 3). All seven incriminated mosquitoes were found fed on human blood. None of the *An. philippinensis/nivipes* could be amplified for the presence of *Plasmodium* parasite. Nested PCR assay revealed that all three (100%) *Plasmodium*-positive *An. minimus* mosquitoes were harbouring *P. falciparum* only, whereas one each (50%) of *An. annularis* and *An. culicifacies* *Plasmodium*-positive mosquitoes harboured *P. falciparum*. In *An. annularis*, the other specimen was found positive for *P. vivax*, while in *An. culicifacies*, the other specimen could not be amplified in nested PCR for the detection of *Plasmodium* species despite several attempts. None of the mosquito assayed was found positive for *P. malariae*.

Discussion

Identification of mosquito host preference is useful in understanding the disease transmission profile in an area.²⁰ However, the accurate interpretation of host preference is possible only when adequate variety of hosts is present in the study area.²¹ Out of the four *Anopheles* vector species tested during peak malaria transmission period in the present study, *An. minimus* showed very high AI as compared to the remaining three species. The other three *Anopheles* species, except *An. minimus*, were somewhat opportunistic in the host selection. *An. minimus* has been an important malaria vector in the region;^{6,14,22,23} however, its density was lower and outnumbered by other three species. This potential vector was found positive for *P. falciparum* only, whereas *An. annularis* was harbouring both *P. falciparum* and *P. vivax*. *An. philippinensis/nivipes* could not be incriminated as

malaria vector; however, earlier studies have detected *Plasmodium* sporozoite among a few specimens of *A. nivipes* in the region.⁵ In *An. culicifacies*, both specimens were positive for *Plasmodium* but only one specimen was detected to have *P. falciparum*, while the *Plasmodium* species present in the other specimen could not be identified in nested PCR, which may be due to mishandling or loss of DNA at some step. Although a few specimens of mosquito species other than *An. minimus* were found positive for *Plasmodium*, their high density throughout the malaria season could help them to emerge as primary malaria vectors by replacing *An. minimus*. *P. malariae* has been reported in some areas of the north eastern region;^{3,24} however, in the present study, none of the *Anopheles* vector was found positive for *P. malariae* parasite.

Many studies have been carried out to detect the host preference and sporozoite in the vector mosquito; however, techniques other than PCR could not receive wide acceptance.^{2,15,18,21} PCR assays have proven to be sensitive, specific, and extremely useful in scanning large number of samples in short time.²⁵ PCR assays are useful in detecting the role of individual mosquito in malaria transmission, and therefore helpful in devising region specific malaria control strategies. Species identification is tedious and may be misleading using traditional methods, causing the epidemiological significance to be missed. PCR methods have been found more effective as compared to microscopic methods to detect the sporozoite at very low level of parasitaemia.¹⁵ The PCR assays used here are highly efficient and reproducible for identifying host and human *Plasmodium* from mosquitoes. We have tested a limited number of *Anopheles* mosquitoes during malaria peak season from a limited area. However, using this method, a large number of *Anopheles* mosquitoes from different areas during different seasons need to be tested to arrive at a concrete conclusion on malaria vector potential of these mosquitoes. The present study with limitation of not detecting daily survival rate of tested mosquito species may offer the opportunity to detect malaria vector mosquitoes of entire northeastern region and contribute for designing better vector control interventions.

Table 3 Detection of human blood meal and *Plasmodium* in vector mosquitoes using PCR

Vector sp.	Collected/ tested	HBF (AI)	<i>Plasmodium</i> sp. (%)	<i>P. falciparum</i> (%)	<i>P. vivax</i> (%)	<i>P. malariae</i> (%)	No amplification in nested PCR
<i>An. philippinensis/nivipes</i>	744/74	34 (45.9%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
<i>An. culicifacies</i>	514/51	19 (37.3%)	2 (10.5%)	1 (50%)	0 (0.0%)	0 (0.0%)	1 (50%)
<i>An. annularis</i>	526/52	14 (26.9%)	2 (14.3%)	1 (50%)	1 (50%)	0 (0.0%)	0 (0.0%)
<i>An. minimus</i>	90/10	9 (90.0%)	3 (33.3%)	3 (100%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Note: HBF: human blood fed; AI: anthropophilic index.

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