J Vector Borne Dis 46, December 2009, pp. 255–260

Antibodies raised against hemolymph of *Anopheles culicifacies* reduce the fecundity and malaria parasite development

Amrita Kumari[a], S.K. Gakhar[b] & Vikas Hooda[c]

[a]*Department of Zoology, Hindu Girls College, Sonepat;* [b]*Advanced Centre for Biotechnology, Maharshi Dayanand University, Rohtak;* [c]*University Institute of Engineering & Technology, Maharshi Dayanand University, Rohtak, Haryana, India*

Abstract

Background & objectives: Several studies have been made to study the effect of antisera raised against different tissues (hemolymh, ovary, midgut and salivary glands) on the fecundity and malaria parasite development in the different species of mosquitoes but there are no reports on the antisera raised against the hemolymph of *Anopheles culicifacies*, the principal malaria vector in India accounting for 65% of malaria cases. Hence, an attempt was made to study the same and evaluate its impact on malaria parasite development.

Methods: Polyclonal and multifactorial antibodies were produced in rabbits against heterogenous mixture of hemolymph proteins. Antibodies against hemolymph proteins were screened for their potential to influence reproductive performance of mosquitoes. Antibody titer in rabbit serum was determined by ELISA and putative candidate antigens were identified in the hemolymph of *An. culicifacies* by western blotting. Cross reactivity amongst various tissues *vis-a-vis* hemolymph protein was also identified. In addition, a significant reduction in oocyst development was also observed in *An. culicifacies* mosquitoes that ingested antihemolymph antibodies along with *Plasmodium vivax*.

Results: The maximum reduction in fecundity (57%) was observed during fourth week, after the last booster and number of oocyts per infected mosquito reduced by 73.35% in the group of mosquitoes that ingested antihemolymph antibodies along with the infected blood meal respectively. However, the ingestion of antibodies against hemolymph proteins did not have significant influence on hatchability. Antisera raised against hemolymph proteins of *An. culicifacies* recognized 11 polypeptides by western blotting.

Interpretation & conclusion: During the present study, 11 putative candidate antigens were identified in the hemolymph of *An. culicifacies,* against which antibodies produced significantly reduced the fecundity by 57%. In addition, a significant reduction in oocyst development was also observed in *An. culicifacies* that ingested antihemolymph antibodies along with *P.vivax*.

Key words *Anopheles culicifacies* – antibodies – hemolymph – malaria parasite – transmission – vaccine

Introduction

Malaria continues to remain a serious public health problem throughout the tropical regions of the world despite decades of international efforts to control the spread of the vector and the disease. This disease is spread by *Plasmodium* — the parasite, which affects ~40% of the global population in ~100 countries around the world[1].

Various studies unequivocally incriminated *Anopheles culicifacies* (Diptera: Culicidae) as the major malaria vector, responsible for transmission of ~65% of malaria cases in India[2]. However, not many stud-

ies have been carried out to block malarial parasite transmission or to induce antimosquito immunity in this major vector of malaria in India. *Anopheles culicifacies* exists as a complex of five sibling species provisionally designated as A, B[3], C[4], D[5] and E[6] .These sibling species are reported to have various biological differences, viz. their distribution, response to insecticides[7], host preferences[6] and vectorial capacity[8]. *Anopheles culicifacies* A and C are primary vectors whereas species B has very little role, if at all, in the transmission of malaria[9].

In the present study, attempts were made to identify putative candidate antigens in the hemolymph of *An. culicifacies* A against which antibodies raised may reduce the fecundity and malaria parasite development in the mosquito.

Material & Methods

Mosquito rearing: The colony of *An. culicifacies* A (obtained from National Institute of Malaria Research, New Delhi) was maintained in the laboratory at relative humidity (RH) 70–80% and temperature 28±2°C[10]. The larvae were reared in the laboratory and mixture of powdered dog biscuits and yeast tablets was provided as larval food in 3:2 ratio at a density of 300 larvae/450 ml of water. The insectary was fitted with a simulated dusk and dawn machine with a photoperiod of 14 h day and 10 h night as per standard procedures[11]. Adult mosquitoes were kept in 30 x 30 x 30 cm organdy cloth cages fixed in an iron frame as described previously[11].

Sample preparation: Sixty newly emerged adult female mosquitoes were anesthetized on ice. The hemolymph was collected as droplets from the wounds of newly emerged adult females by amputation of the antennae and/or by removing the wings/or legs in phosphate buffer saline (PBS) containing phenylmethylsulphonyl fluoride (PMSF)[12]. The hemolymph collected was centrifuged at 10,000 g for 15 min at 4°C in refrigerated centrifugation machine (3 K30 Sigma, Germany) to remove any debris.

Immunization: Trichloroacetic acid (TCA) precipitated hemolymph proteins (170 µg protein in 0.5 ml 0.1 NaOH) were injected subcutaneously at multiple sites by emulsification with equal volume of Freund's complete adjuvant in three groups of rabbits, i.e. each group had one control rabbit which was immunized with PBS and Freund's complete adjuvant (FCA) but without antigens and other experimental rabbit in which antigens were injected along with PBS and FCA. Two weeks later, a first booster injection of antigens from another set of freshly emerged female mosquitoes was injected into three groups of rabbits with the exception that an equal volume of Freund's incomplete adjuvant was used. A week after the first booster, a second booster dose was injected in the same way as the first booster dosage, as described previously[11]. Approval for the animal studies was taken from the Animal Ethics Committee, Maharshi Dayanand University, Rohtak. Care of laboratory animals was made throughout the experimental studies according to the internationally accepted ethical guidelines[13].

In vitro ELISA: Antibody titers in the sera of different groups of rabbits were determined by enzyme linked immunosorbent assay (ELISA) using immunizing antigens (10 µg/ml) to coat the wells as described by Suneja *et al*[14]. Bound antigens were incubated with dilutions of rabbit antisera followed by addition of alkaline phosphate conjugated goat anti-rabbit IgG (1:20,000). Immune complex was detected with TMB/H_2O_2 substrate system.

Immunoblotting: Altogether, 80 mosquitoes were dissected to obtain various tissues (hemolymph, midgut, ovary and salivary glands). The quantity of proteins was estimated by the Bradford method[15]. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)[16] on 10% polyacrylamide gels under reducing conditions. Gels were either silver stained or were used for overnight electrophoretic transfer to 0.45 µm nitrocellulose membrane at 4°C for the western blotting[17]. Nitrocellulose membranes containing trans-

ferred *An. culicifacies* proteins were blocked with 5% non-fat milk for 1 h. Sheets were then incubated with rabbit antisera (1:100) for 1.5 h, washed thrice with PBS containing 0.1% Tween-20 and incubated for 1 h at room temperature with alkaline phosphate conjugated goat anti-rabbit IgG (1:5000). Antibody binding was visualized by NBT-BCIP substrate. Molecular weight of polypeptide was determined from standard marker (GENEI, India). All the reagents used in the experiments were purchased from SIGMA (through IGIB, New Delhi).

Fecundity and hatching: Immunized rabbits boosted with hemolymph proteins were used for blood feeding up to 7 wk. Six sets per week (containing about 20 females/set) were generated to observe the egg laying pattern and hatchability in *An. culicifacies*, as described previously[14].

Parasite invasion blocking assay: Sera from immunized, as well as control rabbits along with *P. vivax* were fed to *An. culicifacies* mosquito using membrane feeding apparatus to screen out the effects of antibodies. Peak titer sera from the rabbit were collected, pooled and stored at –70°C in deep freezer (Thermo, U.S.A.) for the membrane feeding. An equal volume of immune and control sera were mixed with infected sera containing *P. vivax* for the membrane feeding of mosquitoes. All 5-day old female mosquitoes were membrane fed separately on immunized and control sera. Unfed or partially fed females were removed. After eight days, mid-gut was pulled out to count the number of oocysts according to Ponnundurai *et al*[18] and using the formula mentioned below:

$$\frac{\text{MON in control} - \text{MON in antihemolymph} \times 100}{\text{MON in controls}}$$

Where, MON = Mean oocysts number.

Statistical analysis: Data were subjected to Student's *t*-test, to determine the significance of fecundity reduction and difference in oocyst count in experimental and control groups.

Results

High antibody titers ranging from $1:10^4$ in the first week to $1:10^6$ in the fourth week were detected in immunized rabbits, whereas serum and pre-immune serum of control rabbits with only Freund's adjuvant showed a negligible amount of antibodies, i.e. up to 10^2 only. The level of antibody titer continues to rise during the subsequent weeks which declined to a minimum value of $1:10^3$ during the seventh week (Fig. 1).

Immunoblotting of antihemolymph proteins antibodies identified 11 different antigens of molecular weights 171, 132, 119, 97, 82, 75, 62, 44, 33, 29 and 21 kDa (Fig. 2). Six antigens 171, 132, 119, 82, 75 and 33 kDa were exclusively present in the hemolymph; however, two antigens (29 and 62 kDa) showed cross-reactivity with all the four tissues.

Table 1 reveals a high antibody titer level interference with the mosquito biology. A significant reduction in fecundity was observed during the third week (42.8%, $p < 0.05$) and fourth week (57.7%, $p < 0.01$) respectively, after the last booster when the mosquitoes were fed on rabbits immunized with hemolymph antigens. Thereafter, the rate of fecundity declined

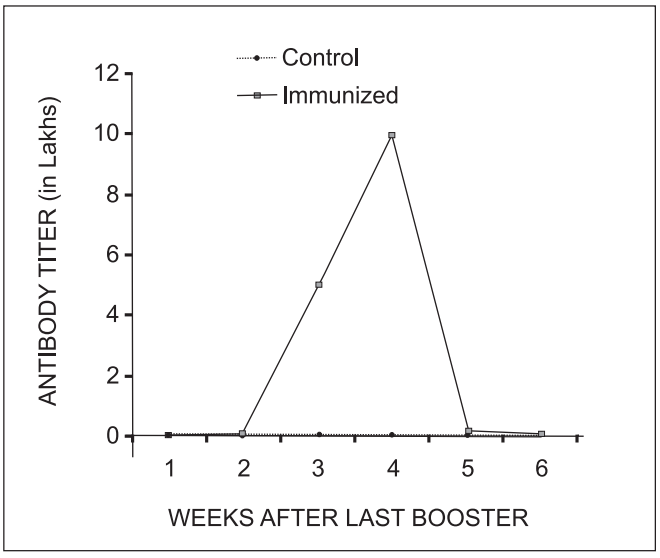

Fig. 1: Antibody titer after last booster measured by antibody capture ELISA

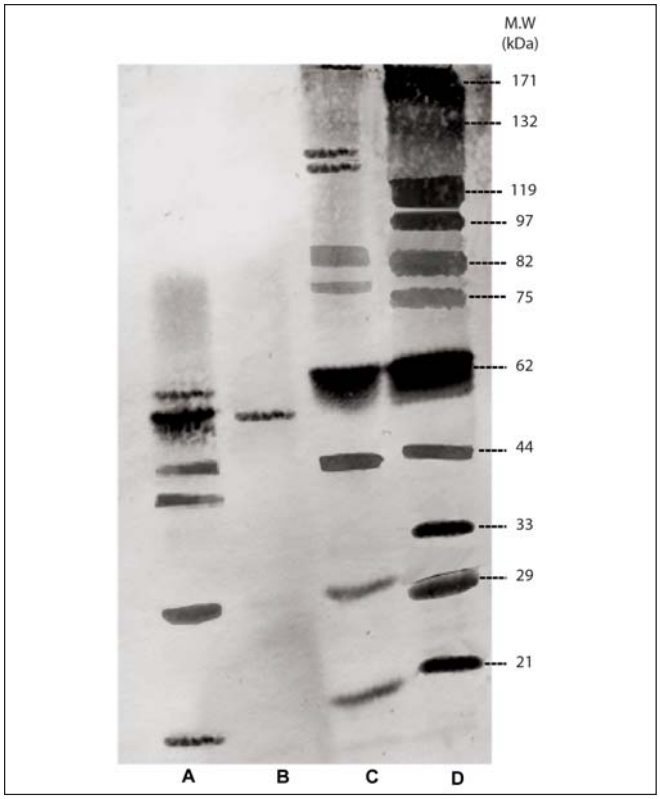

Fig. 2: Western blot analysis of specific tissue expression of antigenic polypeptides (A–Midgut; B–Salivary gland; C–Ovary; and D–Hemolymph)

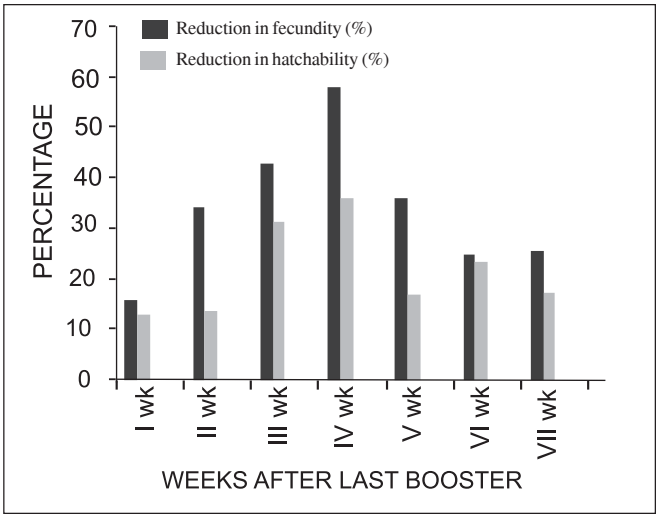

Fig. 3: Effect on fecundity and hatchability of *An. culicifacies* fed on the immunized rabbits with hemolymph antigens of *An. culicifacies*

to 25.53% until seventh week. Total hatchability of eggs laid by female mosquitoes which fed on immunized sera was also reduced by 35.9% during the fourth week (Fig. 3).

A significant reduction in parasite infection was also observed in *An. culicifacies* that ingested anti-hemolymph antibodies along with *P. vivax* as compared to the control. The infection rate of *An. culicifacies* was reduced by 18% after feeding with immunized sera. Mean number of oocysts/mos-

Table 1. Effect of antimosquito antibodies raised against hemolymph of *An. culicifacies* (8 = day old, glucose fed) on fecundity and hatchability of *An. culicifacies*

Weeks after last booster	No. of females fed		Mean eggs laid/ female±S.D.)		Reduction in fecundity (%)	Mean larvae hatched/female±S.D.)		Reduction in hatchability (%)
	C	I	C	I		C	I	
1	35	16	63.94±5.2	53.75±2.6	15.9	53.88±2.4	46.8±2.5	13.1
2	35	16	66.02±6.26	43.56±2.85	34.01	57.71±1.4	49.9±4.1	13.5
3	35	20	65.42±3.4	37.4±2.5	42.8*	56.6±4.2	38.83±0.4	31.3
4	35	16	63.14±3.1	26.68±2.96	57.7**	56.31±2.9	36.08±2.4	35.9
5	35	20	66.74±4.63	42.85±5.02	35.79	57.88±1.5	48.08±1.2	16.9
6	17	10	36.52±7.61	27.5±2.34	24.69	30.82±1.6	23.6±2.3	23.4
7	10	3	64.9±1.9	48.33±4.2	25.53	42.7±3.6	35.33±2.5	17.2

*p <0.05; **p <0.01; C–Control; I–Immunized.

quitoes was found to be drastically reduced in the mosquitoes that ingested antihemolymph antibodies (32.5±2.8) as compared to the control (125±5.13). Hence, the transmission blocking percentage (73.35) was significant at $p < 0.05$.

Discussion

Earlier studies[11,13,14,19,20] made on the development of antibody responses paved the way to attempt the present study for the characterization of antihemolymph antibodies in the malaria vector *An. culicifacies.* The observed rate of reduction in the fecundity was in accordance with the results of previous studies[21, 22]. However, the reduction in fecundity observed in mosquito *An. stephensi* by Almeida and Billingsley[23] was merely 18,16, 21 and 22% when mosquitoes fed on antibodies produced against head, gut, ovaries and fat bodies, respectively. Recently, Manoj *et al*[13] showed 37% reduction in fecundity of *An. culicifacies* when mosquitoes had fed upon antimosquito salivary gland antibodies. The maximum reduction in fecundity during the present study was observed in the fourth week after the last booster, coinciding with the results of ELISA. A high antibody titer ($1:10^5$–$10^{6)}$ is also in accordance with the previous study[23].

This observed reduction in fecundity suggests that humoral antibodies, somehow, interfere with the normal process of oogenesis as the ovarian proteins/ insect vitellogenins are synthesized by the female fat body and are transferred in the hemolymph; these vitellogenins are then selectively sequestered to the developing oocytes by receptor mediated endocytosis and are stored in yolk granules[24]. Therefore, the reduction in number of eggs produced may be attributed to one or the combination of several factors, i.e. specific antimosquito antibodies binding to target antigens which may down regulate vitellogenins synthesis by fat body, inhibition of the uptake of circulatory vitellogenins or reabsorption of contents of some of the developing follicles.

Further, the results of western blotting revealed that antihemolymph antibodies when ingested along with the blood meal by female mosquitoes are indeed capable of binding with hemolymph proteins, and to some extent to other tissues. This cross-reactivity is therefore, attributed to different factors, i.e. antigens or epitopes may be common to other tissues, or nonspecific binding may also occur with low affinity antibodies.

The present study also demonstrated that antibodies against hemolymph antigens, when ingested by mosquitoes along with infected blood, affected the normal development of oocycts in the midgut and the migration of sporozoites to the salivary glands. In addition to this, if we combine the present study with mass spectrometry analysis of proteins together with the data from the recent sequencing of the *An. gambiae* genome it could hopefully give us a lead to identify the novel protein targets in the hemolymph of *An. culicifacies* which may contribute to the development of a transmission blocking vaccine to control the malaria in future.

References

1. Chauhan VS. Vaccines for malaria: prospects and promise. *Curr Sci* 2007; *92:* 1525–34.
2. Sharma VP. Fighting Malaria in India. *Curr Sci* 1998; *75:* 1127–40.
3. Green CA, Miles SJ. Chromosomal evidence for sibling species of the malaria vector *Anopheles* (*Celia*) *culicifacies* Giles. *J Trop Med Hyg* 1980; *83:* 75–8.
4. Subbarao SK, Vasantha K, Adak T, Sharma VP. *Anopheles culicifacies* complex: evidence of new sibling species, species C. *Ann Ent Soc Am* 1983; *76:* 985–90.
5. Vasantha K, Subbarao SK, Sharma VP. *Anopheles culicifacies* complex: population cytogenetic evidence for species D (Diptera: Culicidae). *Ann Entomol Soc Am* 1991; *84:* 531–6.
6. Kar I, Subbarao SK, Eapen A, Ravindran J, Satyanarayana TS, Raghavendra K, Nanda N, Sharma VP. Evidence of a new malaria vector species, species E, within the *Anopheles culicifacies* complex (Diptera: Culicidae). *J Med Entomol* 1999; *36:* 596–600.
7. Raghavendra K, Subbarao SK, Vasantha K, Pillai MKK,

Sharma VP. Differential selection of malathion resistance in *Anopheles culicifacies* A and B (Diptera: Culicidae) in Haryana state. *J Med Entomol* 1992; *29:* 183–7.

8. Subbarao SK, Adak T, Vasantha K, Joshi H, Raghavendra K, Cochrane AH, Nussenzweig RS, Sharma VP. Susceptibility of *Anopheles culicifacies* species A and B to *Plasmodium vivax* and *Plasmodium falciparum* as determined by immuno radiometric assay. *Trans R Soc Trop Med Hyg* 1988; *82:* 394–7.
9. Kaur S, Singh OP, Adak T. Susceptibility of species A, B and C of *Anopheles culicifacies* complex on *Plasmodium yoelii yoelii* and *Plasmodium vinckei petteri* infections. *J Parasitol* 2000; *86:* 1345–8.
10. Gakhar SK, Singh S, Shandilya H. Changes in soluble proteins during the development of malaria vector *An. stephensi* (Diptera: Culicidae). *Proc Natl Sci Acad* 1997; *B63:* 289–98.
11. Gakhar SK, Jhamb A, Gulia M, Dixit R. Anti-mosquito ovary antibodies reduce the fecundity of *An. stephensi* (Diptera: Culicidae). *Jpn J Infect Dis* 2001; *54:* 181–3.
12. Gakhar SK, Shandilya H. *Plasmodium* induced changes in hemolymph proteins during the development and ageing of malaria vector *An. stephensi*. *Insect Sci Appl* 2000; *20:* 141-9.
13. Manoj C, Adak T, Pahwa R, Gakhar SK. Antibodies to *An. culicifacies* salivary glands encumber vector competence to *Plasmodium vivax*. *Asian J Bio Sci* 2008; *3:* 269–74.
14. Suneja A, Gulia M, Gakhar SK. Blocking of malaria parasite development in mosquito and fecundity reduction by midgut antibodies in *An. stephensi* (Diptera: Culicidae). *Arch Insect Biochem Physiol* 2003; *55:* 63–70.
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram-quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; *72:* 248–54.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; *227:* 680–5.
17. Towbin H, Stachelin T, Gordon J. Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci* 1979; *76:* 4350–4.
18. Ponnunduarai T, Van Genert GJ, Bensink T, Loss AWH, Merrwissin JHET. Transmission blockade of *P. falciparum*: its variability with gametocyte number and concentration of antibody. *Trans R Soc Trop Med Hyg* 1987; *81:* 491–3.
19. Gakhar SK, Gulia M. Antimosquito egg antibodies reduces the fecundity and viability in malaria vector *An. stephensi*. *J Immunol Immunopathol* 2001; *3:* 53–6.
20. Gulia M, Suneja A, Gakhar SK. Effect of antimosquito hemolymph antibodies on the fecundity and on the infectivity of malaria parasite *Plasmodium vivax* to *An. stephensi* (Diptera: Culicidae). *Jpn J Infect Dis* 2002; *55:* 78–82.
21. Sutherland GB, Ewen AB. Fecundity decreases in mosquitoes ingecting blood from specifically sensitized mammals. *J Insect Physiol* 1974; *20:* 655–60.
22. Ramasamy MS, Ramasamy R, Kay BH, Kidson C. Anti-mosquito antibodies decreases reproduction capacity of *Aedes aegypti*. *Med Vet Entomol* 1988; *2:* 87–93.
23. Almeida APG, Billingsley PF. Induced immunity against the mosquito *An. stephensi* Liston (Diptera: Culicidae): effects on mosquito survival and fecundity. *Int J Parasitol* 1998; *28:* 1721–31.
24. Hagedorn HH, Kunkel JG. Vitellogenin and vitellin in insects. *Ann Rev Entomol* 1979; *24:* 475–505.

Corresponding author: Amrita Kumari, Department of Zoology, Hindu Girls College, Sonepat–131 001, India.
E-mail: k.amrita9@gmail.com; advance.biotech@gmail.com

Received: 26 May 2009 *Accepted in revised form:* 7 July 2009