

COMPARATIVE SUSCEPTIBILITY OF THREE IMPORTANT MALARIA VECTORS *ANOPHELES STEPHENSI*, *ANOPHELES FLUVIATILIS*, AND *ANOPHELES SUNDAICUS* TO *PLASMODIUM VIVAX*

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ABSTRACT: The 3 laboratory-colonized malaria vectors, i.e., *Anopheles stephensi*, *An. sundaicus*, and *An. fluviatilis*, were studied for their comparative susceptibility to *Plasmodium vivax* sporogony. There was no significant difference in oocyst and sporozoite recruitment by these 3 species, whereas the geometric mean (GM) of the oocyst number per midgut was significantly lower in *An. fluviatilis* as compared with that in the other 2 species. There was no difference in the GM of oocyst between *An. stephensi* and *An. sundaicus*. Adaptability to laboratory conditions and susceptibility to plasmodial infection suggest that *An. fluviatilis* and *An. sundaicus* can also be used as a vector model for vector–parasite interaction studies.

Among 57 *Anopheles* species reported from India, only 6, i.e., *An. culicifacies*, *An. stephensi*, *An. fluviatilis*, *An. minimus*, *An. dirus*, and *An. sundaicus*, are epidemiologically important (Rao, 1984; Sharma, 1998). Of these, *An. stephensi* is a well-established vector of malaria in urban and industrial areas, whereas *An. fluviatilis* exhibits its influence in forested areas in central India and foothills of northeastern India; *An. sundaicus* is the only recognized vector for malaria transmission in the Andaman and Nicobar group of islands (Sharma, 1998). *Anopheles stephensi* comprises 2 races, i.e., type form and var. *mysorensis* and contributes 12% of total malaria cases in India. *Anopheles fluviatilis* sensu lato is an efficient vector of malaria and transmits 15% of malaria cases in the country (Sharma, 1998). It has been recognized as a complex of 3 sibling species, designated as ‘S’, ‘T’, and ‘U’ (Subbarao et al., 1994). In *An. sundaicus*, 3 cytological forms, A, B, and C, have been reported from Thailand and Indonesia (Sukowati and Baimai, 1996), which were subsequently recognized as 3 distinct species (Sukowati et al., 1999). Recently, 1 more cytological form, cytotype D, has been reported from India (Nanda et al., 2004). We have assessed the susceptibility of laboratory-reared *An. stephensi* type form, *An. fluviatilis* species T, and *An. sundaicus* cytotype D against *Plasmodium vivax*, the most predominant malaria species in India. The results of these comparative susceptibility studies are presented in this study.

MATERIALS AND METHODS

Anopheles stephensi type form and *An. fluviatilis* species T have been maintained as cyclic colonies in the insectary at the Malaria Research Centre, Delhi, India, since 1977 and 1992, respectively, whereas the *An. sundaicus* cytotype D colony was established in 2002.

The cyclic colonies of these 3 vector species were established initially by pooling progeny from at least 60–70 isofemale cultures originated from wild-caught females. The mean temperature and relative humidity of the insectary were 28 ± 1 C and $70 \pm 5\%$, respectively, with photoperiod of 14 hr light and 10 hr dark. Adult mosquitoes were held in $30 \times 30 \times 30$ cm cloth cages and were offered water-soaked raisins and 1% glucose-soaked cotton pads as a source of nutrient. Female mosquitoes were fed on rabbits before oviposition.

Thick and thin blood smears from patients seeking treatment at the malaria clinic of Malaria Research Centre, Delhi, were prepared by finger prick, stained in JSB stains (Singh, 1956), and examined under

$\times 100$ oil immersion lens for the presence of malaria parasites. Adult volunteers (ages ≥ 16 yr) having mature *P. vivax* gametocytes (density ranging 0.05–0.5%) were selected for experimental laboratory feeding following a human use protocol approved by the Scientific Advisory Committee and Human Ethical Committee of the Centre. About 2 ml of blood was drawn by venipuncture from consenting volunteers and placed immediately into plastic vials coated with heparin to prevent clotting. All *P. vivax*-positive patients were treated with 600 mg chloroquine once on day ‘0’ and 15 mg of primaquine for 5 consecutive days (adult dose); all *P. falciparum*-positive cases were treated with 1,500 mg chloroquine in divided doses during 3 days, followed by single dose of 45 mg primaquine on day 4. Infants and children were given proportionate doses, following the drug policy of National Anti-Malaria Programme (NAMP), Government of India.

The mosquitoes were fed on *P. vivax* blood isolates through membrane as described by Adak et al. (1999). A total of 54 *P. vivax* isolates were used for different paired-feeding experiments as shown in Table I. In each feeding experiment, cohorts of 75–100 mosquitoes under study were fed on *P. vivax* blood isolates. *Anopheles stephensi* were fed on all the blood isolates. In all feeding experiments, 4- to 6-day-old mosquitoes from each cyclic colony were starved overnight before being used. After feeding, the unfed and partially fed mosquitoes were removed; only fully fed mosquitoes were kept in the insectary for subsequent examination of sporogonic development.

To evaluate the degree of infection, 5–6 mosquito midguts were dissected in normal saline (0.65% NaCl) on day 5 after infective blood meal and stained in a drop of 0.5% mercurochrome (Eyles, 1950). Subsequently, midguts were placed under a coverglass and examined for the presence of oocysts using phase contrast microscopy under a $\times 10$ objective. To assess sporozoites development, salivary glands were dissected in normal saline on day 9 after feeding and examined using phase contrast microscopy with a $\times 40$ objective.

Susceptibility of 3 mosquito species was compared using 3 parameters, i.e., percent gut positivity with oocysts, the mean number of oocysts (geometric mean [GM] among the infected mosquitoes) and percent gland positivity with sporozoites. Differences in gut and gland positivity rates with respect to oocyst and sporozoite, respectively, were compared using a chi-square test. The degree of oocyst load was estimated by calculating the GM number of oocysts. To test the differences between the GM number of oocysts, Student’s *t*-tests were performed using log values of oocyst number of individual mosquito.

Pinned voucher specimens of *An. stephensi* type form, *An. fluviatilis* species T, and *An. sundaicus* cytotype D from the cyclic colonies are available at the Malaria Research Centre, Delhi, India.

RESULTS

The results of comparative susceptibility of *An. fluviatilis*, *An. sundaicus*, and *An. stephensi* are shown in Table I. In paired-feeding experiments there was no significant difference in gut and gland positivity among the 3 species. However, GM number of oocyst was significantly lower in *An. fluviatilis* as compared with *An. stephensi* ($P < 0.001$) and *An. sundaicus* (P

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TABLE I. Comparative susceptibility of *Anopheles stephensi*, *An. fluviatilis*, and *An. sudaicus* to *Plasmodium vivax*.

Species compared	Number of <i>P. vivax</i> isolates*	Gut dissected/positive for oocysts (%)	<i>P</i> value (chi-square)	GM oocyst (fiducial limits)	<i>P</i> value (<i>t</i> -test)	Gland dissected/positive for sporozoites (%)	<i>P</i> value (chi-square)
Paired feedings							
<i>An. fluviatilis</i> vs. <i>An. stephensi</i>	34	181/139 (76.80)	NS	9.66 (7.68–12.16)	<0.0001	112/78 (69.64)	NS
<i>An. sudaicus</i> vs. <i>An. stephensi</i>	31	204/146 (71.57)	NS	23.25 (18.77–28.25)	NS	175/119 (68.00)	NS
<i>An. fluviatilis</i> vs. <i>An. sudaicus</i>	11	157/111 (70.70)	NS	33.30 (25.35–43.74)	<0.05	88/57 (64.77)	NS
<i>An. stephensi</i> vs. <i>An. fluviatilis</i>		162/124 (76.54)		26.59 (20.58–34.36)		152/111 (73.03)	
<i>An. sudaicus</i> vs. <i>An. sudaicus</i>		62/51 (82.25)	NS	13.57 (9.33–19.75)		33/23 (69.69)	NS
<i>An. sudaicus</i> vs. <i>An. sudaicus</i>		60/44 (73.33)		26.36 (17.79–39.06)		29/20 (68.96)	
Pooled Data							
<i>An. fluviatilis</i> vs. <i>An. sudaicus</i>	34	181/139 (76.80)	NS	9.66 (7.68–12.16)	<0.0001	112/78 (69.64)	NS
<i>An. stephensi</i> vs. <i>An. sudaicus</i>	31	157/111 (70.70)	NS	33.30 (25.35–43.74)	NS	88/57 (64.77)	NS
<i>An. fluviatilis</i> vs. <i>An. stephensi</i>	54	317/228 (71.92)	NS	24.69 (20.58–29.61)	<0.0001	262/188 (71.76)	NS
<i>An. sudaicus</i> vs. <i>An. stephensi</i>			NS		NS		NS
<i>An. fluviatilis</i> vs. <i>An. sudaicus</i>			NS		<0.0001		NS

* Eleven isolates were common in all the paired feedings.

GM indicates geometric mean; NS, not significant.

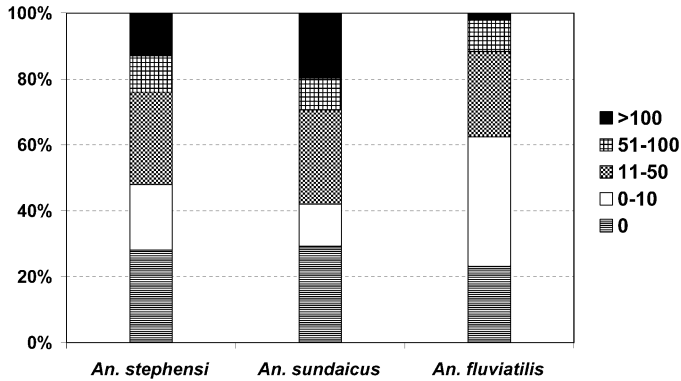


FIGURE 1. Proportion of mosquitoes with different oocyst densities in *Anopheles stephensi*, *Anopheles sundaicus*, and *Anopheles fluviatilis* infected with *Plasmodium vivax*.

< 0.05), whereas, it did not differ between *An. stephensi* and *An. sundaicus*.

The overall infectivity of *P. vivax* isolates to *An. stephensi* that have been fed along with *An. fluviatilis* (34 isolates) and that with *An. sundaicus* (31 isolates) did not differ statistically in term of percent gut–gland positivity and GM number of oocyst, indicating that overall there was no significant difference in average infectivity of *P. vivax* isolates used with both the groups. Therefore, pooled data of both the pair feedings were analyzed for comparative susceptibility of all the 3 species. The analyses have been shown in Table I and described in following sections.

Anopheles stephensi fed more readily in the membrane feeder as compared with *An. fluviatilis* and *An. sundaicus*. Maximum feeding (85.5%) was observed in *An. stephensi* as compared with 52.5% in *An. fluviatilis* and 45.7% in *An. sundaicus*. Among all fed mosquitoes, the overall proportion of mosquitoes with 0, 1–10, 11–50, 51–100, and >100 oocysts for each species and their distribution patterns are shown in Figure 1.

Among all fed mosquitoes, the proportion of mosquitoes that did not support sporogony as evidenced by the absence of oocyst in the midgut on day 6 were more or less similar in all 3 species ($P > 0.05$). More than 70% of the mosquitoes from all 3 species were infected with at least 1 oocyst. More than 20% of *An. sundaicus* had >100 oocysts in the midgut, as compared with approximately 10% in *An. stephensi* and less than 3% in *An. fluviatilis*. Gut infectivity rate and range of oocyst number per gut varied widely in different batches of mosquitoes. As many as 350 oocysts were observed in *An. stephensi*. Among the oocyst-positive mosquitoes, the median number of oocysts was 26, 31, and 32 in *An. fluviatilis*, *An. stephensi*, and *An. sundaicus*, respectively. The gland positivity rate observed in the 3 species ranged between 64.8 and 71.8%. The mean numbers of oocysts were significantly lower in *An. fluviatilis* as compared with *An. sundaicus* and *An. stephensi* ($P < 0.0001$). However, the mean number of oocysts did not differ significantly between *An. sundaicus* and *An. stephensi*. Overall analysis of infectivity of *P. vivax* with reference to gut and gland positivity rates for the 3 species did not reveal any significant difference ($P > 0.05$).

DISCUSSION

In the present study, the relative susceptibility of 3 important malaria vectors of India was determined for *P. vivax*, the predominant malaria species. All the 3 mosquito species were found to be highly susceptible to *P. vivax* infection as evidenced by high gut and gland positivity rates.

Natural variability in malaria susceptibility is well known among mosquito species, depending on the parasite species and strains. Variability also exists between individuals and strains within a vector population, as reported for *An. gambiae*, Giles (Burgess, 1960), *An. maculipennis* Meigen (Ramsdale and Coluzzi, 1975), *An. albimanus* (Collins et al., 1976), and *An. culicifacies* (Adak et al., 1999; Kaur et al., 2000). Curtis and Graves (1983) have also demonstrated a genetic basis for this host–parasite interaction. Natural variability in malaria susceptibility because of differential genetic factors of mosquitoes is not yet clearly understood. In this study, *An. fluviatilis* species T, which was identified as a poor vector species in the field (Sharma et al., 1995; Shukla et al., 1998), was found to be highly susceptible to *P. vivax* infection in the laboratory. It could be ranked at the same level as the 2 other efficient vectors of malaria, *An. stephensi* and *An. sundaicus*, in terms of gut and gland positivity rates. Although the GM number of oocyst in *An. fluviatilis* is significantly lower as compared with the other 2 species, this factor is of little importance in malaria epidemiology because in nature most infected mosquitoes possess just a few oocysts. The high zoophagic nature (zoophagic index >99%) is probably the main factor that determines this species as a poor vector or nonvector (Nanda et al., 1996). It will be important to learn if any behavioral or other biological factors, i.e., its life span, are responsible for its poor vector potential in the field. Because both *An. fluviatilis* and *An. sundaicus* are readily adaptable to laboratory conditions and are highly susceptible to *P. vivax* infection, they could both be used as additional vector models in the conduct of various host–parasite interaction studies.

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