Detection of Mosquito Larval Antigens in a Natural Predator by Using a Simple Immunological Technique

Araya Dharmkrong-at Chusattayanond[a,*], Kosol Roongruangchai[a], Chaweephant Suangkiatikul[b] and Sangvorn Kitthawee[b]

[a] Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand.
[b] Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand.
* Corresponding author.

Received 5 Aug 1999
Accepted 8 May 2000

ABSTRACT A simple technique for seeking natural predators to mosquito larvae was developed. In the present study, adult backswimmers, a natural predator to mosquito larvae, were used as a model. The adult predators were fed with larvae and pupae of *Aedes aegypti* and tested for the presence of antigens of the mosquito larvae at 1, 2, 4, 6 and 24 hr thereafter. The test was performed using an immunological precipitation reaction in gel against immune rabbit serum obtained from 2 rabbits repeatedly immunized with aqueous somatic extract of *A. aegypti* larvae and pupae. One major and 2 to 3 minor precipitin bands were demonstrated from the predators at 1 to 6 hr after feeding. No precipitin reaction was observed at 24 hr. However, a reaction of partial identity was observed between the antigens of A. *aegypti* and those of *Culex quinquefasciatus* larvae. It can be concluded that the immunological precipitation reaction in gel can be used as a simple tool for seeking natural predators to mosquito larvae. Further study is needed to make it possible to identify the predated species of mosquito larvae within the body of the predator.

KEYWORDS: immunodiffusion, mosquito larvae, predator, backswimmer.

INTRODUCTION

Mosquitoes are the most common blood-sucking arthropods. They are the most important vectors for several important communicable diseases such as malaria, lymphatic filariasis, yellow fever and Dengue fever. As they feed on a wide variety of animals including amphibians, reptiles, birds and mammals, they are also known as vectors of zoonotic diseases including the highly virulent Japanese B encephalitis and the pig virus, and the less virulent dirofilariasis caused by *Dirofilaria* spp, the filarial nematode from dogs. In addition to these known diseases, the possible emergence of mosquito-transmitted zoonotic diseases not previously discovered can not be overlooked.

Mosquito-borne diseases bring about death, poverty and debilitating effects. Controls of mosquitoes depends largely on the use of broad-spectrum hydrocarbon insecticides. These chemicals have brought a complex of problems including behavioral and physiological resistance and environmental contamination.

The current trend for mosquito control is to decrease the use of chemical methods, but increase the use of biological methods. Introducing predacious animals such as fish into the habitat of mosquito larvae is one of several approaches that have been tried. However, the mosquito problem is too severe to be tackled by any single type of predators. The use of aquatic carnivorous animals other than fish may be one effective approach. In order to reach this goal, appropriate animals should be sought and recovered from their natural habitat. Detection of mosquito larval and pupal materials within the body of potential predators would be useful in this discovery process. Serological techniques for studying predator and prey relationships have previously been studied.[1-7] This paper reports the possibility of using an immunodiffusion technique as a tool for detecting mosquito larval and pupal antigens in the body of potentially predacious animals. The experiment was done using backswimmers, *Notonecta* sp., a general predator with the larvae and pupae of *Aedes aegypti* and *Culex quinquefasciatus* in a predator-mosquito model system.

MATERIALS AND METHODS

Mosquitoes

Aedes aegypti and *Culex quinquefasciatus* were used in this study. The colonies were obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok and maintained in the insectory of the Department of Biology, Faculty of Science, Mahidol University, Thailand.

The insectary was an air conditioned room

maintained at 24-28°C at 45-75% relative humidity. Adult mosquitoes were maintained in cages provided with a bottle of distilled water and a bottle of multi-vitamin syrup as food. Adult female mosquitoes were fed with mouse blood. The mice were anaesthesized with ether to allow mosquitoes to feed on their blood. Female mosquitoes laid their eggs on a soaked filter paper. Eggs were hatched in the water. After hatching, larvae were fed with a finely ground fish food pellet. The larvae were used in the experiment when they were second and third instars. Pupae were harvested when needed.

Backswimmers

Backswimmers (Hemoptera: Notonectidae), a natural predator of mosquitoes, were collected from pools and other slow running water at Salaya campus, Mahidol University, Nakornpathom Province. The backswimmers collected from field were maintained in the insectary. Waterplants were provided as resting and oviposition sites. Adequate mosquito larvae were fed daily to the backswimmers. Aeration was also provided. The backswimmers were used when they were approximately 3 mm long (small size).

Rabbits

New Zealand White rabbits weighing 1.5-2.0 kg were obtained from and reared by the Animal Centre, Faculty of Science, Mahidol University. The rabbits were fed ad libitum with food pellet and dechlorinated tap water.

Preparation of Mosquito Larval and Pupal Antigens

1. *Preparation of larvae and pupae for antigen extraction*

Larvae and pupae of *Aedes aegypti* and *Culex quinquefasciatus* were collected and maintained separately in dechlorinated tap water in the absence of any kind of food material for 16 hr. The fasted larvae and pupae were blotted on filter paper pads in order to remove the excess water and were processed further as follow.

2. *Antigen extraction*

Aqueous somatic antigens of the larvae and the pupae of *Aedes aegypti* and *Culex quinquefasciatus* were prepared by the technique previously described.[8] Blot dried mosquito larvae and pupae weighing 0.75 gm were homogenized in a ground glass tissue grinder in the presence of 0.85% sodium chloride solution (NSS) and 20 µl each of 10 mM of the protease inhibitors TPCK and PMSF. Subcellular fractions of the homogenate were broken up by sonication in a Soniprep 150 ultrasonic disintegrator. The equipment was operated at the highest frequency for intervals of 10 sec 20 times with a resting period of 20 sec between sonications. The material was handled at low temperature throughout the process. The sonicated material was then allowed to stand at 4°C over night before it was centrifuged at 10,000 rpm. for 45 min. The supernatant containing an aqueous somatic extract of larvae and pupae was obtained and stored at -20°C until used.

Pre-immune Rabbit Serum

Pre-immune rabbit serum was prepared for use as a negative serum control in the experiment. Five ml of blood was collected from the ear vein of each of the 2 rabbits before immunization. The blood was allowed to clot and retract completely by standing at room temperature for 2 hr followed by standing over night at 4°C. Serum was obtained and stored frozen at -20°C.

Immune Rabbit Serum

Immune rabbit serum against *A. aegypti* larvae and pupae antigens was prepared by the technique modified from that described by Boreham and Gill, 1973.[2]

1. *Preparation of a water-in-oil emulsion of antigen*

Mosquito larvae and pupae were fasted for 16 hours, blot dried with filter paper, homogenized in the presence of NSS. The amount (wet weight) of the larvae and the pupae and also the volume of the saline varied according to the immunization schedule described below. The temperature of the materials was maintained at 4°C throughout the process. A water-in-oil emulsion of the homogenized materials was made by mixing them with Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA) using a two-way needle and syringe.

2. *Immunization schedule*

The water-in-oil emulsions of *A. aegypti* antigens were injected subcutaneously into 2 rabbits 3 times on days 0, 14, and 21. The emulsion for the first injection dose contained 750 mg antigen, 3.0 ml NSS and 3.0 ml FCA, while those for the second and the third doses contained 375 mg antigen, 3.0 ml NSS and 3.0 ml IFA. The injections were done using 21Gx11/2 inch needles at 8 sites including 2 axillary and 2 inguinal lymph nodes and the back area of each rabbit.

3. Obtaining the immune rabbit serum

One week after the last injection, the rabbits were bled from the ear vein. Forty ml of blood was collected and serum was obtained by the technique described above. The sera from both rabbits showed the same reactivity at slightly different strength when tested by immunodiffusion. They were pooled and kept frozen at -20°C until used.

Preparation of Backswimmer Antigens

One hundred and twenty adult backswimmers were fasted in dechlorinated tap water for 3 days in order to clear stomach contents. The predators were then divided to 6 groups. Those in groups 1-5 were fed with larvae and pupae for 2 hr. After feeding the predators were separated from their prey and fasted for 1, 2, 4, 6, and 24 hr. Predator group 6 were not fed and thus comprised the negative control group.

The backswimmers from each group were homogenized in 200 µl of NSS using a microgrinder. The homogenate was then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant containing antigen extract was obtained and stored at -20°C until used.

Immunodiffusion Test

The technique used in the present study was modified from the Ouchterlony double diffusion method previously described.[9] To perform this technique, 1% agar (Agar noble, Difco Laboratories) was prepared in NSS in the presence of 0.001% merthiolate. Four ml of the hot-melted agar was layered onto each of 2.5x7.5 cm microscopic glass slides and allowed to cool and set. Wells with a diametre of 0.2 mm were made 2 cm apart using an agar punch. The wells were arranged with one at the centre and six around the centre in a ring. Antigens and antiserum were placed in the wells according to the experimental design. The immunodiffusion reaction was allowed to proceed in a moisture chamber at 4°C for 1-2 days before the precipitin bands were observed. Salt was removed from the gel by soaking in several changes of distilled water. The gel was dried and stained with 0.6% Amido Schwardtz-10 B (E Merck AG).

RESULTS

1. Reactivity of Immune Rabbit Serum against *A. aegypti* Somatic Extract

The immune serum prepared in this study was allowed to react against somatic extract of *A. aegypti* larvae and pupae in the immunodiffusion test. The serum was applied in wells 1 to 6 at undiluted strength and at serially diluted concentrations from 1:2 to 1:32 respectively, while *A. aegypti* antigen was applied in the central well E (Fig 1.). Five precipitin bands consisting of 3 major (No 1,2,3) and 2 minor (No 4,5) bands were demonstrated in the gel (see Fig 1A). Bands 2 and 3 were close together and the most prominent. The reaction was proven to be specific since the pre-immune serum did not show any bands in the test (Fig 1B).

2. Detection of *A. aegypti* Antigens in Somatic Extracts of Backswimmers Fed with *A. aegypti*

In this experiment the immune rabbit serum was used as a tool to detect *A. aegypti* larval and pupal antigens in somatic extracts of *A. aegypti*-fed backswimmers by the immunodiffusion test. The immune serum was applied in the well S and aqueous extracts of backswimmers at 1,4,6 and 24 hrs after feeding with *A. aegypti* larvae and pupae were applied in wells 1,2,3 and 4 respectively. As shown in Fig 2., major precipitin band resemblings those in positions No 2 and 3 in Fig 1. were clearly demonstrated

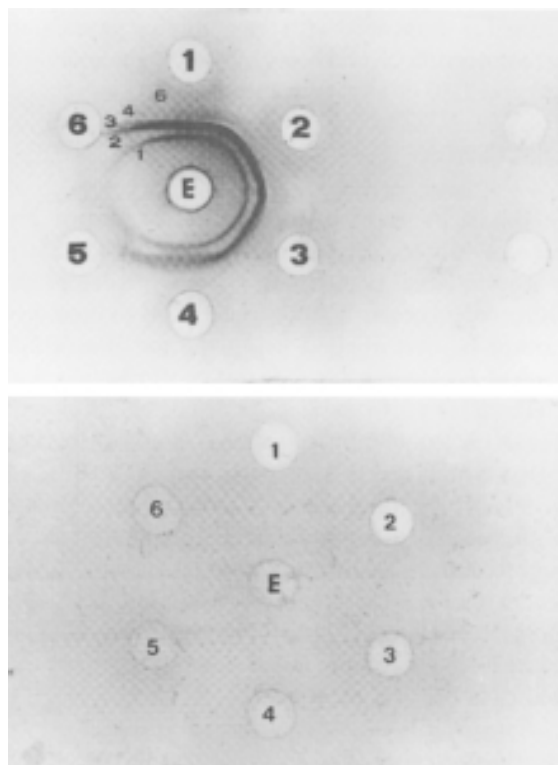

Fig 1. Immunodiffusion tests of rabbit serum at undiluted concentration (1) and at diluted concentrations of 1:2 (2), 1:4 (3), 1:8 (4), 1:16 (5) and 1:32 (6) against somatic extract of *A. aegypti* larvae and pupae (E). The serum was obtained from the same rabbit before (B) and after (A) immunization.

together with 2 minor precipitin bands. The reaction diminished for extracts from 4 and 6 hr after feeding and was absent at 24 hr after feeding. However, the hyperimmune serum was also found to react and form minor precipitin bands with the somatic extract from fasted backswimmers which was applied in well B (Fig 2.). This result indicated the presence of common antigenic components in the somatic extract of *A. aegypti* larvae and pupae and backswimmers. These components, however, could be distinguished from those specific for *A. aegypti*.

3. Reactivity of the Immune Rabbit Serum against *C. quinquefasciatus* Somatic Extract

In order to test the specificity of the immune serum prepared against *A. aegypti* larvae and pupae, it was allowed to react with somatic extract of *C. quinquefasciatus* larvae and pupae in the immunodiffusion reaction. As shown in Fig 3., major precipitin bands were demonstrated between the wells containing the hyperimmune serum (S) and those containing somatic extract of *C. quinquefasciatus* that was undiluted (C) or diluted 1:10 (D). However, the bands were not as prominent and completely formed as those seen with the homologous reaction between serum (S) and *A. aegypti* extracts at undiluted (A) and 1:10 diluted concentrations (B).

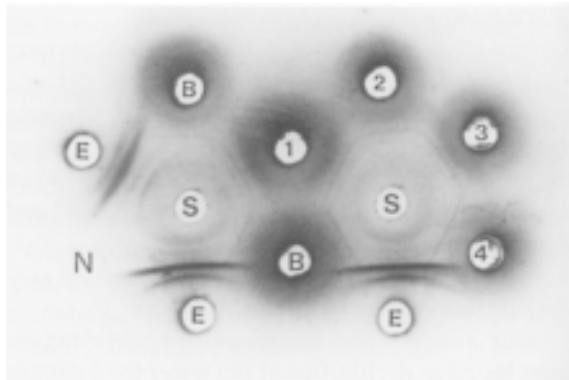

Fig 2. Immuunodiffusion tests of immune rabbit serum at undiluted concentration (S) against somatic extract of backswimmers at 1 hour (1), 4 hours (2), 6 hours (3) and 24 hours (4) after consuming *A. aegypti* mosquito larvae and pupae. Somatic extract of *A. aegypti* mosquito larvae and pupae was used as a positive control (E). NSS (N) and somatic extract of backswimmers that did not consume any mosquito larvae (B) were used as negative controls. The major precipitin bands at the positions 2 and 3 were demonstrated between the antiserum and the backswimmer extracts obtained at 1, 4 and 6 hr after feeding.

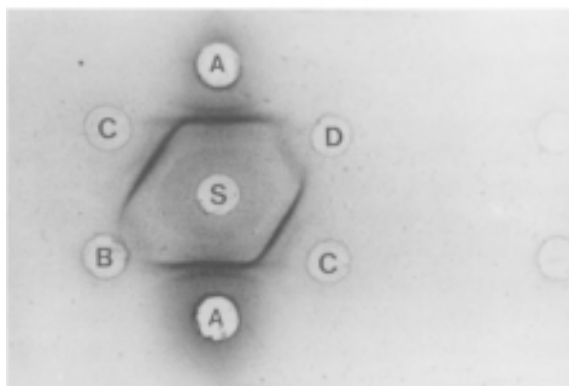

Fig 3. Immunodiffusion test of immune rabbit serum, at undiluted concentration (S) against somatic extract of *A. aegypti* at undiluted (A) and at 1:10 diluted (B) concentrations and also against somatic extract of *C. quinquefasciatus* at undiluted (C) and 1:10 diluted (D) concentrations.

Discussion

Based on field observations, predation by backswimmers seems to significantly regulate mosquito larval populations.[10] Many predators are either solely or partly fluid-feeders and so their diet cannot be investigated using the gut dissection method. Ecologists have used a variety of immunological methods to study predation.[1-7] The methods are based on the common principle that antibodies raised in rabbits against predated species can be used to detect antigens of that species in the gut contents of predators. In determining the predatory role of backswimmers against mosquito larvae and pupae, serum obtained from rabbits hyperimmunized with antigens of mosquito larvae and pupae were used to probe for the mosquito larvae and pupae within the bodies of the backswimmers.

The results obtained from the present study clearly indicated that antigens of the consumed mosquito larvae and pupae could be detected in aqueous fractions of the backswimmer extracts using the hyperimmune rabbit serum. The serum could detect the antigens from as early as 1 hr to as late as 6 hr after larval ingestion. Thus for the test to be applied in the field, suspected predators should be immediately frozen when collected. Choosing an immunological test with increased sensitivity such as passive haemagglutination may be recommended, but any technique with too high sensitivity such as an ELISA (Enzyme-linked immunosorbant assay) may bring out the cross-reactive components shared between the mosquito larvae and the backswimmers, unless the antiserum were pre-adsorbed before use.

Acknowledgements

We acknowledge Professor Timothy W Flegel for his kind assistance in reviewing the manuscript.

REFERENCES

1. Service MW (1973) Study of the natural predators of *Aedes cantans* (Meigen) using the precipitin test. *J Med Entomol* 10, 503-10.
2. Boreham PFL and Gill GS (1973) Serological identification of reptile feeds of Glossina. *Acta Tropica* 30, 356-65.
3. Boreham PFL and Ohiagu CE (1978) The use of serology in evaluating invertebrate prey-predator relationships: a review. *Bull Entomol Res* 68, 171-94.
4. Boreham PFL (1979) Recent developments in serological methods for predator-prey studies. *Entomol Soc America, Misc Pub* 11, 17-23.
5. Frank JH (1979) The use of the precipitin technique in predator-prey studies to 1975. *Entomol Soc America, Misc Pub* 11, 1-15.
6. Sunderland KD (1988) Quantitative methods for detecting invertebrate predation occurring in the field. *Ann Appl Biol* 112, 201-224.
7. Sopp PI Sunderland KD Fenlon JS and Wratten SD (1972) An improved quantitative method for estimating invertebrate predation in the field using an enzyme-linked immunosorbent assay (ELISA). *J Appl Ecol* 29, 295-302.
8. Dharmkrong-at A and Sirisinha S (1983) Analysis of antigens from different developmental stages of *Angiostrongylus cantonensis. Southeast Asian J Trop Med Pub Hlth* 14, 154-62.
9. Crowle AJ (1961) *Immunodiffusion.* Academic Press, New York, N.Y.
10. Kitthawee S Edman JD and Upatham ES (1993) Mosquito larvae and associated macroorganisms occurring in gem pits in Southern Tha Mai District, Chanthaburi Province, Thailand. *Southeast Asian J Trop Med Pub Hlth* 24, 143-51.