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PERSISTENCE OF DENGUE-3 VIRUS THROUGH TRANSOVARIAL TRANSMISSION PASSAGE IN SUCCESSIVE GENERATIONS OF *AEDES AEGYPTI* MOSQUITOES

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Abstract. Progeny of *Aedes aegypti* mosquitoes infected intrathoracically with dengue-3 virus was reared to subsequent generations. In each generation, blood-fed females were confined individually and the eggs obtained from the transovarially infected females were pooled. The seventh generation obtained from the infected parental mosquitoes showed that virus could persist in mosquitoes in successive generations through transovarial passage. The rate of vertical transmission initially increased in the few generations (F_1-F_2), but in subsequent generations it was found to be steady. Parental mosquitoes inoculated with virus showed higher mortality than the diluent-inoculated controls. There was an increase in the larval duration of transovarially infected batches at the seventh generation when compared with uninfected control mosquitoes. The fecundity and fertility of the transovarially infected batches of mosquitoes was also affected when compared with the controls. This is the first report demonstrating persistence of dengue virus in the successive generations of mosquitoes infected through vertical transmission. These observations, which have great epidemiologic importance, suggest that vector mosquitoes may play an important role in the maintenance of virus in nature, and that mosquitoes may act as reservoirs of these viruses.

INTRODUCTION

Dengue is hyperendemic in Southeast Asia, where a more severe form, dengue hemorrhagic fever and shock syndrome, is a major public health concern.[1] Dengue viruses are members of the genus *flavivirus*. They are plus-sense, ssRNA viruses that cause dengue in humans. Their natural history suggests that biologically these viruses are highly adapted to their mosquito hosts and they were most likely mosquito viruses prior to becoming adapted to lower primates and humans.[2] These viruses are maintained in a "human-mosquito-human" cycle. *Aedes aegypti* is considered to be the main vector of these viruses. In addition to the maintenance of dengue virus at a low level of transmission within the human population, transovarial transmission is considered to be an important aspect in its maintenance during inter-epidemic periods. The role of viral maintenance during inter-epidemic periods in species other than its primary vectors has also been suggested.[3]

There are many reports of transovarial transmission of dengue viruses.[3–5] However, in the Rajasthan State of India, it has been shown to be a common, natural phenomenon in *Ae. aegypti*.[4] In a recent study, Mourya and others[5] have shown that transovarially infected mosquitoes can orally transmit the virus. This study also suggested that when eggs obtained from infected females are hatched after several weeks of incubation at room temperature, the rate of vertical transmission increases. It has been suggested that at room temperature the virus has an opportunity to multiply and increase its copy number in the quiescent embryos. Occurrence of this phenomenon in nature may be advantageous for maintenance of this virus. These studies indicate that prior to summer if mosquitoes gets an opportunity to feed on a viremic host and females lay eggs in microniches where some proportion of the eggs survive through the inter-epidemic season, the emerging adults may initiate the "human-mosquito-human" cycle. The present study also envisages these observations in understanding the possible role of *Ae. aegypti* in the maintenance of virus through transovarial passage over different generations.

MATERIALS AND METHODS

Mosquitoes. The mosquitoes used in the present study were obtained from the laboratory colony maintained at the Desert Medicine Research Centre (Jodhpur, India). This colony originated from the mosquitoes collected in Jodhpur City in Rajasthan State, and has been maintained for 10 years.

Virus. The dengue-3 virus strain (633798) used in the study was obtained from National Institute of Virology (Pune, India). It was originally isolated from a febrile patient with dengue fever in Thailand in 1963, and was used at 21st mouse passage level.

Infection of mosquitoes. Four-to-five day-old *Ae. aegypti* females were infected with virus by intrathoracic inoculations.[6] The dose of inoculum each mosquito received was 1.2 logs/0.2 µl of the 50% mean infective dose (MID_{50}). The infected mosquitoes were held in small cages covered with mosquito netting, and incubated at 25 ± 4°C and a relative humidity of 80 ± 5%. Three days after inoculation, the mosquitoes were provided a blood meal on healthy chickens and then held for egg laying. The procedure used for obtaining F_1 generation adults reared from eggs of the first, second, and third gonotropic cycles (G1, G2, and G3) was the same as that previously reported.[7] The eggs were allowed to hatch after conditioning of 3–4 days and larvae were reared to adults. After the G4 cycle eggs were laid, some of the parental females were tested for the presence of virus by screening head squashes by an indirect fluorescent antibody (IFA) test.[8]

Raising of progeny from transovarially infected mosquitoes. Eggs obtained from all gonotrophic cycles were pooled and allowed to hatch. The F_1 progeny obtained were reared to adults. Male mosquitoes (3–4 days after emergence) were tested for the presence of dengue antigen using IFA tests as described earlier. Females were allowed to feed on fresh 1–2-day-old chickens, 200 randomly selected, fed, female mosquitoes were confined individually for egg laying. Head squashes of the surviving spent females were tested for the presence of dengue antigen. Eggs of the females found positive for virus antigen were pooled and allowed to hatch. The F_2 progeny

TABLE 1

Percent mortality in virus-inoculated mosquitoes*

Days post-infection	Dengue-3 virus inoculated†	Diluent inoculated
1	12.0	3.0
3	3.0	1.0
5	4.7	2.0
7	3.7	0.7
9	3.3	1.7
11	7.0	3.3
13	2.7	1.7
Total	36.4	13.4

* N = 300 in both batches. $P = 0.01732$, by Fisher's exact test, when virus-inoculated mosquitoes and controls were compared.
† 1.2 logs/0.2 µl of a 50% mean infective dose (MID_{50}) in mosquitoes.

were reared similarly. This procedure was used up to the seventh generation.

Fecundity and fertility of transovarially infected mosquitoes. Batches of 200 first instar larvae obtained from the inoculated F_7 generation and first instar larvae obtained from the colony stock were placed in 500-ml beakers, each containing 250 ml of water. The larvae were fed as follows: 25 mg of feed/day/beaker for four days, followed by 50 mg for two days and 100 mg for the remaining period. Fresh feed was added to each beaker after changing the water every day. A daily count of larvae, pupae, and emerging adults was made. Emerging adults were kept in small cages for 4–5 days to ensure mating.

Fifty females were removed from these cages and weighed to get average unfed weight. After weighing, they were transferred to plastic vials (height = 5 cm, diameter = 3 cm) with nylon netting at the top. These were then allowed to feed on chickens through the nylon netting. Immediately after feeding, the weight of individual fed females was determined after anesthetizing them on ice. Each vial was provided with 10% glucose in cotton pads and a piece of wet blotting paper for egg laying.[9] After 3–4 days of feeding, an egg count was made. Females that did not lay eggs were scored as non-layers and were removed. The spent females were screened for the presence of antigen by an IFA test. The eggs obtained were conditioned in desiccators and allowed to hatch separately to assess the hatching rates. This procedure was also used for normal colony stock of mosquitoes as controls.

Confirmation of virus obtained by transovarial passage. The pools of some of third to fourth instar larvae obtained from the F_7 generation were triturated in phosphate-buffered saline containing 0.1% CHAPSO (Sigma, St. Louis, MO). These suspensions were centrifuged at 10,000 × g for 30 min. These pools were checked for the presence of virus by inoculating the suspensions into mosquitoes by intrathoracic inoculations.[6] An IFA test was performed on the inoculated mosquitoes on day 12 post-infection to detect viral antigen.

RESULTS

Results showed that there was a higher mortality in the virus-inoculated mosquitoes when compared with that of the diluent-inoculated control. The IFA test performed on the F_1 generation mosquitoes showed that 2.8% of the mosquitoes were positive for viral antigen, while parent females were cent percent positive.

When the progeny of the transovarially positive mosquitoes were selected by individually confining the females and pooling of eggs of only positive mosquitoes, there was an increase in the positivity of the mosquitoes. However, the positivity of mosquitoes in subsequent generations (F3 to F7) did not show any increase and was constant. It was interesting to note that females had higher rate of antigen positivity than males.

TABLE 2

Percentage of mosquitoes positive for dengue virus antigen in successive generations of *Aedes aegypti* mosquitoes*

	Males		Females		
	Tested	Number positive (%)	Tested	Number positive (%)	Total Percent positive
F_1	123	3 (2.4)	167	5 (3.0)	2.8
F_2	158	12 (7.6)	144	14 (9.7)	8.6
F_3	258	22 (8.5)	173	34 (19.7)	13.0
F_4	122	8 (6.6)	125	21 (16.8)	11.7
F_5	154	14 (9.1)	166	23 (13.9)	11.6
F_6	145	13 (9.0)	120	18 (15.0)	11.7
F_7	157	16 (10.2)	176	26 (14.8)	12.6

* P = 0.0088, by Fisher's exact test, when proportions for males and females were compared.

The pools of third-to-fourth instar larvae processed for confirmation of the presence of virus antigen showed that 5% (1 of 20) of the pools were positive and the head squash positivity of the inoculated mosquitoes was 35% (12 of 34).

Mortality in the immature stages and adults was higher in transovarially infected batches. Fertility and fecundity in transovarially infected batches were found to be low when compared with the control mosquitoes. To compare these parameters, we used a ratio (eggs laid/blood ingested)/(eggs hatched/eggs laid) reported by Mourya and others.[9] This showed that fertility and fecundity were significantly reduced ($P < 0.01$) in transovarially infected batches when compared with the controls.

DISCUSSION

Previous reports had considered the possibility of transovarial transmission of dengue virus.[10,11] However, since this phenomenon could not be demonstrated due to unavailability of sensitive methods, it did not receive the proper attention. Frier and Rosen[12] suggested that the entry of virus into the developing eggs takes place in the genital chamber of the

TABLE 3

Larval duration, fecundity and fertility in transovarially infected and control *Aedes aegypti* mosquitoes*

	Controls	Transovarially infected
Larval duration (days)	11.5 ± 0.83	12.6 ± 0.94
Larval mortality (%)	3.52	13.43
Average unfed weight of females (mg)	2.16	1.72
Average fed weight of females (mg)	3.22 ± 0.77	3.15 ± 0.73
Average blood ingested (mg)	1.06 ± 0.77	1.43 ± 0.73
Average number of eggs laid	86 ± 29	48.5 ± 19.4
Average number of eggs hatched	70.5 ± 28	27.7 ± 14.4
Eggs laid:blood ingested (EL:BI)	81	34
Egg hatched:eggs laid (EH:EL)	0.82	0.57
(EL:BI)/(EH:EL) ratio†	99 (48)	60 (40)

* Values are the mean ± SD. $P = 0.009$, $\chi^2 = 1.4$, by chi-square test, when the EL/BI and EH/EL ratio were compared.
† Values in parenthesis are the number of individuals.

females as the mature eggs are fertilized during oviposition. However, Graham and others[13] have recently identified the genetic basis of transovarial transmission of La Crosse virus in *Ae. triseriatus*. They showed that selection of a mosquito strain for a low rate of transovarial transmission was faster in its development than the strain with higher a higher rate of transovarial transmission. Data presented here showed that the virus could be detected up to the seventh generation if a selection pressure of transovarially infected females was maintained. The refractoriness of *Ae. aegypti* mosquitoes to both *flaviviruses* (yellow fever) and alphaviruses (chikungunaya) is a dominant genetic trait.[14,15] Although the situation with La Crosse virus in *Ae. triseriatus* cannot be generalized for dengue virus in *Ae. aegypti*, it can be surmised that in absence of such selection pressure it would not have been possible to detect virus in successive generations due to occurrence of high heterozygosity and a dominance of a low rate of transovarial transmission as shown by Graham and others[13] in the case of La Crosse virus.

It is known that susceptibility of *Ae. aegypti* to *flaviviruses* and alphaviruses is polygenic and a quantitative trait.[14,15] It has also been recently shown that susceptibility of *Ae. aegypti* to dengue virus is associated with vertical transmission rates, since higher transovarial transmission in a strain could be due to the presence of a higher number of susceptible, infected females.[5] In the present study, we have detected dengue viral antigen up to the seventh generation, and have observed an increase in the number of virus-positive mosquitoes in initial generations up to the F_2, which was then stabilized in subsequent generations. We believe that this also could be due to the selection of progeny from virus-positive mosquitoes. No additional increase in this factor in subsequent generations indicates that there may be genetic factors involved.

In recent studies by Mourya and others,[5] these investigators indicated that it is difficult to raise a dengue-susceptible isofemale mosquito line while ensuring that there is no transovarial transmission of virus occurring during the selection. The present study clearly suggests that vertical transmission of virus in successive generations is the most important factor involved in the maintenance of virus in nature. Mosquitoes can act as reservoirs for dengue virus. However, this phenomenon suggests that these were originally viruses of mosquitoes, which subsequently adapted to lower primates.[2] Conversely, we have observed mortality in the virus-inoculated and transovarially infected mosquitoes. Larval duration and fertility of the mosquitoes was also found to be affected in transovarially infected mosquitoes. It is difficult to explain how mortality in the adult mosquitoes was due to pathogenesis caused by the virus. Much work has been done on the pathogenesis of these dengue virus serotypes in mosquitoes.[16] However, most of the experiments used dengue virus serotype-2, probably because it appears to be more adapted to mosquitoes and because it is the most widely circulating serotype. A phylogenetic tree of these serotypes shows that serotype 3 is slightly different than serotype 2. It is also known that pathogenesis also depends on the virus strain, with less-adapted strain causing higher pathogenesis. Genetic distance studies suggest that the dengue-3 serotype is probably less adapted then the dengue-2 serotype. However, deleterious effects observed in present studies cannot be attributed to the virus strain alone. Additional studies in this area are needed.

It has been observed that a genetically engineered dengue virus strain in an intracellularly immunized, transgenic *Ae. aegypti* strain showed multiplication in midgut cells.[17] Tissue tropism of a virus strain may be an important factor responsible for survival of virus. During transovarial transmission, particularly when virus multiplies in quiescent embryos, the organ involved in virus multiplication may be an important factor in the longevity and survival of that host. A large number of innate immune responses of *Ae. aegypti* to different pathogens are known.[18] Studies on *Anopheles gambiae* have shown that when they are challenged with pathogens, immune-responsive genes are activated. It is interesting that none of the known immune-responsive genes are expressed in the ovary of these mosquitoes.[19] However, information on such innate responses to viruses in these mosquitoes is still scanty. It appears that fewer defense responses are made to viruses in the ovary, and that transovarial transmission is a suitable mechanism for their maintenance in nature. However, dengue virus does not undergo latency inside the eggs (embryos) as previously thought. This was believed to be the mechanism for persistence of virus during inter-epidemic situations. Virus undergoing transovarial transmission gets an opportunity to multiply in the quiescent embryo. It is possible that the multiplication of virus in different organs during embryogenesis or in later stages of life might vary in individual mosquitoes due to tissue tropism, virus strain, and genetic makeup of the host. These factors individually or cumulatively could contribute to the differential larval duration, fecundity, fertility, and to some extant mortality in transovarially infected mosquitoes.

Joshi and others[4] have reported a high rate of transovarial transmission of dengue-3 virus in mosquitoes collected from Jodhpur (Rajasthan State), India. Mourya and others[5] suggested that this could be due to the fact that these field-collected mosquitoes originated from eggs that had remained conditioned and quiescent in the field for a longer time. The rate of transovarial transmission may depend on the serotype and strain of virus and on the species or the geographic strain of mosquito. Therefore it is important to evaluate the competence of local vectors in each geographic area.[20] However, we believe that in the present situation the mosquito strain could have also played an important role, which supports higher rates of transovarial transmission. The mosquitoes used in the present studies were originally collected from Jodhpur area.

Although transovarial transmission of dengue virus has been demonstrated in the laboratory and in the field, the actual epidemiologic importance of this is not clearly understood. The data presented here suggest that the persistence of transovarial transmission in successive generations of mosquitoes is an important mechanism in the maintenance of virus during non-mosquitogenic situations. It has been demonstrated that mosquitoes that become infected by the transovarial route can actually transmit virus orally,[5] and the present data suggest that mosquitoes may act as reservoirs of these viruses. Moreover, the results are also indicative that in nature within heterogeneous population of infected and uninfected *Ae. aegypti*, there has been never a situation in which more than 20% of the female progeny from 100% infected females were infected by the transovarial route. Consequently, whereas the transovarial transmission maintains the

virus in nature only in optimum numbers, it never dominates the replacement pattern of infected mosquitoes by uninfected ones in a heterogeneous population (infected and uninfected), thus facilitating virus as an efficient parasite (non-lethal to persistence of vector population).

In India, transovarial transmission of Japanese encephalitis virus suggests that it is one of the mechanisms by which virus can survive through the inter-epidemic seasons.[21] Recent long-term studies carried out in southern India showed that the minimum infection rates of this virus in mosquito progeny are directly correlated with the activity of virus in that area (Thenmozhi VCRC, unpublished data). More work is needed to evaluate the role of transovarial transmission during inter-epidemic periods and its association with cases of dengue in the subsequent mosquitogenic season. This may be one of the supporting factors in predicting dengue outbreaks in a specific area.

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