VECTORIAL ROLE OF *ANOPHELES SUBPICTUS* GRASSI AND *ANOPHELES CULICIFACIES* GILES IN ANGUL DISTRICT, ORISSA, INDIA

Swati Kumari, Sarat Kumar Parida, Nitisheel Marai, Asima Tripathy, Rupenansu Kumar Hazra, Santanu Kumar Kar and Namita Mahapatra

Regional Medical Research Centre (Indian Council of Medical Research), Orissa, India

Abstract. Malaria transmission by *Anopheles subpictus* Grassi, 1899 and *Anopheles culicifacies* Giles, 1901 was studied from March 2004 to February 2007 in Angul District, Orissa, India, which is highly endemic for malaria. Adult mosquitoes were collected from human dwellings using sucking tubes and a mechanical aspirator. After identification, some *An. subpictus* and *An. culicifacies* specimens were subjected to a precipitin test to determine their anthropophilic index and the remaining samples were preserved in isopropyl alcohol for sporozoite detection by nested PCR. *An. subpictus* was the most prevalent (29.0%) anopheline species detected, followed by *An. culicifacies* (11.6%). The anthropophilic index for the *An. subpictus* was higher than *An. culicifacies* and was highest during the summer season. Malaria sporozoite rates of 0.52% and 1.82% were detected for *An. subpictus* and *An. culicifacies*, respectively. Sporozoites were detected during the summer in *An. subpictus* and during the rainy season and winter in *An. culicifacies*. The slide positivity rate (SPR) was high during the summer. The high anthropophilic index and presence of sporozoites in *An. subpictus* during the summer indicate *An. subpictus* is a contributory factor for the high SPR during the summer, and *An. culicifacies* is a contributory factor for the high SPR during the rainy and winter seasons, along with other anophelines. In the present study *An. subpictus* has been incriminated as a vector of malaria for the first time in Orissa.

INTRODUCTION

Anopheles subpictus Grassi is widely distributed and found in abundance in the Oriental region. It is found to the west of India in Afghanistan, Pakistan and Iran and to the east in New Guinea and in the Marinas islands. It is also found in Southern Sri Lanka and northern China. In India it is found throughout the mainland and on Lakshdweep islands, but not on the Andaman and Nicobar Islands (Rao, 1984). *Anopheles culicifacies* Giles has wide distribution in India and extends up to Ethiopia, Yemen, Iran, Afghanistan and Pakistan in the west and Bangladesh, Myanmar, Thailand, Cambodia and Vietnam in the east. It is also found in Nepal and southern China to the north and extends to Sri Lanka in the south (Rao, 1984).

Among Indian anophelines, *An. culicifacies* has been regarded as an important vector of malaria (Subbarao, 1988; Surendran *et al*, 2000). Malaria sporozoites were not detected in *An. subpictus* until 1981

Correspondence: Namita Mahapatra, Regional Medical Research Centre (ICMR), Bhubaneswar-23, Orissa, India.
E-mail: namita_rmrc@rediffmail.com

in India. Paniker *et al* (1984) detected malaria sporozoites in *An.subpictus* in a coastal village of Tamil Nadu. Sporozoite positive specimens were also found in Baster District of Madhya Pradesh by Kulkarni (1983). Tyagi and Yadav (2001) in Thar District, Rajasthan found *An. subpictus* amongst eight anopheline species collected from four villages which were endemic for malaria parasites. In Orissa *An. culicifacies* was established as a malaria vector by Nagpal and Sharma (1986). Until now no study has been conducted to evaluate the role of *An. subpictus* in malaria transmission in Orissa. This study has been conducted to determine the role of *An. subpictus* and *An. culicifacies* in malaria transmission in Angul District, Orissa, India.

MATERIALS AND METHODS

Study area

Orissa is the third most malaria endemic state in India. It has four geographical regions: a northern plateau, a central tableland, a coastal belt, and an eastern step. Malaria is prevalent in all these geophysiographical regions of Orissa. The study was conducted in Angul District, Orissa, India, from March 2004 to February 2007. The study area lies between 20º 30' and 21º 30' latitude and between 84º 30' and 85º 15' longitude and is highly endemic for malaria.

Mosquito collection

The study villages were visited once a month for entomological collections. Indoor resting mosquitoes were collected from human dwellings in highly malaria endemic villages of Angul District between 6:00 AM and 9:00 AM using a sucking tube and mechanical aspirator. Species identification was done using taxonomic keys of Christophers (1933). Anophelines were collected and per man hour density (PMHD) was determined per the formula given below:

PMHD = Number of mosquitoes collected (particular species) / (number of persons involved in collection) x (actual time spent in collection)

The blood meals were collected on no.1 Whatman filter paper then a gel-diffusion method was carried out followed by a precipitin test (Collins *et al*, 1983; Parida *et al*, 2006). Each blood meal was tested against human, cow, bird, dog, pig, goat, rat and snake antisera. Adult unfed specimens and the heads and thoraxes of fully fed malaria specimens of *An.subpictus* and *An.culicifacies* were kept in individual vials for the detection of sporozoites by nested PCR technique. Malaria data were collected from primary health centers (PHCs) and slide positivity rates (SPR) were calculated which can be defined as the number of slides found positive out of one hundred slides examined.

DNA template preparation

Total DNA from individual mosquitoes was extracted following a modified method proposed by Collins *et al* (1987). Briefly, the mosquitoes were homogenized in 100 µl of extraction buffer (pH 9.1) containing 0.1M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl, 0.05 M EDTA, then phenol chloroform isoamyl extraction was carried out. Finally, the DNA was precipitated using ethanol and dissolved in 50 µl of deionized water.

Nested polymerase chain reaction for the detection of sporozoites

Amplification of genus and species specific *Plasmodium* was done using the primers: rPLU5 (5'CCTGTTGTTGCCTTAAACTT C3'), PLU6 (5'TTAAAATTGTTGCAGTT AAAACG3'), rFAL1 (5'TTAAACTGGTTTGG GAAAACCAAATATATT3'), rFAL2 (5'ACA CAATGAACTCAATCATGACTACCCGTC3'), rVIV1 (5'CGCTTCTAGCTTAATCCACATA ACTGATAC3') and rVIV2 (5'ACTTCCAA GCCGAAGCAAAGAAAGTCCTTA-3') as described by Snounou *et al* (1993a,b) and

Mahapatra *et al* (2006). Each 20 µl reaction mixture for nest1 amplifications contained 12 µl of template DNA, 250 nM of each primer (rPLU5 and rPLU6), 4 mM $MgCl_2$, PCR buffer (50 mM KCl, 10mMTris-HCl), 200 mM of each dNTPs and 0.4 units of Taq DNA polymerase. The PCR conditions (nest1) were as follows: 94ºC for 4 minutes; 94ºC for 30 seconds, 55ºC for one minute; extension at 72ºC for one minute; 35 cycles and final extension at 72ºC for 4 minutes. About 8 µl of the nest 1-amplification products served as the DNA template for each 20 µl of second PCR (nest 2) amplifications. The concentration of the nest 2 primers and other constituents were identical to the nest 1 amplification, except that 0.3 units 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 58ºC for the species-specific primer. The PCR products were analyzed after electrophoresed in 1.5% agarose gel and stained with ethidium bromide.

Statistical analysis

Pearson's correlation coefficient was applied to see the correlations if any between PMHD of *An. subpictus* and *An. culicifacies* with the SPR during different seasons of the study period.

RESULTS

A total of 7,700 anopheline specimens comprised of 8 species were caught during the study period. They were *An. subpictus* (29.0%), *An. vagus* Donitz, 1902 (19.7%), *An. nigerrimus* Giles (4.9%), *An. culicifacies* (11.6%), *An. annularis* Van der Wulp, 1884 (9.92%), *An. varuna* Iyengar, 1924 (11.6%) *and An. fluviatilis* James, 1902 (8.4%) of the total number collected. *An. subpictus* was the predominant species among the anophelines. Other known malaria vectors were comparatively fewer in number than *An. culicifacies*. The seasonal prevalences of *An. subpictus* and *An. culicifacies* are depicted in Fig 1. The density of *An. subpictus* was higher than *An. culicifacies* in all three seasons, however, the difference was significant only during summer. These findings led us to screen *An. subpictus* for the presence of malaria sporozoites.

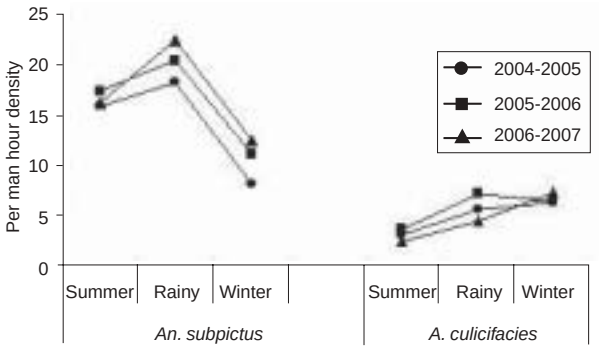

Fig 1–Seasonal prevalences of *An. subpictus* and *An. culicifacies* from March 2004 to February 2007.

Fig 2–Detection of sporozoites in *An. subpictus* by nested PCR. Lane M, 100bp ladder; lanes 1-4, test sample; lane 5, *P. vivax* positive sample; lane 6, negative control. Lane 2, *P. vivax* sample positive for *An. subpictus* showing an amplification product of 120 bp.

Table 1
Malaria sporozoites in *An. subpictus* and *An. culicifacies* by nested PCR method.

Species	No. tested	No. of positive samples	Parasite species tested		Sporozoite rate (%)	Sporozoite positive in season
			Pf [a]	Pv [b]		
An. subpictus	1,158	6	0	6	0.5	Summer
An. culicifacies	716	13	10	3	1.8	Rainy and Winter

Pf [a], *Plasmodium falciparum*
Pv [b], *Plasmodium vivax*

Table 2
Seasonal anthropophilic index of *An. subpictus* and *An. culicifacies*.

Season	Species	No. of blood meals studied	No. of blood meals positive in humans	Anthropophilic index (%)
Summer	*An. subpictus*	56	20	35.7
	An. culicifacies	57	12	21.1
Rainy	*An. subpictus*	58	17	29.3
	An. culicifacies	56	15	26.8
Winter	*An. subpictus*	54	15	27.8
	An. culicifacies	57	16	26.1

Six of 1,158 *An. subpictus* specimens were found positive for *Plasmodium vivax* sporozoites by nested PCR and showed a sporozoite rate of 0.5%. The sporozoite positive specimens for *An. subpictus* were found during the summer only (Table 1, Fig 2). With *An. culicifacies*, 13 of 716 specimens were positive for malaria sporozoites (10 for *P. falciparum* and 3 for *P. vivax*) and showed sporozoite rate of 1.8%. Malaria sporozoites positive specimens of *An. culicifacies* were found during rainy and winter seasons (Table 1).

Blood meals of *An. subpictus* and *An. culicifacies* were tested against human, bovine, caprine and avian serum by precipitin test. None of the blood meals were found positive for bovine, caprine, or avian serum. The anthropophilic indices were 31.0 % and 25.3% for *An. subpictus* and *An. cuilicifacies*, respectively. The seasonal anthropophilic indicies for *An. subpictus* and *An. culicifacies* are presented in Table 2. Anthropophilic index for *An. subpictus* was comparatively higher than *An. culicifacies* for the three seasons, but was highest during the summer (35.7%).

The seasonal prevalences of SPR are shown in Fig 3. Though the SPR were high throughout the study period, it was significantly higher during the summer. There was a positive correlation between the PMHD for *An. subpictus* and SPR during the summer ($r = 0.846$, $p < 0.001$) and for *An. culicifacies* during the rainy ($r = 0.789$, $p < 0.001$) and winter seasons ($r = 0.833$, $p < 0.001$).

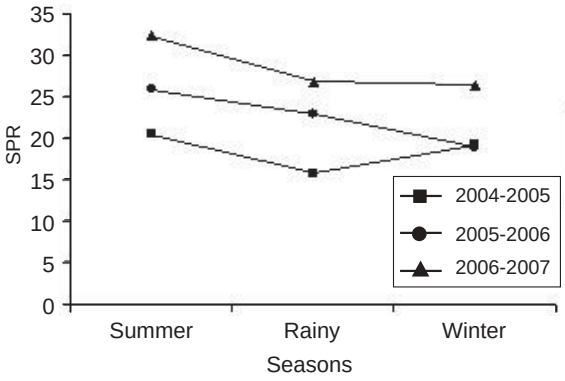

Fig 3–Slide positivity rate (SPR) during the study period.

DISCUSSION

The results of the present study clearly showed the predominance of *An.subpictus* (29.0% among anophelines. *An. culicifacies* comprised 11.6% of the total species collected. *An. subpictus* was the most abundant species in the study (Paniker *et al*, 1981; Kulkarni *et al*, 1987; Premasiri *et al*, 2005). In a study carried out by Singh *et al* (2003) in central India, *An. culicifacies* was the predominant species, accounting for more than 75% of the total mosquitoes, followed by *An. subpictus* (11.4%). In the present study, both *An. subpictus* and *An. culicifacies* showed the highest density during the rainy season (July to October). In Madhya Pradesh, Kulkarni *et al* (1987) found both species to be prevalent from July to September. Feeding habits and seasonal prevalence of *An. culicifacies* vary greatly in different parts of India, but *An. subpictus* is predominant during the irrigation season (Rao, 1984).

Malaria sporozoite detection by nested PCR showed sporozoite rates of 0.5% and 1.8% in *An. subpictus* and *An. culicifacies*, respectively. In Sri Lanka, Yapabandra and Curtis (2004) found malaria sporozoite rates in *An. subpictus* and *An. culicifacies* of 3.8% and 1.9%, respectively. *An. subpictus* was established as a primary vector of malaria for the first time in West Bengal with a sporozoite rate (*P. vivax*) of 0.3% (Chatterjee and Chandra, 2000). In the present study, *An.subpictus* samples positive for malaria sporozoite were found during the summer and *An. culicifacies* samples positive for malaria sporozoites were found during rainy and winter seasons. Kulkarni (1987) observed malaria sporozoites in *An. subpictus* in February and July and in *An. culicifacies* in March and September. Paniker *et al* (1984) found malaria sporozoites in the gut and salivary glands of *An. subpictus* during the months where more malaria cases occurred in Pudukuppam village, Pondicherry.

An. subpictus was found to be the predominant mosquito species biting man (Paniker and Rao, 1984). The same was also found in the present study with an anthropophilic index of 31.0%. Kulkarni (1987) reported an anthropophilic index for *An. subpictus* of 35.6% in Madhya Pradesh. An anthropophilic index of 41.0% was found for *An.subpictus* in Tarakeswar, West Bengal (Chatterjee and Chandra, 2000). It has also been reported to be highly anthropophilic in Western Orissa (Collins *et al*, 1991). The anthropophilic index for *An. culicifacies* was found to be 25.3% in this study and 22.7% and 29.4% in 1991-1991 and 2000-2001 respectively, in Gujarat (Bhatt *et al*, 2008).

A high anthropophilic index (35.7%) and presence of malaria sporozoites were observed during the summer season in *An. subpictus. An. culicifacies* was found to have an anthropophilic index of 21.1% during the summer. During the same period the SPR was also reported to be very high in the study areas (Fig 3). The density of *An. subpictus* was higher than *An. culicifacies* during the summer. Hence, the higher SPR during the summer in the study area is probably due to *An. subpictus* since other vector species, such as *An.culicifacies*, were low in number, though *An. culicifacies* contributes

to malaria transmission during the rainy and winter seasons along with other vector species. A positive correlation between PMHD and the SPR during summer was found for *An. subpictus* ($p<0.001$) and for the rainy ($p<0.001$) and winter seasons ($p<0.001$) for *An. culicifacies* were found in the present study.

In the present study *An. subpictus* was incriminated as a vector for malaria for the first time in Orissa. Due to ecological changes some species of mosquitoes though previously reported as a non-vectors are changing their vectorial capacity and becoming vectors thus contributing to the high malaria incidence. Thus, prevention planning must take into consideration the local vectors. Further study of *An. subpictus* species complex and their prevalence by barcoding method is being carried out, which should give greater insight into *An. subpictus* biology.

ACKNOWLEDGEMENTS

The authors are thankful to the Indian Council of Medical Research, New Delhi and the Director of the Regional Medical Research Centre, Bhubaneswar for his constant encouragement and valuable suggestions during the study, as well as for providing the necessary facilities. We also thank the staff members of the Division of Medical Entomology, Regional Medical Research Centre, Mr GD Mansingh, Mr B Pradhan, Mr CS Tripathy, Mr SS Beuria and Mr G Simachallm for their cooperation.

REFERENCES

Bhatt RM, Srivastava HC, Rajnikant, Yadav RS. Dynamics of *An.culicifacies* transmitted malaria in absence of effective zooprophylaxis in a riverine settlement in Gujarat. *Curr Sci* 2008; 95: 82-8.

Chatterjee S, Chandra G. Role of *Anopheles subpictus* as primary vector of malaria in an area in India. *Jpn J Trop Med Hyg* 2000; 28: 177-81.

Christophers SR. The fauna of British India –including Ceylon and Burma, Diptera. Vol.4 Family Culicidae, Tribe Anopheline. Landon: Taylor and Francis, 1933: 271.

Collins RT, Das MK, Agarawala RS, Dhal WB. An adaptation of the gel diffusion technique for identifying the source of mosquito blood meals. *World Health Organ Vector Biol Control* 1983; 83: 1-10.

Collins FH, Mendez MA, Rasmussen PC, Mehaffey PC, Besansky NJ, Finnetry V. A ribosomal RNA gene probe differentiates member of species of *Anopheles gambiae* complex. *Am J Trop Med Hyg* 1987; 37: 37-41.

Collins RT, Narasimham MVVL, Dhal KB, Mukherjee BP. Gel diffusion analysis of Anopheles blood meals from 12 malarious study villages of Orissa state, India. *J Am Mosq Control Assoc* 1991; 7: 595-603.

Kulkarni SM. Detection of sporozoite in *An.subpictus* in Bastar District, Madhya Pradesh. *Indian J Malariol* 1983; 20:159-60.

Kulkarni SM. Feeding behavior of Anopheline mosquitoes in area endemic for malaria in Bastar District, Madhya Pradesh. *Indian J Malariol* 1987; 24:163-71.

Mahapatra N, Marai NS, Ranjit MR, Hazra RK. Detection of *Plasmodium falciparum* in Anopheles mosquitoes from Angul district; Orissa, India .*J Vector Borne Dis* 2006; 43: 191-4.

Nagpal BN, Sharma VP. Incrimination of *Anopheles culicifacies* as vector of malaria in Orissa. *Indian J Malariol* 1986; 23: 57-9.

Paniker KN, Geetha Bai M, Bheema Rao US, Viswam K, Suryanarayana Murthy U. *Anopheles subpictus* vector of malaria in coastal villages of South East India. *Curr Sci* 1981; 50: 694.

Paniker KN, Rao USB, Rajagopalan PK. Persistent malaria transmission in Pudukuppam a coastal villages of Pondicherry. *Indian J Med Res* 1984; 80: 51-6.

Parida SK, Hazra RK, Marai N, Tripathy HK, Mahapatra N. Host feeding patterns of malaria vectors of Orissa, India. *J Am Mosq Control Assoc* 2006; *22*: 629-34.

Premasiri DA, Wickremasinghe AR, Premasiri DS, Karunaweera N. Malarial vectors in an irrigated rice cultivation area in southern Sri Lanka. *Trans R Soc Trop Med Hyg* 2005; 99: 106-14.

Rao TR. The Anophelines of India. New Delhi: Malaria Research Centre. *Indian Counc Med Res* 1984: xvi + 518.

Singh N, Mishra AK, Shukla MM, Chand SK. Forest malaria in Chhindwara, Madhya Pradesh, Central India. A case study in a tribal community. *Am J Trop Med Hyg* 2003; 68: 602-7.

Snounou G, Pinheiro L, Goncalves A, Fonseca L, Dias F, Brown KN. The importance of sensitive detection of malaria parasites in the human and insect hosts in epidemiological studies, as shown by the analysis of field Sample from Guinea Bissau. *Trans R Soc Trop Med Hyg* 1993a; 87: 649-53.

Snounou G, Vriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Molec Biochem Parasitol* 1993b; 58: 283-92.

Subbarao SK. The *Anopheles culicifacies* complex and control of malaria. *Parasitol Today* 1988; 4: 72-5.

Surendran SN, Ramasamy MS. *Anopheles culicifacies* Y chromosome dimorphism indicates sibling species B & E with different malaria vector potential in Sri Lanka. *Med Vet Entmol* 2000; 14: 437-40.

Tyagi BK, Yadav SP. Bionomics of malaria vectors in two physiographically different areas of the epidemic-prone Thar Desert, north-western Rajasthan (India). *J Arid Environ* 2001;47: 161-72.

Yapabandara AMGM, Curtis CF. Vectors and malaria transmission in a gem mining area in Sri Lanka. *J Vector Ecol* 2004; 264-76.