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Detection of *Plasmodium falciparum* infection in *Anopheles* mosquitoes from Keonjhar district, Orissa, India

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The determination of the presence of malaria sporozoites in wild caught *Anopheles* mosquitoes remains an integral component in understanding the transmission dynamics in area-specific malaria epidemiological studies¹. Mosquito infection rate, together with concurrent human-landing density data provide vulnerable parameters for estimating the intensity of transmission and entomological inoculation rates that serve as a relative measure of disease risk among exposed human populations². Detection of advance stage sporozoites in mosquitoes also provides compelling evidence of incriminating a particular vector species³.

Since the earliest days of research into the transmission of malaria, light microscopy has been used to determine sporozoite rates of *Plasmodium* species in mosquitoes. However, availability of skilled microscopists, for processing large number of samples to detect the sporozoites and identification of sporozoite species are the main limitations of this method⁴. Hence, a method with greater sensitivity and specificity has been looked for^{5–8}. The polymerase chain reaction (PCR) is highly sensitive and specific⁹ and its application to both blood meal identification and sporozoites detection has demonstrated how the prevalence of malaria parasites can be underestimated by light microscopy⁴. In the present study, we have used this technique to find out the sporozoite rate in ano-

pheline mosquitoes collected from a malaria endemic area of Orissa and compared its efficiency with the conventional microscopic method.

The study was conducted in Ghatgaon Public Health Centre (PHC) of Keonjhar district of Orissa (India). The study area lies between 21° 24' 39" N latitude and 85° 54' 48" E longitude and highly endemic for malaria and reported high malaria deaths (40) in the state during five years (1998–2002) with a slide positivity rate (SPR) ranging from 13.5 to 21.2%. *Plasmodium falciparum* is the predominant species (> 95%).

Indoor resting mosquitoes were collected from human dwellings between 0600 to 0900 hrs by mechanical aspirator from November 2002 to January 2003. They were brought to the laboratory and identified. Some of the female *Anopheles* mosquitoes were dissected in normal saline and examined for oocyst and sporozoite infections in gut and salivary gland respectively. The rest of the female mosquitoes were processed for detection of sporozoites by PCR technique.

DNA was extracted from individual mosquitoes as follows: after removing the wings and legs, the tissues (head and thorax) were macerated by a sterile pipette tip and mixed with 100 µl of DNAase free water. The homogenate was then boiled in water-bath at 95°C for 45 min, the DNA containing supernatant

was separated by centrifuging at 12000 rpm at room temperature for 20 min. Amplification of genus and species-specific *Plasmodium* was done by using the primers: rPLU5 (5'-CCTGTTGTTGCCTTAAACTTC-3'), rPLU6 (5'TTAAAATTGTTGCAGTTAAACG-3'), rFAL1 (5'TTAAACTGGTTTGGGAAACCAAATATATT-3'), rFAL2 (5'-ACACAATGAACTCAATCATGACTACCCGTC-3'), rVIV1 (5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3') & rVIV2 (5'CTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3') as described by Snounou *et al*¹⁰. Each 20 µl reaction mixture for nest-1 amplifications contained 12 µl of template DNA, 250 nM of each primer (rPLU5 & rPLU6), 4 mM MgCl₂, PCR buffer (50 mM KCl, 10 mM Tris-HCl), 200 µM of each dNTPs and 0.4 units of Taq DNA polymerase. The PCR conditions (nest-1) were as follows: step-1: 94°C for 4 min; 94°C for 30 sec, 55°C for one min; extension at 72°C for one min; 35 cycles and final extension at 72°C for 4 min. About 8 µl of the nest-1 amplification products served as the DNA template for each of the 20 µl of second PCR (nest-2) amplification. The concentration of the nest-2 primers and other constituents were identical to nest-1 amplification, except that 0.3 unit of Taq DNA polymerase was used. The second PCR (nest-2) amplification conditions were identical to those of first PCR (nest-1) except that the annealing temperature was 58°C for the species-specific primer. The PCR products were analysed after electrophoresis in 1.5% agarose gel

and stained with ethidium bromide.

A total of 565 those, anophelines were collected from the study area. Out of 523 anophelines were processed for the detection of sporozoites (*An. annularis*, *An. culicifacies*, *An. subpictus*, *An. vagus* and *An. hyrcanus*). *An. annularis* was found to be the dominant species (Table 1). Dissection and microscopic examination of the salivary glands of *An. annularis* (132), *An. culicifacies* (14), *An. subpictus* (45), *An. vagus* (52) and *An. hyrcanus* (10) revealed no sporozoite. Rest of the mosquitoes from all the species were processed by PCR, of these only 7 *An. annularis* out of 204 were found positive for sporozoite, thus giving a sporozoite infectivity rate of 3.4%. The nested primer pair used for the detection of *P. falciparum* show band in 205 base pair (bp) (Fig. 1).

Detection of sporozoites in *Anopheles* mosquitoes by PCR method has been attempted by Harada *et al*¹¹ and Wilson *et al*¹². The sporozoite rate of *An. annularis* by microscopic method found to range between 0.012 and 0.2% in the various regions of India¹³⁻¹⁵ but the species of the malaria parasite could not be identified. In our study no sporozoite was found in routine dissection method. However, by PCR technique sporozoite rate was detected to be 3.4% and the species was identified to be *P. falciparum*. By using PCR technology four fold increase of the sporozoite rate (0.62 to 2.2%) was observed by Wilson *et al*¹² in

Table 1. Detection of sporozoite by dissection and PCR method

Species	Total no. collected (%)	No. of mosquitoes processed for detection of sporozoites			
		Dissection method		PCR method	
		Nos.	+ve	Nos.	+ve
<i>An. annularis</i>	345 (61.1)	132	-ve	204	7
<i>An. culicifacies</i>	227 (4.8)	14	-ve	8	-ve
<i>An. subpictus</i>	78 (13.8)	45	-ve	20	-ve
<i>An. vagus</i>	93 (16.4)	52	-ve	30	-ve
<i>An. hyrcanus</i>	22 (3.9)	10	-ve	8	-ve

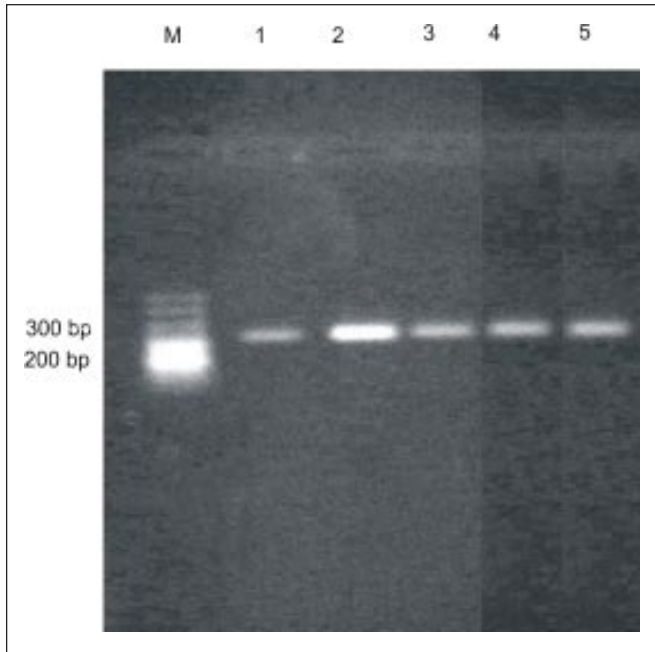


Fig 1: Detection of sporozoite by PCR method in *Anopheles annularis* [Lane M: 100 bp ladder; Lane 1 to 4: Test samples of *An. annularis*; Lane 5 : Positive *Pf* sample]

An. gambiae s.s. in Ghana *P. falciparum* was detected in 15.2% of *An. farauti* in Solomon Islands using PCR method by Harada *et al*¹¹. Tassanakajon *et al*¹⁶ could detect four out of five wild caught *An. dirus*, which were negative by ELISA but found positive for the presence of *P. falciparum* sporozoite by PCR (80% infectivity rate). Snounou *et al*⁴ found 10.6% infectivity rate in *An. gambiae* complex for *P. falciparum* infection by PCR method in Guinea Bissau population. All the above studies show the high epidemiological significance of use of PCR for the detection of sporozoite. The present study demonstrated that by depending on conventional dissection method alone, the epidemiological significance of this species would be missed. Due to PCR technology we could detect the *P. falciparum* sporozoite in *An. annularis*. In the study area, >95% of the malaria cases are due to *P. falciparum*. The predominance of *An. annularis*, with sporozoite rate of 3.4% for *P. falciparum* along with the high *P. falciparum* infection in the community, suggests that it is the vector of importance in this region particularly in winter.

Therefore, it warrants for in-depth studies on the bio-nomics of *An. annularis* for developing location-specific control programme.

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