Effect of Midgut Bacterial Flora of *Aedes aegypti* on the Susceptibility of Mosquitoes to Dengue Viruses

by

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Abstract

Aedes aegypti mosquitoes collected from the field were surface sterilized and microbial isolations were made from the midguts. Isolates belonging to various genera were obtained, of which the one most abundant isolate was Aeromonas culicicola. This was first isolated and described as a new species from Culex quinquefasciatus mosquitoes and during this study frequent isolations of this bacteria were also obtained from *Aedes aegypti* mosquitoes. It was selected to study its effect on the susceptibility of *Aedes aegypti* mosquitoes to dengue (DEN) viruses. The isolate was incorporated in the blood meal of mosquitoes supplemented with the DEN virus. This resulted in the increased susceptibility of mosquitoes to the DEN virus. To further confirm this observation the experiments were repeated with E. coli, which also resulted in an increased susceptibility of mosquitoes to virus. To further investigate the effect of isolate on the susceptibility of mosquitoes to the virus, mosquitoes were treated with antibiotics to reduce the internal flora of mosquitoes. The treatment of antibiotic and incorporation of bacterial isolates in the blood meal did not show such an increase in the susceptibility of mosquitoes to the virus. The results suggest that there is a possibility that the quality of water in which *Aedes aegypti* mosquitoes breed and the bacterial flora they carry in their midgut might also have a role in determining their susceptibility to the virus.

Keywords: *Aedes aegypti*, Aeromonas culicicola, antibiotics, dengue virus, midgut flora, susceptibility.

Introduction

Mosquitoes serve as the obligate intermediate hosts for numerous diseases that collectively are a major cause of human mortality and morbidity worldwide. In India, *Aedes aegypti* is considered to be an important vector of dengue[1]. Mosquitoes show varied susceptibility to different pathogens that they transmit. Recently, it has been shown that mosquitoes offer both cellular and humoral responses to pathogens. There has been very little work done to understand such responses in mosquitoes with regard to arboviruses. The susceptibility of mosquitoes to arboviruses is

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considered as quantitative trait loci and various intrinsic and extrinsic factors, including genetic factors, have been ascribed to this[1].

Mosquitoes harbour a large number of bacteria in their midgut. The midgut barrier is considered to be one of the major factors which governs the susceptibility of mosquitoes to arboviruses. Any pathogen that enters a mosquito through blood meal comes in contact with the midgut where it interacts with the epithelial cells and the resident bacteria of the midgut. There is very little information available on the effect of the resident microbial flora on the susceptibility of mosquitoes to viruses. It had been shown that certain antibiotics reduced the bacterial flora in the mosquito midgut, which in turn increases the infectivity of *Plasmodium falciparum* in the *Anopheles* mosquitoes[2]. Such effects have not been investigated for the susceptibility of mosquitoes towards the viruses. Therefore, the present study was conducted to understand the effect of bacteria on the susceptibility of mosquitoes towards the viruses. The most abundant and frequent bacterial isolate obtained from *Aedes aegypti* was studied to determine its effect on the susceptibility of mosquitoes to the dengue virus.

Materials and methods

Mosquito species

The *Aedes aegypti* mosquito species used in the experiments were obtained from laboratory colonies maintained at the National Institute of Virology, Pune. The mosquitoes were maintained at a temperature of 28ºC (± 2ºC) and relative humidity of 80 (± 5%).

Antibiotics treatment

The resident gut flora of the *Aedes aegypti* mosquitoes was cleared using the protocol described by Toure et al[3]. Briefly, the cages were wiped with 70% alcohol before placing the pupae. Emerging adult mosquitoes were fed daily with a mixture of penicillin (10 microgram/ml), streptomycin (10 microgram/ml) and gentamycin (15 microgram/ml) in a 10% sterile sucrose solution on sterile cotton balls for three consecutive days. Sugar pads were removed and mosquitoes were starved for 24 hours before receiving blood meal containing bacterial isolate. After feeding, mosquitoes were given 10% sterile sugar solution on sterile cotton balls daily till they were dissected.

Virus strain

Dengue-2 (DEN-2) virus strain (9012384) was isolated from a patient suffering from dengue haemorrhagic fever during an epidemic, which occurred at Chirimiri, India. Viral stocks used for experiments were prepared in mice.

Bacterial strains

E. coli DH 5 alpha was used as control. *Aeromonas culicicola* MTCC 3249 was isolated earlier from the midgut of *Culex quinquefaciatus*[4].

Infection of mosquitoes through membrane

Dilutions of DEN virus were made in defibrinated white leghorn fowl blood. Four to five-days-old female *Aedes aegypti* were fed on the DEN-infected blood through an artificial membrane, Parafilm (American

National Can, Greenwich, CT 06836, U.S.A.) as described by Harada, Matsuoka & Suguri[5]. After feeding, mosquitoes were maintained for 10 days on 10% glucose solution in distilled water. The presence of antigen was checked after 10 days.

Detection of virus in mosquitoes

Detection of the DEN viral antigen in the head squashes of the mosquitoes was done on the tenth post-infection (PI) day using the indirect immunofluorescence antibodies (IFA) technique[6].

Results and discussions

Effect of bacterial isolates on the susceptibility of mosquitoes to viruses

Mosquitoes were fed with blood meal, which contained the DEN virus and bacterial isolate *Aeromonas culicicola* or *E. coli*. The presence of the viral antigen was determined on the tenth day. Similar experiments were repeated where mosquitoes were treated with antibiotics so as to eliminate the normal bacterial flora of the midgut. They were treated with antibiotics as mentioned earlier. In both the experiments, control was the batch of mosquitoes which did not receive the bacterial isolate in the blood meal containing the respective virus.

Effect of bacterial isolate and E. coli on susceptibility of Aedes aegypti to DEN virus

The untreated *Aedes aegypti* carrying normal gut flora, when infected orally with the dengue virus, showed that 12.5% of the mosquitoes were positive for the antigen in head squashes on the tenth day. There was more than a two-fold increase in the overall susceptibility to the dengue virus with the incorporation of bacterial isolate and *E. coli*. However, the increase in susceptibility was not seen when the antibiotic-treated mosquitoes were used (Table).

Table: *Susceptibility of Aedes aegypti mosquitoes to dengue virus*

	Percent mosquitoes positive (SD)	Fold increase in susceptibility
Untreated		
A. Control	12.5 (4.2)	–
B. Aeromonas culicicola	27.8 (9.6)	2.2
C. E. coli	27.8 (4.8)	2.2
Antibiotics treated		
A. Control	15.3 (2.4)	–
B. Aeromonas culicicola	9.7 (6.4)	–
C. E. coli	13.9 (4.8)	–

Number of bacilli used; B $4.2x10^7$/ml; C: $9x10^7$/ml
Post-feeding titre range in three replicates: 5.5 – 6.0 log MID_{50}/0.02ml mouse i.c.
In batch 2, mosquitoes were treated with antibiotics.
Average data of three replicates

[The mosquitoes were fed with bacterial isolate during the infection with dengue virus by oral feeding with blood. The susceptibility of mosquitoes to the virus was checked by the presence of antigens in the head squashes using immunofluorescence assays]

Recently, it has been shown that the dengue viral entry through adsorption and penetration leading to infection is accomplished within two hours and carbohydrate residues may contribute to binding and penetration of the virus into the mosquito cells[7]. It is known that the entry of arboviruses into the gut cells is receptor-mediated. However, in the case of mosquitoes when they take blood meal, the formation of the peritrophic membrane starts within 20-30 min, which later on surrounds the blood bolus. Therefore, it is assumed that the entry of virus particles into the midgut epithelial cells must take place before the formation of the peritrophic membrane. In the orally-infected mosquitoes, soon after the blood meal the activity of the proteolytic enzymes starts, which also destroy the virus particles present in the blood bolus.

Earlier, it had been reported that *Cx. bitaeniorhynchus* treated with tetracycline showed an increased susceptibility of mosquitoes to the Japanese encephalitis (JE) virus[8]. The larvae of this mosquito species are found in slow running or stagnant water bodies and feed only on algae at immature stages. It is presumed that these mosquitoes have heavy resident microbial flora in the gut, which helps them in the digestion of algae. The treatment of antibiotics reduces their survival and also reduces the heavy load of normal gut flora. This probably increased the probability of the JE virus particles to bind to the receptors, which in normal course compete or block these receptors. In the past, similar observations had been made by Beier et al[9] that the reduction of normal bacterial flora in the mosquito midgut increased the *Plasmodium falciparum* infection rates.

It has also been reported that the incorporation of bacterial flora through the blood meal get established in the midgut as resident bacteria[3]. In the present work when bacterial isolates were incorporated in the blood meal of *Aedes aegypti* mosquitoes supplemented with dengue virus, they produced a significant change in their susceptibility to the DEN viruses, of which they are a natural vector. *Aedes aegypti* mosquitoes are fresh-water breeders and are likely to have less load of microbial flora. The incorporation of these isolates has produced a very high change in their susceptibility to the DEN virus.

It has been shown recently that certain species of mosquitoes produce a defense response when challenged with bacterial or protozoal pathogens[10]. At the present juncture it is difficult to explain whether the differences in the susceptibility observed by the incorporation of isolates caused different degrees of defense responses, which might result in an altered susceptibility of mosquitoes to viruses. However, the data suggest that there is a possibility that the quality of water in which mosquitoes breed and the bacterial flora they carry in their midgut might also have some cumulative effect on the various intrinsic and extrinsic factors which contribute to determining their susceptibility to the virus.

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