HORIZONTAL AND VERTICAL TRANSMISSION OF DENGUE VIRUS TYPE 2 IN HIGHLY AND LOWLY SUSCEPTIBLE STRAINS OF *AEDES AEGYPTI* MOSQUITOES

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Summary. – Isofemale lines of *Aedes aegypti* mosquitoes highly and lowly susceptible to dengue type 2 (DEN-2) virus (DEN(h) and DEN(l), respectively) were established by oral feeding and individual rearing. The susceptibility at F13 generation was found to be 61% and 25% for the DEN(h) and DEN(l) line, respectively. The virus-infected mosquito females were allowed to probe on bovine albumin phosphate saline pH 7.2 (BAPS) through membrane feeders. The presence of virus in the probed BAPS was determined either by ELISA or by intrathoracic (i.t.) inoculation of mosquitoes or by both methods. The rate of oral transmission of virus was found to be 2 times higher in the DEN(h) isofemale line than in the DEN(l) one. Similarly, vertical transmission rate of the virus was found to be 7 times higher in the DEN(h) line. When batches of eggs obtained from infected female mosquitoes were allowed to hatch after two months the vertical transmission rate of the virus was very high. It is possible that, at room temperature, the virus gets an opportunity to multiply and increase its copy number in the quiescent embryos. The progeny obtained from the infected mosquitoes was found to be capable of transmitting the virus horizontally when allowed to probe on BAPS through the membrane feeder. This is the first report demonstrating horizontal transmission of DEN-2 virus by mosquitoes infected through vertical transmission. The higher vertical transmission rate of the virus in the progeny obtained from the eggs dessicated for a longer time and the horizontal transmission of the virus from the progeny is of very high epidemiological significance.

Key words: Aedes aegypti; dengue virus type 2; isofemale lines; horizontal transmission; transovarial transmission; vertical transmission

Introduction

Dengue (DEN) is a serious health problem in many tropical countries of the world. This disease is hyperendemic in southeast Asia, where more severe forms, DEN hemorrhagic fever and shock syndrome, are of major public health concern (Halstead, 1980). The origin of DEN viruses is unknown. Their natural history suggests that, biologically, this virus is highly adapted to their mosquito hosts and they have been most likely mosquito viruses prior to adaptation to lower primates and humans (Gubler, 1997).

Mosquito populations in general exhibit variation in their susceptibility to arthropod-borne viruses. Studies on the selection of DEN virus-susceptible and -refractory lines of *Aedes aegypti* mosquito have shown that virus susceptibility is controlled genetically and it is polygenic and recessive (Miller and Mitchell, 1991). Several studies in the past have also shown the occurrence of transovarial transmission of DEN viruses (Joshi *et al.*, 1996; Ahmad *et al.*, 1997; Rodhain and Rosen, 1997). The aims of the present study were (i) to determine the association of oral susceptibility of *A. aegypti*

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Abbreviations: BAPS = bovine albumin phosphate saline pH 7.2; DEN = dengue; i.c. = intracerebral(ly); IFA = immunofluorescence assay; i.t. = intrathoracical(ly); p.i. = post infection

Table 1. Susceptibility of different generations of DEN(h) and DEN(l) lines of *A. aegypti* to DEN-2 virus given by oral route

Generation	Feeding		*A. aegypti*		
	Suspension (log LD_{50}/0.02 ml)	n	DEN(1)	DEN(h)	P values[a]
F1	3.3[b]	35	4/36 (11.1)[c]	9/48 (18.7)	0.513
F3	3.1	56	4/16 (25.0)	10/24 (41.6)	0.456
F5	3.5	78	8/39 (20.5)	14/42 (33.0)	0.295
F7	3.7	91	4/32 (12.5)	15/60 (25.0)	0.254
F8	3.3	110	3/48 (6.3)	17/71 (23.9)	0.024
F10	3.4	141	6/29 (20.7)	19/57 (33.3)	0.332
F12	4.4	192	7/33 (21.2)	22/41 (53.6)	0.009
F13	4.1	ND	7/28 (25.0)	24/39 61.5)	0 006
Total			43/261 (16.5)	130/382 (34.0)	

[a]Differences between DEN(l) and DEN(h) lines.
[b]Titer for infant mice by i.c route.
[c]Positive/total (%).
n = No. of females used for next progeny in both lines.
ND = selection not done.

mosquitoes to DEN viruses with their virus transmission capabilities and with vertical transmission rate of the virus, and (ii) to find whether mosquitoes, which become infected by transovarial passage can transmit the virus horizontally.

Materials and Methods

Mosquitoes

DEN(h) line of A aegypti. The mosquitoes employed for selection of this line were collected from Balagola District, Karnataka, India. The isofemale line was developed by individually confining the females fed on blood-virus mixture. After oviposition, head squashes of individual females were screened for the presence of DEN-2 virus antigen using the indirect immunofluorescence assay (IFA) (Mourya *et al.*, 1994; Ilkal *et al.*, 1984). The progeny obtained from the DEN-2 virus-positive mosquitoes were pooled. These mosquitoes were again infected with DEN-2 virus as described above. The procedure of selecting the DEN-2 virus-positive mosquito progeny was followed for 12 generations.

DEN(l) line of A. aegypti. The mosquitoes employed for selection of this line were from a laboratory colony maintained in this laboratory since the last 25 years. This line had lower susceptibility to DEN virus as compared to the DEN(h) line. The DEN(l) isofemale-line was developed similarly using the progeny obtained from the DEN-2 virus-negative females. All the mosquitoes were reared in an insectary maintained at 28 ± 2°C and 70–80% relative humidity.

Virus. DEN-2 virus, Jammu strain was isolated from a patient suffering from DEN hemorrhagic fever during an epidemic which occurred in Jammu, India. Stock used in this study was prepared in infant mice by intracerebral (i.c.) inoculation and used at 8th passage level.

Infection of mosquitoes through membrane. Dilution of virus was made in defibrinated chicken (white leghorn) blood. Four- to five-day-old female mosquitoes were fed on the infected blood through an artificial membrane (Parafilm, American National Can, USA) as described by Harada *et al.* (1996). First a stock of feeding suspension was prepared and then it was distributed into different feeding cups to feed different lines of mosquitoes simultaneously.

Infection of mosquitoes by i.t. inoculation. Four- to five-day-old female mosquitoes were inoculated i t. with approximately $10^{2\cdot1}$ MID_{50} in 0.2 µl (Rosen and Gubler, 1974).

Detection of virus in mosquitoes. DEN-2 viral antigen in head squashes and salivary glands of mosquitoes was detected by IFA (Ilkal *et al.*, 1984).

Detection of horizontal transmission of virus in mosquitoes. At different days post infection (p.i.) mosquitoes of each line were allowed to probe in batches of 25 on mini-feeders. To determine the oral transmission of virus the feeders were loaded with 100 µl of BAPS. After probing, presence of viral antigen was detected in BAPS by ELISA or by i.t. inoculation of mosquitoes or by both methods (Mourya *et al.*, 2000). Immediately after probing, head squashes of the mosquitoes were prepared for detection of viral antigen.

Detection of vertical transmission of virus in mosquitoes. Mosquitoes were infected with virus either by oral or i.t. route. The inoculated mosquitoes at day 3 p.i. were allowed to have the 1st, 2nd, 3rd and 4th bloodmeal on uninfected chickens and lay eggs. Thus obtained eggs of G1, G2, G3 and G4 cycle were conditioned for 3 to 4 days. These were then allowed to hatch by immersing in water. Some of the G2 cycle eggs obtained from the females were stored at room temperature in desiccators and allowed to hatch after one and two months of egg laying The pools of III to IV instar larvae and emerging adults were triturated in PBS containing 0.1% CHAPSO (Sigma). The obtained suspensions were centrifuged at 10,000 x g for 30 mins and the supernatants were tested for DEN-2 virus antigen by an antigen capture ELISA using a flaviviruses cross-reactive MAb (Hx-2) as described by Gore *et al.* (1990). The ELISA-positive pools were inoculated in uninfected *A aegypti* mosquitoes by i.t. route.

Table 2. Horizontal transmission of DEN-2 virus by two lines of *A. aegypti* mosquitoes infected by oral or i.t. route

Route of infection	Day p.i.	Transmission in *A. aegypti* lines[a]	
		DEN(l)	DEN(h)
Oral[b]	10	–	+
Oral	10	–	–
Oral	14	+	+
Oral	14	–	+
Oral	18	+	+
Oral	18	–	–
Oral	24	–	–
i.t.[c]	14	+	+
i.t.	14	+	+

[a]Mosquitoes positive by IFA at day 10 p.i., which were inoculated with BAPS after probing by infected mosquitoes.
[b]Post feeding virus titer of $10^{4.4}$ LD_{50}/0.02 ml in infant mice by i.c. route.
[c]Virus dose of $10^{1.2}$ MID_{50}/0.2 µl in mosquitoes.

Detection of horizontal transmission of virus in mosquitoes infected by transovarial route. In some experiments, the progeny obtained from the infected mosquitoes was maintained in the insectary up to day 24 post eclosion. The females (progeny) were allowed to probe on membrane feeders at various days p.i. to detect oral transmission of virus.

Results

The susceptibility of two isofemale lines, DEN(h) and DEN(l), to DEN-2 virus is shown in Table 1. Initially, the differences in the susceptibility of different filial generations were not significant but they increased to significant levels in the F-8 generation. We assume that during the process of isofemale line selection, a small proportion of increased susceptibility in the DEN(h) line could arise due to transovarial transmission of the virus since the eggs (G2) of virus-positive females were pooled every time. With DEN(l) line this might not be the case since the progeny were taken from the mosquitoes which were found virus-negative by head squash examinations.

The horizontal virus transmission potential of both the lines, determined by membrane feeder method, showed that only 2 of 7 batches in the DEN(l) line realized transmission, while there were 4 of 7 in the case of DEN(h) line (Table 2). These experiments were conducted at F-12 generation when the susceptibility of these two lines was high (Table 1). However, no difference in the horizontal transmission of the virus in these two lines infected by i.t. route was noticed (Table 2).

Vertical transmission experiments showed that 1.2% of pools were positive in the DEN(l) line while it was 7.1% in the case of DEN(h) line. This happened when the progeny was obtained from the eggs of females infected by oral route at F-13 generation and the eggs were hatched within 3 to 4 days of egg laying (Table 3). On the other hand, there was no such difference in vertical transmission rate of the virus when the progeny was obtained from the females infected by i.t. route (Table 4). The pools, which were found positive by ELISA, were also found positive by the i.t. inoculation method.

The larvae obtained from the eggs after 30 and 60 days of incubation at room temperature, whose parent females were infected by i.t. route, showed about 50–63% of pools positive (Table 4). It was also interesting to note that the progeny of mosquitoes, obtained from the orally infected females, transmitted virus orally when incubated for different periods (Table 5).

Table 3. Vertical transmission rate of DEN-2 virus in two lines of *A. aegypti* mosquitoes infected by oral route

Gonotropic cycle	Sex	Total No. of mosquitoes/ No. of pools	No. of positive pools[a]
A aegypti DEN(1) infected by oral route (110)			
G1	L	350/6	0
G2	L	350/6	0
G3	L	350/6	0
G4	L	200/4	0
G1	M	550/8	0
G2	M	750/11	1
G3	M	600/11	0
G4	M	200/4	0
G1	F	300/5	0
G2	F	550/8	0
G3	F	400/7	0
G4	F	350/6	0
Total		4950/82	
A. aegypti DEN(h) infected by oral route (100)			
G1	L	300/5	0
G2	L	350/6	0
G3	L	350/6	0
G4	L	350/6	0
G1	M	350/6	0
G2	M	400/7	1
G3	M	450/7	1
G4	M	400/5	2
G1	F	250/5	1
G2	F	350/6	1
G3	F	350/6	0
G4	F	325/5	0
Total		4225/70	

The F-13 generation of mosquitoes was used. Numbers of parent females are in parentheses. L = larvae; M = males, F = females.

Table 4. Pools (%) positive for vertical transmission of DEN-2 virus in two lines of *A. aegypti* mosquitoes infected by i. t. route

	DEN(l) line			DEN(h) line		
Eggs hatched	3-4 days	30 days	60 days	3-4 days	30 days	60 days
Larvae (G1-G4)	7.7	50.0	56.0	7.1	50.0	63 0
Males (G1-G4)	0.0	ND	ND	22 2	ND	ND
Females (G1-G4)	8.3	ND	ND	0.0	ND	ND
Total	5.9	50.0	56.0	8 8	50 0	63.0

The F-13 generation of mosquitoes was used. Pools were positive by ELISA and the i t. inoculation.
ND = not done.

Table 5. Horizontal transmission of DEN-2 virus by membrane-feeder in two lines of *A. aegypti* mosquitoes infected by transovarial route

Day p.i.	Transmission[a]	
	DEN(l) line	DEN(h) line
10	–	–
10	–	–
14	–	–
14	+	–
18	–	+
18	–	–
24	–	–
24	–	+

Twenty-five females were allowed to probe on BAPS in each experiment. The F12 generation of mosquitoes was used.
[a]Mosquitoes positive by IFA at day 10 p i. which were inoculated with BAPS after probing by infected mosquitoes.

Discussion

The results show that it is difficult to raise an isofemale line highly susceptible to the virus ensuring that it does not have a history of transovarial transmission of the virus. The difficulty in selection of such an isofemale line is acute every time unselected males are allowed to mate with selected female progeny. Theoretically, the possibility of increased susceptibility due to some of the individuals becoming virus-positive via transovarial route does exist. Our DEN(h) line had higher number of virus-positive mosquitoes; perhaps due to this fact the transmission rate was higher.

Oral virus transmission in the DEN(h) line was higher than that in the DEN(l) line. Midgut factor might have also played an important role due to which higher number of mosquitoes were virus-positive in the DEN(h) line, which ultimately resulted in a higher vertical transmission rate of the virus. The mosquitoes infected by i.t. route did not show such a difference. The role of the mesentronal barrier was also observed in the case of Chikungunya virus (Mourya *et al.*, 1998).

It was interesting to note that vertical transmission rate was very high in the progeny obtained from eggs stored at room temperature for one or two months. In almost all the earlier laboratory studies the progeny obtained from the infected mosquitoes have been processed for detection of vertical transmission of the virus within a couple of days of egg laying. It is probable that when progeny were obtained within 3–4 days from the eggs, perhaps many of the individuals contained a small amount of virus. That is why a low vertical transmission of virus has always been detected. Eggs of *A. aegypti* contain fully-grown larvae inside. It seems that the virus undergoing transovarial passage, gets an opportunity to multiply in the quiescent embryo, therefore many of the larvae which harbored virus by this route, had detectable level of virus. Therefore the virus titer in these pools reached such a level that ELISA and the i.t. inoculation test could detect it.

Recently, Joshi *et al.* (1996) have reported a high minimum infection rate for DEN-3 virus in the field-collected mosquitoes. Such high minimum infection rates have never been observed earlier. This could be possibly due to the fact that these field-collected mosquitoes originated from the eggs which had remained conditioned and quiescent in the field for a longer time. DEN viruses perhaps do not undergo latency inside the eggs (embryos) as it was thought to be the mechanism of persistence of the virus during inter-epidemic periods. Summing up, transovarial transmission of the virus is an important mechanism for its persistence during non-mosquitogenic periods, and the virus keeps multiplying in the embryos inside the eggs, perhaps at a slow rate due to very slow rate of metabolism.

Recently, Graham *et al* (1999) have shown the genetic basis of transovarial transmission of La Crosse virus in *A. triseriatus* mosquitoes Namely they have noticed that the selection of a mosquito line with low rate of transovarial transmission of the virus was quicker then the selection of a

mosquito line with high rate of transovarial transmission. It is difficult to say at this moment whether the selection of a line with high oral susceptibility to the virus is directly related to the selection of a line with higher transovarial transmission rate, or it is dual selection. A higher transovarial transmission of the virus in a line could be due simply to the presence of a higher number of susceptible infected females.

Allthough the existence of the phenomenon of transovarial transmission of DEN viruses has been demonstrated both in the laboratory and in the field, its actual epidemiological importance has not been clearly understood. All the earlier studies have only shown either virus surviving transovarial passage or demonstrated the presence of antigen in the progeny. Our present studies clearly demonstrated that the transovarial passage of DEN-2 virus is reality.

The rate of vertical transmission of the virus may depend on the species and strain of the virus and on the species or the geographic strain of the mosquito. Further work is needed to evaluate the role of this phenomenon during inter-epidemic periods in nature. If transovarial transmission of DEN viruses is very important factor for their persistence during inter-epidemic period the control of the immature stages of vectors and the elimination of breeding sources must be given priority.

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