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Antibodies to *Anopheles culicifacies* salivary glands encumber vector competence to *Plasmodium vivax*

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Anopheles culicifacies A and C are responsible for 65-70% of malaria cases in India. Species B is least susceptible to parasite and plays very little role, if at all, in the malaria transmission. Three sets of rabbits were immunized with the salivary gland proteins from these three members (A, B and C). High titer antibodies were detected in the sera when characterized by *in vitro* ELISA. Western blotting and in vivo ELISA were conducted to gauge the cross reactivity of these antibodies with other tissues as well as salivary glands from other sibling species. Salivary glands and midgut exhibited highest cross reactivity. 97 kDa polypeptide was recognized exclusively by antibodies against salivary glands of species-A and C (primary vectors). Similarly, two immunogenic polypeptides (29 and 21 kDa) were present only in species-B. Fecundity was reduced significantly (37%) and number of oocysts per infected mosquito was reduced by 69% in the group of mosquitoes that ingested anti-salivary gland antibodies along with infected blood meal. Proportion of infected mosquitoes was significantly low as compared to control.

Key words : *An. culicifacies*, Salivary glands, *P. vivax*.

INTRODUCTION

Mosquitoes are unquestionably the most medically important arthropod vectors of diseases. Mosquito-borne diseases like malaria are responsible for significant human morbidity and mortality through out the world. Mosquito control through environmental perturbation and pesticide application, used to be primary strategy for controlling mosquito-borne diseases, but environmental and human health concerns, as well as development of pesticide resistance, limit the usefulness of these traditional approaches in our modern day world. Genetic plasticity of pathogen and drug resistant strains of parasites has significantly contributed to the present situation. No promising strategies have been designed on the horizon for the control of malaria. Most anti-malarial strategies have primarily targeted the infection in humans, whereas we know plasmodium development in mosquitoes is more elaborate than in the vertebrate host (Shahabuddin and Costero, 2001). Development of malaria vaccines that block transmission of parasites by mosquito vectors remains one such pragmatic approach that can complement or replace existing control methods.

Transmission blocking vaccines have long been the subject of intensive research, and although they have yet to be realized clinically, they are at least now becoming a technical possibility. Various vector incrimination studies have unequivocally established that *An. culicifacies* contributes to about 70% of malaria cases in India, but is still ignored in this context.

An. culicifacies exists as a complex of five sibling species provisionally designated as A, B (Green and Miles, 1980), C (Subbarao *et al*., 1983), D (Vasantha *et al*., 1991) and E (Kar *et al*., 1999). These species are reported to have various biological differences *viz.* their distribution, response to insecticides (Raghvendra *et al*., 1992) host preferences (Joshi *et al*., 1988) and vectorial capacity (Subbarao *et al*., 1988). Some of the mosquitoes are immunologically superior to others. *An. culicifacies* A and C are primary vectors whereas, species B has very little role if at all, in the transmission of malaria (Kaur *et al*., 2000).

Salivary glands and midgut are the two different epithelial barrier that the parasite has to overcome for successful completion of sporogony. Since long the role of mosquito salivary gland for the expression of the genes specifically connected to the blood feeding has been recognized but in addition to that, the mosquito salivary glands are also the final destinations of the insect inhabiting stages of many parasites before transmission to a new vertebrate host, playing a critical role in parasite transmission. It itself indicates prime importance of this organ to contribute towards the development of parasite and antibodies directed towards it, might adversely effect parasite transmission and also reproductive performance

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of mosquitoes.

Earlier studies have indicated that vector internal organs (concealed antigens) can induce an artificial immune response in various species *viz*., *An. stephensi* (Alger and Cobrea, 1972; Almeida and Billingsley, 1998; Suneja *et al*., 2003). Barreau *et al*. (1995) demonstrated that polyclonal antibodies raised against *Aedes aegypti* salivary gland proteins block penetration by *P. gallinaceum* sporozoites. Gulia and Gakhar (2004) also described that anti-mosquito salivary glands antibodies hamper the reproductive performance of the mosquitoes and synonymously inhibit parasite development in the mosquito midgut.

Presence of some common antigenic peptides has already been indicated in different species of mosquitoes and also various tissues (*viz*. salivary gland, midgut and haemolymph) of the same mosquito species (Gulia and Gakhar, 2004; Dinglasan *et al*., 2003; Gulia *et al*., 2002). However, no such study appears to be documented on different members of a sibling species complex of any mosquito.

The present study was designed to identify putative candidate antigens in the salivary glands of blood fed *An. culicifacies* sibling species complex that possibly determine the differential vectorial capacity in the members of sibling species complex.

Materials and Methods

Mosquito rearing, antigen preparation and immunization:

Cyclic colonies of *An. culicifacies* species A (Dhera Strain), B (Ladpur strain) and C (Raurkela strain) were maintained in an insectary at 28± 2°C and 70-80% RH and fitted with a simulated Dawn and dusk machine to maintain a photoperiod of 14 h light and 10 h dark. Salivary glands were removed from 150 females belonging to each sibling species of *An. culicifacies* as described by Blacklock and Wilson (1942). Immunizations were carried out according to Suneja *et al*. (2003). Approval for the animal studies was sought from the Animal Ethics Committee, M.D. University, Rohtak. Throughout the experiments, animals were processed following the internationally accepted ethical guidelines for the care of laboratory animals.

In vitro and in vivo ELISA:

Antibody titer was measured by two fold serial dilution of antisera by *in vitro* ELISA for salivary gland proteins from *An. culicifacies* sibling species complex as described by Suneja *et al*. (2003). The cross reactivity of salivary gland antibodies with other tissues (haemolymph, midgut, ovary and also salivary glands from other members of sibling species complex) from immunized and control female mosquitoes were also checked by *In-vivo* ELISA as described by Brennan *et al*. (2000). In case of *in vivo* ELISA, 96 well plates were coated with the salivary glands of mosquitoes already fed with immunized blood, however, antigens from freshly emerged mosquitoes were coated on 96-well plates and then treated with primary antibodies (antisera raised) for *in vitro* ELISA.

Immunoblotting:

Soluble proteins were separated by SDS-PAGE and were transferred electrophoretically to 0.45 nm nitro-cellulose sheet for western blotting (Suneja *et al*., 2003). The molecular weight of polypeptides was determined from standard markers (GENEI, India).

Immunized blood feeding, fecundity, mortality and longevity:

Egg-laying pattern was studied in *An. culicifacies* sibling species complex fed on immunized rabbits (immunized with salivary glands from mosquitoes belonging to three sibling species). Different sets of mosquitoes were allowed to feed on immunized rabbits for eight consecutive weeks after the last booster. Each set constituted of about 120 female mosquitoes. The procedure to evaluate the percentage reduction in fecundity, hatchability, mortality and longevity has been described earlier (Suneja *et al*., 2003).

Parasite invasion blocking assay:

For this assay, membrane feeding was used to examine the effect of anti-salivary gland antibodies on the development of the parasite in *An. culicifacies*. Here the antiserum from the sensitized rabbit (collected during the third week after the last booster) was mixed with the blood drawn from human hosts infected with the gametocytes of *P. vivax*. The gametocytes of *P. vivax* were obtained from patients visiting National Malaria Research Institute, Delhi. In each experiment immune serum containing 100µg of IgGs was mixed with infected sera containing *P. vivax* for membrane feeding of mosquitoes. 200 mosquitoes (5-day old) were membrane fed separately on immunized and control rabbit sera. Unfed or partially fed females were removed. After 6 days, midguts were dissected out to count the number of oocysts. Similarly, after 12 days, salivary glands were dissected out to observe the presence of sporozoites. Percentage transmission blocking was determined by the

method described earlier (Kar *et al.*, 1999). These experiments were conducted in triplicates.

$$\text{Percentage inhibition} = \frac{\text{Mean oocyst no. (control)} - \text{Mean oocyst no. (immunized)}}{\text{Mean oocysts no. in control}} \times 100$$

Statistical analysis:

Mann-Whitney U test (one-tailed) was applied by using the GRAPH-PAD Prism software, to determine the significance of fecundity reduction and difference in oocyst count in between experimental and control groups.

Results and Discussion

In vitro ELISA studies revealed that antibody titers in the rabbits of three sets (immunized with the antigens of the salivary glands from *An. culicifacies* species complex) reached at least 1: 2, 048, 000. The antibody titers reached their peaks during the third week after the last booster, then declined to the minimum level during the sixth week (Fig.1).

Anti-salivary glands antibodies were capable of binding to various tissues *viz.*, midgut, haemolymph and ovary and also to the salivary glands of other sibling species, revealed by *in vivo* ELISA (Table 1). However, the highest cross reactivity was observed between the salivary glands and midgut. These results exhibited that antibodies fed to the mosquitoes traverse through the midgut wall and reach their target tissues undigested. These finding are in complete accordance with the previous findings demonstrating that IgG antibodies are able to pass through midgut and reach mosquito's haemolymph, and subsequently to other target tissues (Vaughan Azad, 1988).

Table 1 : Quantitative estimation of cross reactivity in between salivary glands and various tissues from same species by In vivo ELISA

Sr. No.	Tissue	O.D. of Ag-Ab complex	
		Control	Immunized
1.	Salivary glands	0.186 ± 0.121	1.650 ± 0.194
2.	Haemolymph	0. 164± 0.148	0.712 ± 0.284
3.	Ovary	0.129 ± 0.142	0.175 ± 0.116
4.	Midgut	0.154 ± 0.176	1.046 ± 0.178

Immunogenic polypeptide pattern of An. culicifacies salivary glands:

Homologous antigens (peptides having common epitopes) were recognized in the salivary glands from different sibling species *e.g.* three immunogenic peptides (66, 39 and 29 kDa) were present in salivary glands of mosquitoes from all three sibling species. 97 kDa polypeptide is exclusively present in the salivary glands of *An. culicifacies* A, C mosquitoes (primary vectors). Therefore, it is speculated to be involved in mediating parasite-receptor interaction at the apical surface of midgut or basal surface of salivary gland's distal lobes. However, 36 kDa immunogenic polypeptide could be explored in the salivary glands of *An. culicifacies* A and

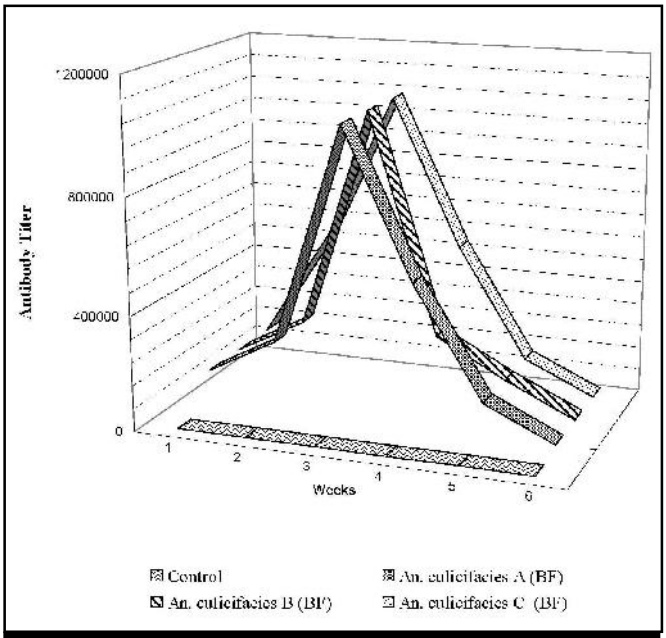

Fig. 1: Antibody titer of sera raised against salivary glands proteins of *An. culicifacies* sibling species complex

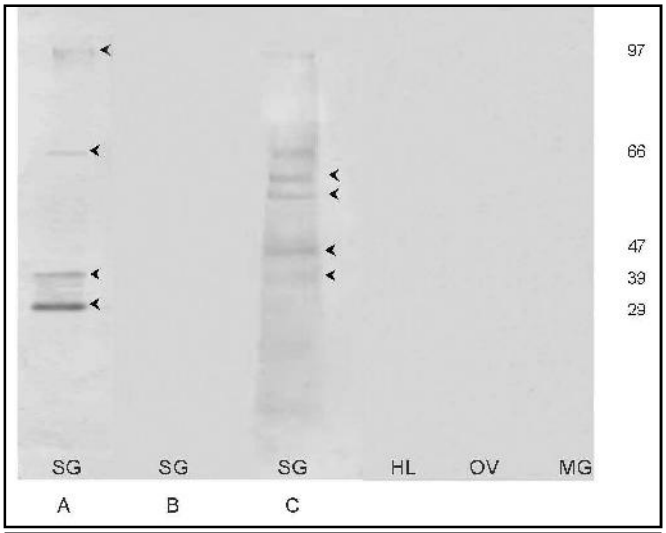

Fig. 2 : Tissue and species specific immunogenic polypeptides recognized by the anti-mosquito antibodies raised against the salivary glands of An. culicifacies A blood fed mosquitoes. SG-Salivary Glands; MG-Midgut; HL-Haemolymph; OV-Ovary

B mosquitoes.

Sibling species specific immunogens from An. culicifacies salivary glands:

Even some peptides specific for a single sibling species could be identified. Seven immunogenic polypeptides (90, 84, 78, 72, 58, 52, 47 kDa) could be resolved only in salivary glands of An. culicifacies C mosquitoes, these peptides can eventually serve as candidates for immunodiagnostics probes. Interestingly, two immunogenic polypeptides (27 and 21 kDa) were exclusively present in salivary glands

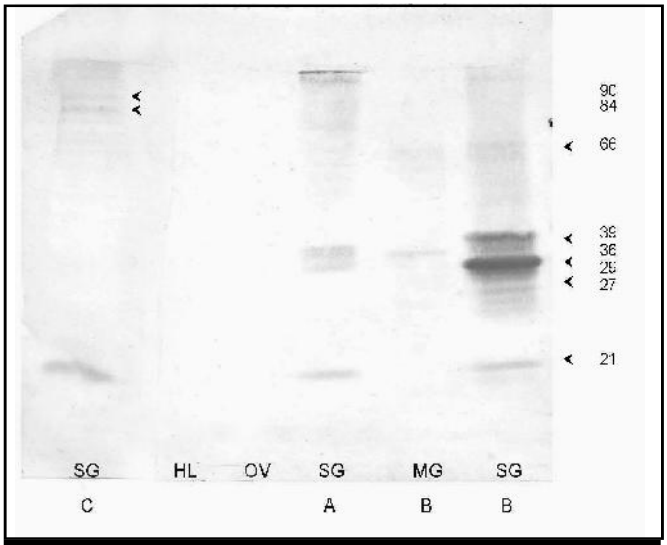

Fig. 3 : Tissue and species specific immunogenic polypeptides recognized by the anti-mosquito antibodies raised against the salivary glands of An. culicifacies B blood fed mosquitoes. SG-Salivary Glands; MG-Midgut; HL-Haemolymph; OV-Ovary

of *An. culicifacies* B (poor vector), therefore, probably account for its refractoriness.

Identification of common epitopes shared by different tissues and salivary glands from other sibling species:

In order to demonstrate cross reactivity, salivary glands antibodies were also treated with the antigens from the other tissues *viz*. midgut, haemolymph and ovary of same species vis-à-vis salivary glands of other sibling species. Polyclonal sera raised against salivary gland did not react with the antigens from other tissues except the midgut of same species. Antibodies raised against the salivary glands of *An. culicifacies* A mosquitoes reacted with 66 and 39 kDa polypeptides present in salivary glands of *An. culicifacies* A and C. Similarly salivary glands of *An. culicifacies* B mosquitoes recognized 36 and 29 kDa polypeptides present in salivary glands of *An. culicifacies* A and B. Polyclonal antibodies raised against salivary glands of An. culicifacies C exhibited maximum cross reactivity against *An. culicifacies* A and recognized seven immunogenic polypeptides (97, 90, 84, 78, 72, 66, 39, 29 kDa) present in the salivary glands of *An. culicifacies* A and C. Cross-reactivity may be attributed to the presence of some conserved epitopes in these epithelial tissues,

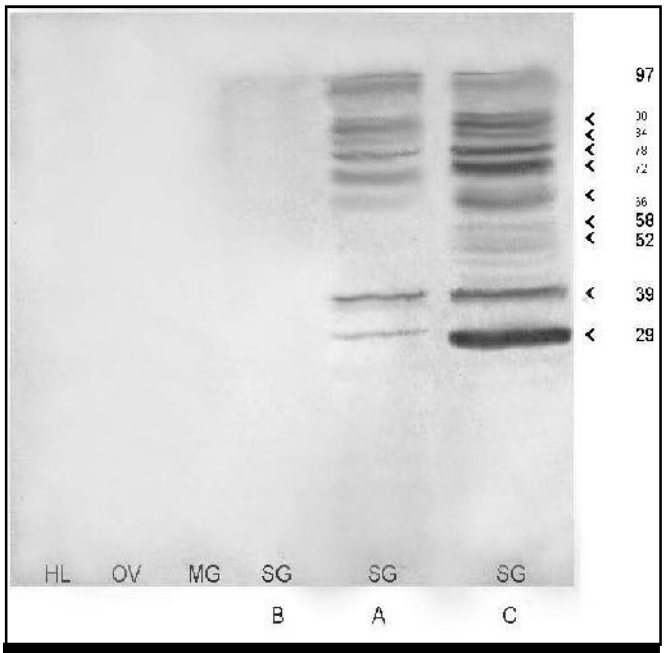

Fig. 4: Tissue and species specific immunogenic polypeptides recognized by the anti- mosquito antibodies raised against the salivary glands of An. culicifacies C blood fed mosquitoes. SG-Salivary Glands; MG-Midgut; HL-Haemolymph; OV-Ovary

and/or non-specific binding by low affinity antibodies.

Effect of anti-mosquito salivary glands antibodies on reproductive physiology of mosquitoes:

Significant reduction in fecundity was observed after ingesting anti-salivary gland antibodies as compared to the control. Maximum reduction in fecundity (37%, $P<0.01$**) was observed in the mosquitoes fed on rabbit immunized with salivary glands during the third week after the last booster (Fig. 5), when the antibody titer was highest.

The present investigations demonstrate that the ingestion of anti-salivary glands antibodies had no statistically significant effect on the mosquito mortality and longevity. Mosquitoes earlier fed on sensitized rabbits, when offered normal blood meal for the second time engorged lesser amount of blood as compared to the group of mosquitoes fed on normal blood on both the occasions, but the effects were found to be statistically insignificant.

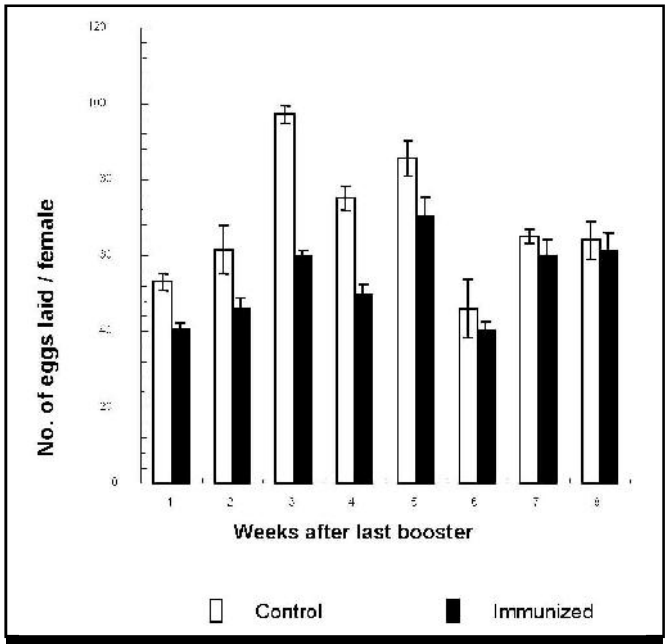

Fig. 5 : Effect of anti-salivary gland antibodies on fecundity of *An. culicifacies* mosquitoes

The marked differences in the fecundity of *An. culicifacies* mosquitoes fed on the blood of control rabbit, immunized rabbit suggests that humoral antibodies, somehow, interfere with the normal process of oogenesis. Reduced fecundity does not seem to be related to any decline in the rate of oviposition. A few female mosquitoes from each feeding set were dissected after they were fed on sensitized or control rabbit. All dissected mosquitoes seemed to have laid their full complement of eggs. The reduction in the number of eggs produced, therefore, may be attributed to specific anti-mosquito antibodies binding to target antigens and disrupting the normal physiology of mosquito. However, the mechanism of the anti-mosquito response is not yet known but could be one or combination of several factors *i.e.* fat body synthesis of vitellogenins may be down regulated, the uptake of circulatory vitellogenins may be inhibited or the content of some of the developing follicles may be reabsorbed. Alternatively, the presence of antibodies in ingested blood might act very quickly to irritate the gut, reducing the total blood intake and nutrients available. Moreover, it is yet to identify the change in vitellogenesis (Kay and Kemp, 1994).

Effect of anti-mosquito salivary glands antibodies on the parasite development:

Significant reduction in parasite infection was observed in *An. culicifacies* that ingested anti-salivary gland antibodies along with the *P. vivax* as compared to control (Table 2). The infection rate of *An. culicifacies* was reduced by 36 % after feeding with immunized sera. The mean number of oocysts per infected mosquito were drastically reduced by 69% ($p<0.0001$*) after they ingested polyclonal antibodies raised against salivary glands of blood fed mosquitoes along with parasitized blood. Antibodies were also able to interrupt translocation and/or development of sporozoites, as very few sporozoites appeared in the salivary glands of mosquitoes which ingested anti-salivary glands antibodies along with infected blood, however, innumerable sporozoites were visible in the salivary glands of mosquitoes fed with infected blood. For the anti-mosquito antibodies to affect the parasite, they must either inhibit a physiological process essential to the parasite or block the normal ookinetes-midgut interaction that may or may not be cell-specific (Vernick *et al*., 1999). The results are consistent with the previous studies (Brennan *et al*., 2000).

Jacobs-Lorena and his colleagues described a peptide SM1, which binds to both the apical surface of the midgut and the basal surface of the salivary gland's distal lobes, suggesting the presence of similar receptors on these organs. Transgenic mosquitoes expressing SM1, developed fewer oocysts, and concomitantly fewer sporozoites, compared to the control (Ito *et al*., 2002). Therefore, the reduction in the number of oocysts developed in the midgut might be due to two reasons: First, antibodies against salivary glands might have similar target sites in the midgut for the parasite to recognize and invade, therefore, might competitively inhibit the parasite. Second, these antibodies might adhere and lead to allosteric alteration in the receptor recognition site in the midgut and hence could deny ookinetes to recognize and adhere to the target site.

To conclude, the present study assertively implicated the salivary glands as the likely source for candidate antigens; however, the final formulation should be designed

Table 2 : Effect of antibodies raised against salivary gland of blood fed *An. culicifacies* mosquitoes on malaria parasite development

No. of mosquitoes fed/ group		Infected Mosquitoes (%)		Mean no. of oocysts /mosquito		Transmission blocking (%)
Control	Anti-SG	Control	Anti-SG	Control	Anti-SG	Anti-SG
90	72	58	22	120±5.126	38.50±2.048	69.4*

concocting multiple antigens from multiple tissues.

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