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Short Communication

Anti-Mosquito Ovary Antibodies Reduce the Fecundity of *Anopheles stephensi* (Diptera:Insecta)

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SUMMARY: Rabbit antibodies to five antigens (AJ** 29, 35, 43, 64, and 80 kDa) derived from the ovaries of *Anopheles stephensi* tended to reduce the number of eggs produced. Ingestion of anti-mosquito ovary antibodies did not show a detectable effect on the mortality of mosquitoes. Antisera raised against *An. stephensi* ovaries showed cross-reactivity in other tissues and in the ovaries of other *Anopheles* spp. by Western blotting. The results indicate that anti-mosquito ovary antibodies have the potential to disrupt the reproductive physiology of mosquitoes, and indicate the need for further studies with target antigens.

Mosquito antigens that are not normally introduced into a vertebrate host when a mosquito feeds (i.e., antigens of the midgut, ovary, etc.) can induce an artificial immune response (1-3). Specific IgG antibodies also cross the midgut epithelium of *Aedes aegypti* (4). Ingested anti-mosquito antibodies have been shown to increase mortality of *An. stephensi* (1) and *Ae. aegypti* (5) and also reduce fecundity in *Ae. aegypti* (2) and *Culex quinquefasciatus* (3). However, in most of these earlier studies (6) antigens were prepared either from the head/thorax or abdomens or by using the whole body. Efforts need to be directed towards the use of defined antigens from various tissues of mosquitoes. Therefore in the present study, it was demonstrated that mosquito ovary immunogens induce the production of anti-mosquito ovary antibodies that reduce fecundity and also the viability of progeny of *An. stephensi* obtained from the blood meal of immunized rabbit.

The culture of *An. stephensi* (Delhi strain) was maintained in our insectary at 28 ± 2°C and 70 - 80% relative humidity (RH) as described earlier (7). Specially prepared cages (3 feet by 3 by 3 high) made up of muslin cloth were used for the adult mosquitoes.

Fully engorged female *An. stephensi* were dissected out in normal saline to collect ovaries because the hemoglobin content of the blood provides the major protein source for egg development. Ovaries pooled from 50 females were homogenized in phosphate buffered saline (PBS) containing phenylmethylsulfonyl flouride (PMSF). The homogenate was centrifuged at l0,000 g for 15 min. The supernatant (0.5 ml, 150 μg proteins) was injected subcutaneously at multiple sites in the three rabbit groups after being emulsified with an equal volume of Freund's complete adjuvant (8). After a 2 week interval, a first booster of protein antigens was injected in the same manner with the exception of incomplete Freund's adjuvant. After 1 week, a second booster injection was administered as in the first booster dosage. It was given in both sides of the upper arm, with 0.5 ml at each site.

Antibody titers in rabbit serum were determined by enzyme-linked immunosorbent assay (ELISA) using immunizing antigens to coat the wells. Bound antigens were incubated with dilutions of rabbit sera, followed by peroxidase conjugated sheep anti-rabbit IgG (8). The immune complex was detected with 2,2′-azinobis, 3-ethyl benz-thiozoline-6-sulphonic acid, and H_2O_2.

Ovarian extracts from each species were prepared for polyacrylamide electrophoresis under reducing conditions (9). The soluble proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were either silver stained (10) or transferred electrically to 0.45 μm nitrocellulose sheets for Western blotting. Nitrocellulose sheets containing separated *An. stephensi* ovary proteins were blocked with 1% nonfat milk in PBS, and the sheets were incubated with pre-immune sera in PBS, washed, and then incubated in the following antisera. Following washing, antibody binding was visualized using a peroxidase substrate system (11).

Immunized rabbit boosted with ovary antigens was used for blood feeding up to 6-7 weeks after the last immunization. Six replicates were made each week to observe the egg-laying pattern in *An. stephensi*. Each set contained about 50 females. The females were taken just after blood feeding. Eggs were laid on wet filter paper. The ovaries of females were also examined for the presence of any retained eggs after egg laying. The total number of egg production in the first gonotrophic cycle was then calculated by summing up oviposited and unlaid eggs of immunized as well as control (normal) blood-fed females. The number of eggs hatched in each set was counted and hatching percentage was also calculated. Differences in mean egg production, mean number of larvae hatched, reduction percentage in fecundity and mortality were also calculated. All the data was subjected to Student's *t* test.

The antibody titer in rabbit measured against immunizing mosquito ovary antigens was very high (1:10^5-10^8). Recently, Almeida and Billingsley (12) have shown the progression of immune responses in mice during five sequential imnunizations with *An. stephensi* mosquito extracts. Control rabbits injected with Freund's adjuvant without mosquito antigens followed by PBS with antigen showed nonspecific reactions (titer up to 10 only). This could be due to a cross-reaction of rabbit antibodies due to the adjuvant. No antibodies were detected in the control rabbits. Serum from control rabbits gave no

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**The antigens identified have been named after one of the co-authors who left for heavenly abode.

precipitin lines with any antigen in a double diffusion test. However, whole ovary antigens gave at least two precipitin lines with antiserum. The anti-*An. stephensi* antiserum was reacted with ovary extract of *An. stephensi* recognizing up to five antigens: AJ 29, 35, 43, 64, and 80 kDa (Fig. 1). It was interesting that AJ 29 kDa antigen was expressed in all of the four tissues (additional hemolymph, midgut, and salivary gland) examined. However, AJ 43 and 80 kDa were also present in hemolymph and midgut, respectively. Two antigens AJ 35 and 64 kDa were exclusively present in the ovary of *An. stephensi*. Antisera raised against *An. stephensi* ovaries were also used to identify cross-reactive immunogens in ovary extract for other *Anopheles* spp. (*An. culicifacies* and *An. fluviatilis* only). Only one cross-reactive antigen (AJ 64 kDa) was identified by Western blotting (Fig 2).

There was no statistically significant difference in the corresponding body weight after engorgement in control and immunized rabbits. However, very significant differences in fecundity were observed between mosquitoes fed on rabbit blood injected with ovary antigens and mosquitoes fed on control rabbits. The number of eggs laid per female was reduced by about 57% ($P < 0.01$), when the females were fed with anti-mosquito ovary antibodies (Table 1). The marked reduction in fecundity in *An. stephensi* females that fed on immunized rabbits compared to those fed on normal or control

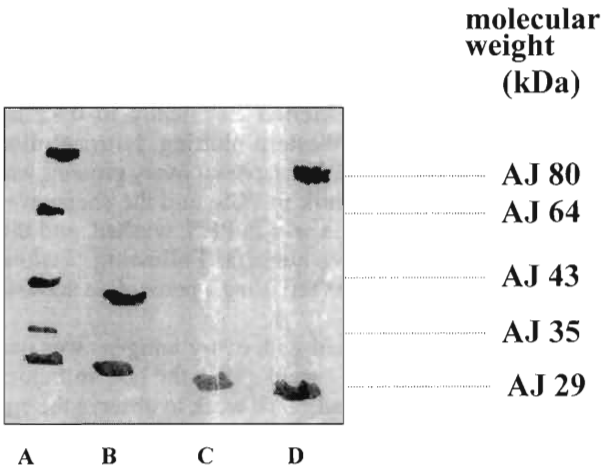

Fig. 1. Western blot analysis of specific tissue expression of antigenic polypeptides.
A: Ovary, B: Hemolymph, C: Salivary glands, D: Midgut.

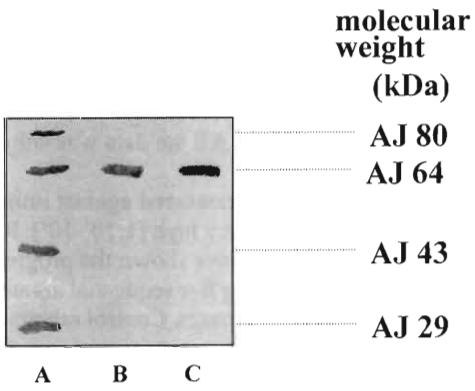

Fig. 2. Western blot analysis of species-specific expression of antigenic polypeptides.
A: *An. stephensi*, B: *An. culicifacies*, C: *An. fluviatilis*.

Table 1. Reduction percentage of eggs laid and egg mortality in *Anopheles stephensi* fed on rabbits immunized with ovary antigens

Feeding after last booster (weeks)	Reduction of eggs laid (%)	egg mortality (%)	
		control	experimental
1	57.7	9.6	9.0
2	53.3	10.0	14.2
3	46.8	30.8	36.0
4	44.1	26.7	26.3
5	45.1	17.0	17.7
6	35.4	22.5	18.3

rabbits suggests that humoral antibodies somehow interfere with the normal process of oogenesis. This lower fecundity rate does not seem to be related to the reduction in oviposition. The dissection of a few females from each feeding, 3 days after they had fed on immunized rabbit blood, showed that no egg development had occurred in these female mosquitoes.

Our observations are consistent with the fecundity reduction seen in *Ae. aegypti* ingesting anti-mosquito antibodies (2,4). However these studies used crude preparations of antigens using either the whole body or the head, thorax, and abdomen separately.

IgG antibodies have been detected in the hemolymph of Anophelines up to 48 h after blood feeding (13). Ramasamy et al. (4) have demonstrated that monoclonal and polyclonal antibodies to vitelline alone did not affect the fecundity of *Ae. Aegypti*, indicating that other target antigens are also involved. These polyclonal antibodies produced from crude preparations were not very effective in reducing fecundity in *An. tessellatus*. Ramasamy et al. (3) had reported that the reduction of fecundity was just 15%, 23%, and 20%, when mosquitoes fed on antibodies to the head, thorax, and abdomen, respectively. However, Srikrishnaraj et al. (14) have shown that anti-midgut antibodies also reduce vector competence for the malarial parasite and are also capable of inhibiting the formation of the peritrophic membrane (PM) in the posterior midgut of *An. tesselatus* (15). Similarly they showed that mosquitoes fed on rabbits with antibodies to mosquito midgut also reduced the fecundity by 25%. The high reduction in the number of eggs laid as observed during the present investigations could be attributed to specific anti-mosquito antibodies binding to target antigens in the ovary, interfering with the normal process of egg maturation and development.

The antibodies' mode of action is also unknown but could involve or a combination of several factors, i.e., inhibition of the metabolism or transport of vitellogenic proteins in insect hemolymph or in their uptake by developing oocysts, upset the hormone balance, or induce the resorption of mature or immature oocytes. Alternatively, the presence of antibodies in ingested blood may irritate the gut, reducing the total blood intake and available nutrients. The amount of blood was not assessed in the present study, but the females that fed appeared to engorge fully. Serological tests indicated a strong response to injected antigens.

The activities of these antibodies in terms of their effects on egg laying pattern was also observed for about 6 weeks. There was no significant mitigation of the effect of antibodies by the end of sixth week as compared to control experiment (Fig. 3). The number of eggs laid/females increased when same antisera was fed during subsequent weeks.

High titered antibodies against a specific combination of antigens may be more effective in bringing about a disruption

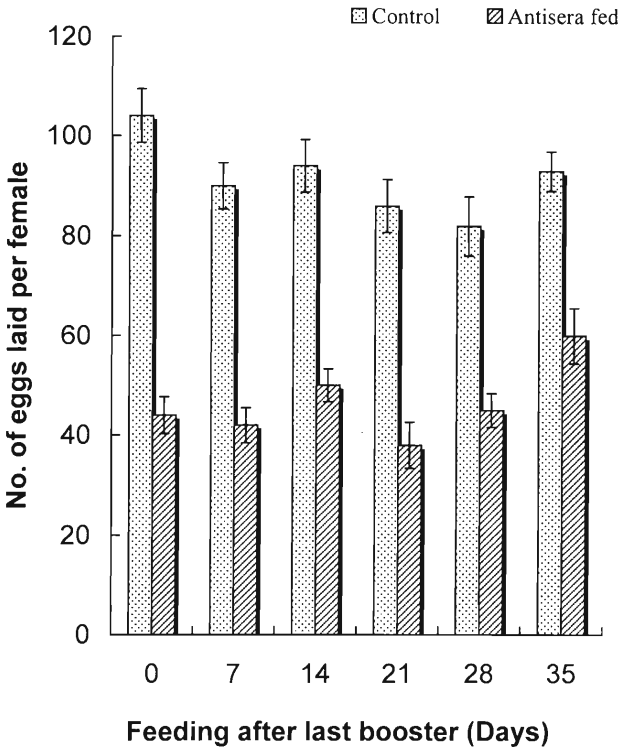

Fig. 3. Fecundity of *Anopheles stephensi* fed on the blood of rabbits immunized with ovary antigens of *An. stephensi*.

of mosquito reproductive physiology. This possibility requires further investigation using a combination of monospecific polyclonal and monoclonal antibodies produced against purified or synthetic antigen. The antibodies did not appear to be present in rabbit in effective concentrations at 5-6 weeks after the last antigen injection. When the same rabbit was used 6 weeks later to infect *An. stephensi*, the increase in the number of eggs was attributed to a decline in antibody titer below an effective threshold.

The percentage of larva hatched from eggs laid by females fed on imnunized rabbit showed that the difference in mortality rates was greater during first 3 weeks. However, this difference was reduced to an almost insignificant level, i.e., the mortality rate was almost same in the control and experimental (immunized) mosquitoes during the subsequent 3 weeks. This indicated that the effect of immunization had declined by the end of the sixth week.

We also did not observe increased mortality among female mosquitoes that had fed on immunized rabbits, in contrast to the findings of Alger and Cabrera (1). It must be noted that the mortality rate in their study was significant only among those insects that fed on rabbits immunized with mosquito midgut. Our experiments showed that the next generation was not affected by their parents having fed on immunized rabbits. Several hundreds of eggs produced by all feedings were reared through next generation and the percentages of egg laying and hatching were calculated. These values did not differ significantly between control and experimental mosquitoes.

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