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Enhanced esterase activity in salivary gland and midgut of *Aedes aegypti* mosquito infected with dengue-2 virus

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Mosquitoes were infected by intrathoracic inoculation. About 95% head squashes were positive for dengue virus antigen on the 15th post infection day (PID). Esterase activity was determined in the homogenates prepared from the salivary glands and midguts on different PIDs of dengue virus inoculated and control mosquitoes showed that it was consistently higher in the virus-infected batches.

Virus dissemination in vector mosquito from midgut and its multiplication in the salivary glands are important in disease transmission. Studies on Japanese encephalitis antigen distribution in the salivary glands of its vector showed that the number of salivary gland area positivity varies irrespective of post infection days in individual mosquitoes. The salivary gland area positive for virus may be an important factor in transmission since hosts may receive varying quantum of viral inoculum[1]. There are some other studies, which have shown physiological differences in salivary glands in mosquito populations, which may have role in disease transmission. Differences in haemagglutinin activity (HA) in the salivary glands of the susceptible and refractory strains of mosquitoes due to varying carbohydrate specificities have been reported by Nayar and Knight[2]. HA may be involved in controlling the activation of defense mechanisms, such as activation of the PpO cascade[2]. Expression of certain immunoresponsive genes in the salivary glands due to the penetration of malaria parasite into the midgut epithelium has been reported[3].

There may be several other responses in the midgut and salivary gland of mosquito due to the pathogens entry in to the body. Basic problem in understanding response to pathogen challenge is the small size of mosquitoes that limits the quantity of materials, which is required for cellular and biochemical studies. When mosquitoes are infected with chikungunya virus, the levels of esterase enzyme increase in response to virus multiplication[1]. These studies were based on estimation of enzyme activity on whole mosquito body. The present studies have been undertaken to understand esterase enzyme activity specifically in the salivary glands and midguts in virus infected mosquitoes.

Mosquitoes — Mosquitoes used for the experiments were from a laboratory colony of *Ae. aegypti* maintained in this Institute since 1994. These were maintained in insectory. Larvae were fed on mixture of dog biscuit and yeast powder (1:1). Adults had continuous access to a diet of 10% glucose solution and were not allowed to blood feed after eclosion.

Dengue-2 virus (9012384) (Jammu strain) was isolated from a patient suffering from dengue haemorrhagic fever during an epidemic, which occurred at Jammu, India. Stock used for experiments was prepared in mice through ic route of inoculation. The virus used was at 9th mouse passage level.

Infection of mosquitoes by intrathoracic inoculation —Two-day old female mosquitoes were inoculated intrathoracically with approximately 0.2 μl virus suspension prepared in Bovine albumin phosphate saline (BAPS) (2.1dex / 0.2 μl MID_{50}), as per Rosen and Gubler[4].

Detection of virus in mosquitoes — Detection of dengue viral antigen in the head squashes of the mosquitoes was done using indirect immunofluorescence antibody (IFA) technique[5].

Enzyme assays were performed on the midguts and salivary glands dissected out from the female mosqui-

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toes. In each experiment batches of mosquitoes were dissected for midguts and salivary glands from virus inoculated and BAPS inoculated (controls) groups up to the 15th post infection day (PID). Generally, the dissections were carried out immediately. In some cases the mosquitoes were stored at –70°C and dissected on the next day. Twenty-five glands were homogenized in 80 µl phosphate buffer saline (PBS) *p*H 7.2 in "U" bottom microtitre plates with the help of glass rods and homogenates were centrifuged at 4000 *g* for 5 min at 4°C. Similarly, 25 midguts were triturated in 120 µl PBS to obtain supernatants for various assays.

Protein assays—The protein content was estimated in 40 µl supernatant fluid from each individual homogenate by the method of Lowry *et al.*[6]. A reference standard protein curve was prepared using Bovine serum albumin fraction 5.

Esterase quantitative assay—Assays were performed by the method of Hemingway[7], with 20µl aliquots of supernatant from each individual, using α- and β-naphthyl acetate as substrates. After incubation for 10 min at 37°C, the reaction was stopped by the addition of stain, (fast blue B in 5% sodium dodecyl sulphate solution) and the end-point absorbancy measured at 490 nm in an UVmax microplate reader. Results were converted to absolute units by analysis against standard curves for α- and β-napthol and adjustment for protein concentration.

Head squashes prepared from the dengue-inoculated mosquitoes showed that about 95% of them were positive for dengue virus antigen on the 15th PID.

Esterase activity determined in the homogenates prepared on different PIDs from the salivary glands and midguts of control inoculated and dengue virus inoculated mosquitoes showed that it was consistently higher in the batches, inoculated with virus. There was significant difference in both the cases when paired *t*-test was applied to compare the enzyme activity on different PIDs in normal and virus infected salivary glands and midguts ($P = < 0.01$). Averages of three replicates of esterase activity in the midguts and salivary glands are depicted in Figs 1 and 2.

Mosquito-borne pathogens, which are transmitted via saliva, modify salivary function and thus indirectly influence biting behavior[8]. Studies on *An. gambiae* have shown that when mosquitoes take malaria parasite-infected blood meal the development of parasite in the gut causes expression of certain immune-responsive genes in the salivary glands[3]. Nayar and Knight[2] have studied the role of immune response of mosquitoes to filarial parasite. They found that hemagglutinins in the salivary glands showed specificities for a broader range of carbohydrate moieties than other body fluids.

Mourya and Mishra[1] in a recent study on JE antigen distribution in three vector species showed that a large number of mosquitoes on various PID had lower number of salivary gland areas positive. The lower number of salivary gland areas positive for virus may

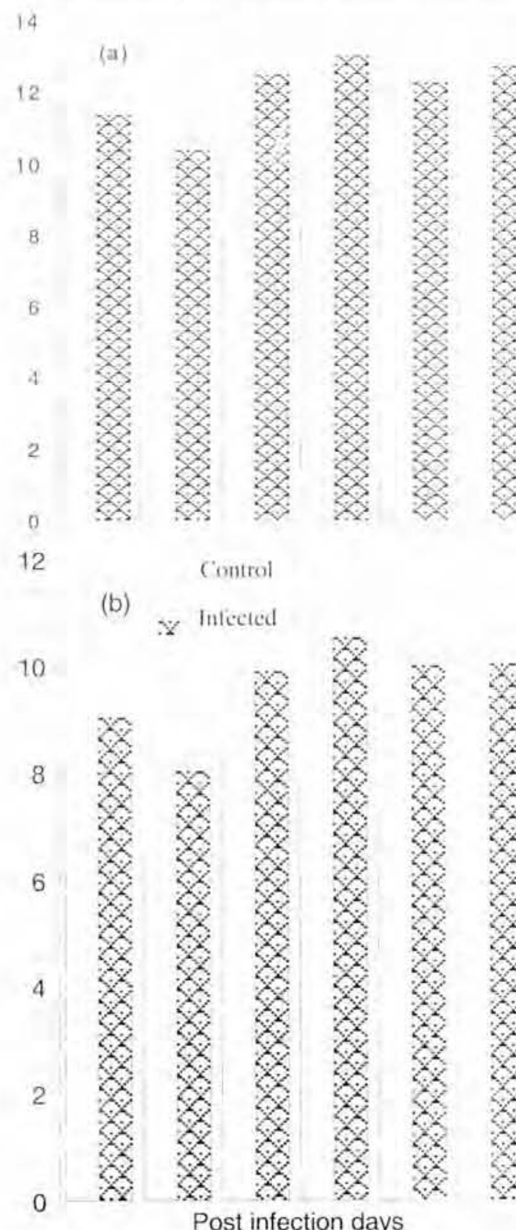

Fig. 1—General esterase activity in midguts (a) and salivary glands (b) extracts of control and dengue virus inoculated mosquitoes.

result in the injection of very low quantum of virus during subsequent blood meal. It was surmised that this may be one of the factors responsible for causing a large proportion of subclinical infections in humans.

When mosquitoes take blood due to presence of anticoagulant "Apyrase" in saliva affects the normal clotting time in the midgut. The very fast clotting of blood in the midgut is said to be main factor for refractivity to microfilaria in *Armigeres subalbatus* mosquitoes. During this period about 80-85% of microfilaria get trapped in the clot and die and a few which are on the surface of the blood-bolus getting an opportunity to bore through the midgut and cross it. These are destroyed by immune responses in haemocoel and thorax[9]. In a similar study, blood clotting time in the midgut of *Aedes aegypti* mosquito is considered to have role in the selective and gradual increase of dengue hemorrhagic fever cases[10].

There is no other information on the association of esterase enzyme activity with virus multiplication. In the present situation it appears that the enhanced esterase activity in the midguts and salivary glands is associated with the multiplication of virus. Further detailed studies are required to surmise that higher expression of this enzyme is due to defense response to multiplication of virus in these organs.

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