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Research Articles

Vector competence of two Indian populations of *Culex quinquefasciatus* (Diptera: Culicidae) mosquitoes to three West Nile virus strains

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ABSTRACT

Background & objectives: Culex quinquefasciatus is one of the principal vectors of West Nile virus (WNV). The mosquito also acts as a bridge vector as it feeds on both birds and humans. In the background of the recent reports of WNV activity in Kerala and Assam with fatalities, a study was initiated to determine the growth kinetics and transmission mechanisms of three strains of WNV in two populations of *Cx. quinquefasciatus*.

Methods: Mosquitoes were infected by oral feeding and growth on different post-infection days was determined with the three strains. Horizontal transmission was determined by confirming sickness and mortality in infant mice after infected mosquito bite. F_1 generation eggs, larvae, pupae and adults of experimentally infected mosquitoes were screened for WNV to determine vertical (transovarial) transmission. Trans-stadial transmission was determined by detecting WNV in adult mosquitoes emerged from infected larvae.

Results: Both the mosquito populations replicated and maintained WNV for a prolonged period with high titers ($\geq 5\log_{10}$ PFU/ml). WNV could be detected in saliva from Days 2 to 32 post-infection. Horizontal transmission by both the populations could be established but no vertical transmission was observed. However, parenterally infected larvae transmitted WNV to adults.

Interpretation & conclusion: WNV has been isolated from >10 mosquito species from India, however, vector competence of none of the species has been studied. The present study demonstrates efficient transmission of WNV by *Cx. quinquefasciatus* mosquitoes. With its country wide prevalence and high vector competence, the mosquitoes could create grave consequences especially when virulent strains with potential to cause acute flaccid paralysis and death are circulating.

Key words: *Culex quinquefasciatus;* horizontal transmission; trans-stadial transmission; vector competence; vertical transmission; West Nile virus

INTRODUCTION

West Nile virus (WNV), a *Flavivirus* of family *Flaviviridae* has gained importance as a major pathogen of public health importance since its introduction to USA in 1999 where it caused mortality to more than a thousand people in the ensuing decade[1-3]. The virus has expanded its geographic range reaching Canada, Latin American countries, Caribbean Islands and Europe making it one of the most widespread arboviruses of public health importance[4-7]. The broad avian host range, susceptibility and transmissibility by a number of mosquito species helped the virus to establish in newer areas rapidly[5-7]. Recent outbreaks of WNV in Europe were characterized by neuro-invasive disease making it a neurotropic arbovirus[8-9]. Though, the infection remains clinically asymptomatic in majority of the cases, 5–10% cases develop neurological complications, *viz*. meningitis, encephalitis and neurological sequelae. WNV has been detected throughout the nervous system, *i.e.* spinal cord, cerebrum and cerebellum and even in the olfactory bulb of patients[10]. Recent studies in US have shown that approximately 51% of the reported cases were neuroinvasive which accounts for the highest number of neuroinvasive cases by an arbovirus[8]. However, no change in viral genetic factors could be associated with the high magnitude of the outbreak[2-3]. Circulation of different lineages of WNV in Europe and USA, especially lineages 1 and 2, which are highly neurotropic, poses a major threat.

WNV is a zoonotic virus maintained in nature between mosquitoes and birds. Horses and humans become incidental hosts upon the bite of infectious mosquitoes. A large number of avian species are found to be susceptible to WNV and develop viraemia, high enough to infect mosquitoes during blood meal. WNV infection has been detected in 326 avian species in US alone[11].

WNV has also been isolated from rodents in Nigeria[12]; fruit bat, *Rousettus leschenaulti* in India[13] and from Lake Frog, *Rana ridibunda* in Russia[12]. Experimental studies have also demonstrated viraemia in juvenile Alligators, *Alligator mississippiensis* sufficient enough to infect mosquitoes[12]. WNV is a unique arbovirus which is vectored by a broad range of mosquito species, as 62 mosquito species could be infected by feeding on a WNV infected host and >30 species could transmit the virus experimentally[12, 14-15].

In India, though WNV sero-prevalence was detected since 1950s, WNV has not been implicated for any major outbreaks involving case fatalities until the recent outbreaks in Assam and Kerala[16-18]. Human cases, which yielded virus isolations, were having only mild infections with flu like symptoms. Apart from humans, WNV has been repeatedly isolated from mosquitoes and once from a fruit bat[16]. The recent activity of WNV in Assam and Kerala, however, has caused concern in the background of circulation of virulent strains with the potential to cause acute flaccid paralysis and death[17-18]. Though, the probability of a WNV outbreak is low, should that happen, the consequences would be grave due to the prevalence of highly competent vector mosquitoes and amplifying hosts in the country. The present study is focused to determine the potential of two Indian populations of *Cx. quinquefasciatus*, an incriminated vector, in the replication and transmission of WNV.

MATERIAL & METHODS

Place of study

The study was carried out at the Microbial Containment Complex, National Institute of Virology, Pashan, Pune, India. All the experiments were carried out in a BSL-2 laboratory with facility to contain infected mosquitoes.

Cx. quinquefasciatus (Pune strain)

Pune strain of *Cx. quinquefasciatus* mosquitoes were procured from a cyclic colony maintained at the National Institute of Virology (NIV), Pune, India, for the last 20 years. The mosquito larvae were fed with a mixture of dog biscuit and yeast tablets in the ratio 3:1, while adults were fed on 10% glucose solution and maintained at 28±2°C with 80±5% relative humidity and 12:12 h light : dark regime.

Cx quinquefasciatus (Kerala strain)

This strain of mosquitoes was collected from Alappuzha district of Kerala state, India where WNV activity has been detected recently. Immature stages were collected and transported to NIV, Pune where a laboratory colony was established and mosquitoes from third generation onwards were used for experiments. The larval diet and maintenance conditions were similar to that described for the Pune strain.

Virus strains

Three WNV strains of lineage 1, *i.e*. Eg101, the prototype strain originally isolated from human serum during the 1951 outbreak in Egypt (Accession No. AF260968); G22886 (Accession No. AY944241), a mosquito isolate from a pool of *Cx. vishnui* collected from Sathuperi district, Tamil Nadu in 1958 and 68856 strain (Accession No. AY944239), isolated from a fruit bat (*Rousettus leschenaulti)* in 1968[13], were used in the study. All the strains had undergone several passages in mice and 3–4 passages in Vero E6 cell line before commencement of the study.

Determination of growth kinetics in mosquitoes

Three to four days-old mosquitoes (n=50 of each population) were allowed to feed overnight on infant Swiss albino mice (n=8) showing acute sickness (previously infected with WNV). Fully engorged mosquitoes were separated and maintained on a diet of 10% glucose. Five mosquitoes of each population were harvested randomly on every alternate day from 0 to 15-day post-infection (PI) and were processed to determine virus titer of each day PI by titration in Vero E6 cell line as described earlier[19]. In brief, individual pools were triturated in 1 ml MEM containing 2% FBS using a hand held homogenizer using sterile pestles (Sigma, USA), Millipore filtered (pore size = 0.22 µm), inoculated over confluent monolayer of Vero E6 cell line in quadruplicate and incubated for 1 h at 37ºC. Cultures were rocked every 15 min and after the completion of incubation, the inoculum was drained, washed with 1× PBS twice and fed with overlay medium. Cultures were incubated in a CO_2 incubator for four days, stained with amido black and the plaques were counted and determined the plaque forming units (pfu). Animal experiments were conducted according to the guidelines laid down by Institutional Animal Ethics Committee.

Determination of vector competence: Saliva from WNV infected mosquitoes (n=10) was extracted every alternate day PI artificially as described by Sudeep *et al*[19]. In brief, legs and wings of the mosquito were removed and proboscis was placed in a capillary tube containing chilled MEM supplemented with 2% FBS and allowed the mosquito to excite for 30 min. The contents of the

capillary tube was immediately transferred to 1 ml chilled MEM; Millipore filtered (0.22 µm) and titrated in Vero E6 cell line to determine virus titer. Saliva of five mosquitoes was pooled to determine virus titer.

Determination of horizontal and vertical transmission: Three to four day old mosquitoes (n=50) were infected either by infection on viraemic mice or by intrathoracic inoculation (IT) as described by Rosen and Gubler[20] and incubated for eight days at 28°C with 80–85% relative humidity. Five mosquitoes of each population were harvested randomly and determined the presence of WNV by indirect immunofluorescent assay (IFA) on Day 5 PI following the procedure described earlier[19]. In brief, the mosquito head squashes were prepared on glass slides, incubated with anti-WNV serum raised in mice (in-house) followed by incubation with secondary antibody (anti-mouse) tagged with FITC. Squashes were screened under a Nikon TS 120 microscope (Japan) for fluorescence. After observing 100% positivity in the tested mosquitoes, the rest of the mosquitoes were fed on two-days old infant mice (Swiss albino) to determine horizontal transmission. Mice were observed for sickness and brains of sick mice were harvested, screened and confirmed by RT-PCR targeting a 558 nucleotide fragment of E-gene. Fully-fed mosquitoes were allowed to oviposit and egg rafts from individual mosquito was kept in 50 ml glass beaker containing water with a pinch of larval food for hatching. A portion of eggs (~2000), IV instar larvae (n=2000), pupae (n=500) and adults (n=600) of each batch were stored and processed in Vero E6 cells to determine the presence/titer of WNV as described by Sudeep *et al*[19]. The adult mosquitoes, larvae and pupae were pooled at the rate of 20 per pool while ~50 eggs constituted a single pool. Individual pools of adult mosquitoes/eggs/larvae/pupae were homogenized and determined the presence of virus by RT-PCR[16] and plaque assay. Parent female mosquitoes used for vertical transmission were also screened individually following the same method. Experiments were conducted in triplicate (biological replicates) with each strain of WNV in the two mosquito populations. The procedure for determining horizontal and vertical transmission after oral infection is same except for the infection procedure where 3–4 days old mosquitoes (n=50 each) were starved for 12 h and allowed to feed overnight on WNV-infected Swiss albino mice (n=8 per group) showing acute sickness.

Determination of trans-stadial transmission (Infection by parenteral inoculation): Fourth instar larvae of *Cx. quinquefasciatus* mosquitoes were inoculated with WNV (Eg101) and grown to adults. Procedure for larval inoculation used was similar to adult inoculation as described earlier[20] with slight modifications. In brief, larvae were immobilized by keeping them on a Whatman filter paper above a block of wet ice and virus was inoculated through thoracic region with a fine capillary needle under a binocular dissection microscope. The larvae were immediately placed in water and fed on larval diet and grown to adults in the laboratory. The adult mosquitoes (n=41) were harvested on Day 7 of emergence, pooled (two pools; one with 20 adults and the other with 21 adults) and determined virus titer as described.

Trans-stadial transmission by oral feeding: Second instar larvae were fed on fragmented WNV (Eg101) infected larvae and allowed to become adults. Infection of larvae was done as described earlier, chopped to fine pieces on Day 2 of inoculation and fed to larvae. The larvae were maintained at 28°C and allowed to develop to adults. On Day 7 of emergence, the adults (n=46) were pooled and processed for virus detection in Vero E6 cells as described earlier. Of the two pools, one had 20 and the other had 26 adult mosquitoes. Statistical significance was determined by Dunnet's test using ANOVA.

RESULTS

Growth kinetics of different WNV strains in mosquitoes Eg101 strain

Studies with the strain demonstrated an identical pattern of growth in both the mosquito populations (Fig. 1). A rapid increase in virus titer was observed during the first three days followed by a plateau phase up to Day 9 PI. Following blood meal, viral load (mean value) increased to reach a maximum of $10^{4.44}$ ($10^{0.83}$) and $10^{4.5}$ ($10^{0.27}$) in *Cx. quinquefasciatus* Kerala and Pune populations at Day 5 PI respectively (Fig. 1). Using two-way factorial ANOVA we found no statistically significant difference in WNV titers in the two populations of *Cx.*

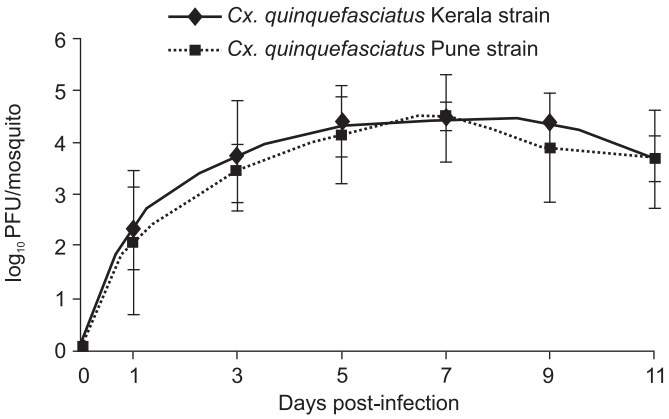

Fig. 1: Growth kinetics of WNV (Eg101) in two populations of *Cx. quinquefasciatus* mosquitoes.

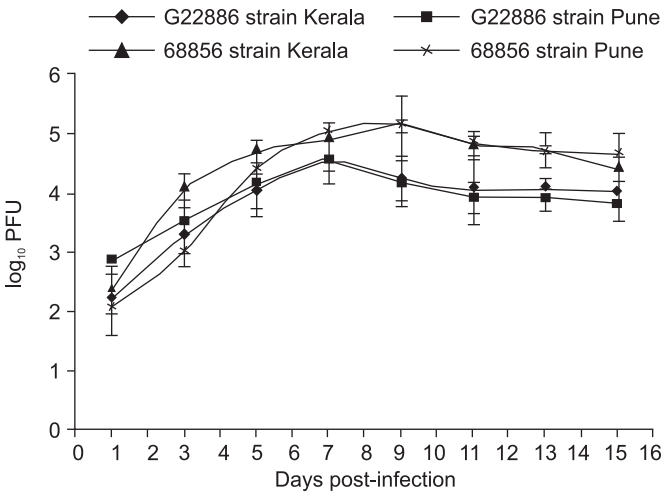

Fig. 2: Growth kinetics of G22886 and 68856 strains in the two populations of *Cx. quinquefasciatus* mosquitoes.

quinquefasciatus (F = 0.408; p = 0.528). This indicates that there is no measurable difference in WNV titers for any specific *Cx. quinquefasciatus* populations at different time points due to WNV infection.

Studies with G22886 and 68856 strains of WNV: G22886 strain showed an identical pattern of growth in both the mosquito populations yielding the highest titer on Day 7 PI (Fig. 2). The plateau phase has been extended up to Day 15 PI maintaining a titer of ~3log approximately. With 68856 strain, the Kerala population showed an exponential growth till Day 5 PI followed by a plateau phase. The Pune population, however, have replicated the virus slowly and reached the maximum titer during 7–9 days PI and maintained the titer till the end of the study period with slight decrease (Fig. 2).

In the study with the two WNV strains, using two-way factorial ANOVA, no statistically significant difference in titers could be observed (F = 1.381, p = 0.254). This indicates that there is no measurable difference in titers for any specific WNV strain at different time points of infection in *Cx. quinquefasciatus* mosquitoes. However, titers of the two WNV strains were significantly changed in due course of virus infection (F = 50.690; p<0.0001). Similarly, the nested effect of PI day and virus strain showed statistically significant difference in virus (F = 2.754; p =0.047), whereas statistically significant difference in virus titers was not observed when only virus and PI day were compared (Table 1).

Using two-way factorial ANOVA, when all the variables were considered together, no statistically significant difference in titers of the two WNV strains could be found (F = 0.011; p = 0.915). This indicates that there is no measurable difference in titers for any specific WNV strain at any time points of infection in the *Cx. quinquefasciatus* species. No significant difference in virus titer of two WNV strains in the two mosquito populations was observed (F = 0.036; p = 0.850). The titers of two strains were significantly changed in due course of infection (F = 49.304; p<0.0001). Similarly, post-infection day*virus interaction in titers was found statistically significant (F = 4.630; p=0.034). However, in post-infection day*mosquito population interaction (F = 0.001; p = 0.979) and virus*mosquito population interaction (F = 0.630; p=0.429) was not significantly differed (Table 2).

Table 1. Type III sum of squares analysis

Source	DF	Sum of squares	Mean squares	F	Pr > F
Post-infection day	1	22.042	22.042	50.690	< 0.0001
Virus	3	1.802	0.601	1.381	0.254
Post-infection day*virus	3	3.593	1.198	2.754	0.047

*Denotes the nested effect; DF–Degree of freedom; F–F distribution value; Pr–Probability.

Table 2. Type III sum of squares analysis

Source	DF	Sum of squares	Mean squares	F	Pr > F
Post-infection day	1	22.042	22.042	49.304	< 0.0001
Virus	1	0.005	0.005	0.011	0.915
Mosquito population	1	0.016	0.016	0.036	0.850
Post-infection day*virus	1	2.070	2.070	4.630	0.034
Post-infection day*mosquito population	1	0.0	0.0	0.001	0.979
Virus*mosquito population	1	0.282	0.282	0.630	0.429

*Denotes the nested effect.

Presence of virus in saliva

WNV in saliva could be detected from Day 5 PI onwards in both the populations of mosquitoes. The titer observed in Kerala and Pune populations on Day 5 PI was 1.4 and 1.17 pfu respectively. The maximum titer obtained was on Day 11 PI yielding ≥ 2.4 pfu in both the populations. Random collection of saliva from infected mosquitoes has shown the presence of virus even on Day 32 PI in the laboratory.

Presence of virus in inoculated and orally fed mosquitoes at the time of oviposition

Head squashes made of five mosquitoes from each population harvested on Day 5 PI in the IT inoculated

Table 3. Range of virus titer observed in individual mosquitoes at the time of oviposition

Virus strain	Mode of infection	Virus titer in parent at oviposition ♀♀ (log pfu/ml) (Pune) (Range)	Virus titer in parent at oviposition ♀♀ (log pfu/ml) (Kerala) (Range)	Virus titer in F_1 generation			
				Eggs	Larvae	Pupae	Adults
Eg101	Oral feeding	4.2–5.2	4–5.4	Virus could not be detected either by plaque assay or by RT-PCR			
Eg101	IT inoculation	4.9–5.3	4.9–6.2				
G22886	IT inoculation	3.2–4.2	3.5–4.2				
68856	IT inoculation	5.1–5.4	4. 7–4.9				

and orally fed mosquitoes were found positive for WNV by IFA. The titer of female mosquitoes of the two populations infected with different WNV strains after oviposition is given in Table 3. No significant difference was detected in the number of eggs laid by infected and uninfected (control) mosquitoes. Similarly, hatching rate of eggs was also found comparable.

Horizontal transmission of WNV in Cx. quinquefasciatus mosquitoes

The viraemic mice (n=8) had virus titer ranging from 5.2–5.6 and 4.2–5.1 pfu/ml of WNV (Eg101) in blood and brain respectively at the time of mosquito feeding. Virus titer in mosquitoes fed on viraemic mouse ranged from 3.5–4.2 and 3–3.3 pfu/ml on Day 8 PI in Kerala and Pune populations respectively in majority of the mosquitoes. Subsequent feeding on newborn mice by WNV-infected mosquitoes on Day 8 PI demonstrated horizontal transmission by both populations of mosquitoes as the mice become sick and died due to infection (Fig. 3). *Cx. quinquefasciatus* (Kerala) caused 100% mortality of mice on Day 4 PI while in *Cx. quinquefasciatus* (Pune), 100% mortality was observed only on Day 6 PI though sickness was seen on Day 3 PI in both groups (Fig. 3). Horizontal transmission to infant mice was also demonstrated by G22886 and 68876 strains of WNV by IT inoculated mosquitoes (Data not shown).

Vertical transmission of different strains of WNV in Cx. quinquefasciatus mosquitoes

Oviposition commenced from Day 5 post-feeding and continued till Day 17. Mode of infection, virus strains used and range of virus titer in each batch of mosquitoes at the time of oviposition, *etc* are given in Table 3. WNV could not be detected either in eggs laid by infected mosquitoes or in immatures (larvae and pupae) and adult mosquitoes of F_1 generation by RT-PCR and plaque assay despite processing ~8000 eggs and ≥ 12000 immatures and adults of F_1 generation.

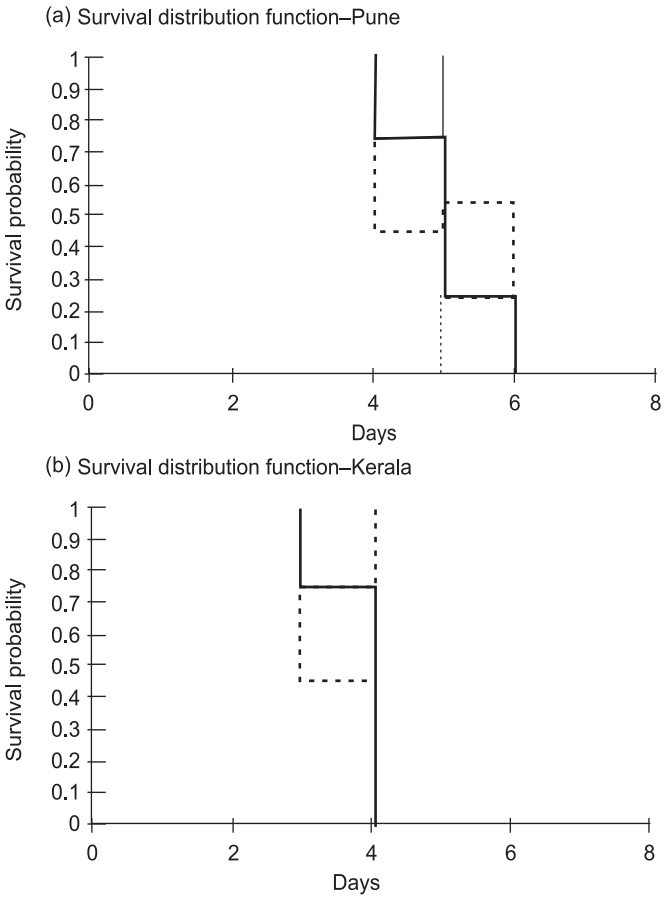

Fig. 3: Survival curve (Kaplan–Meier plot) showing mice mortality during horizontal transmission of WNV (Eg101) by (a) Pune, and (b) Kerala populations of *Cx. quinquefasciatus* mosquitoes.

Trans-stadial transmission of WNV in Cx. quinquefasciatus mosquitoes

Parenterally exposed mosquito larvae replicated WNV (Eg101) and transmitted the virus horizontally to adult mosquitoes (n=41). The two pools of mosquitoes processed had 2.8 and 3.4 pfu/ml on seventh day of emergence. However, *Cx. quinquefasciatus* larvae (n=46) fed on fragments of WNV infected larvae failed to transmit the virus trans-stadially to adults.

DISCUSSION

The epidemiology of WNV has changed during the last few decades, probably due to global warming, modern transportation means and demographic changes[1-2]. However, the mechanism of virus introduction and spread to new places is not yet fully understood[21]. Recurring outbreaks and prevalence of the virus in both USA and Europe demonstrates endemicity of the virus in both the continents. The number of WNV cases in 2012 has increased substantially in US and several European countries[3, 7-8].

In the present study, an evaluation of susceptibility, replication and transmission potential of two populations of *Cx. quinquefasciatus* mosquitoes were conducted using three strains of WNV. Laboratory reared mosquitoes were used for comparison as they were maintained in the laboratory for the last 20 yr; away from exposure to insecticides and other environmental factors that probably affect the susceptibility and vector competence of the mosquitoes. Since WNV cases were reported from Kerala, a population from the affected district was evaluated with laboratory reared mosquitoes to determine whether any changes in virus susceptibility and vector competence had occurred in the former. Rapid replication of the virus was observed in both the populations and the mosquitoes maintained the virus titers (≥5 pfu) for prolonged periods. Growth pattern and virus yield was found to be almost identical with the three strains in the two mosquito populations (Figs. 1 and 2). In a subsequent study, infectivity of the mosquitoes could be demonstrated up to Days 32 PI with Eg101 strain in the laboratory demonstrating the potential of the mosquitoes to be infective for life once infected with WNV (Sudeep unpublished data).

Horizontal transmission of WNV by different vector mosquitoes has been reported by several workers[22-26]. In the present study, both the mosquito populations were found competent to transmit the three WNV strains horizontally as virus could be detected in saliva from Day 5 PI. Successful transmission to infant mice by bite was also demonstrated after eight days of incubation. The mosquitoes demonstrated their potential to pick up virus during feeding on viraemic mice, replicate to >3 pfu on Day 8 PI and transmit to suckling mice. Though, both the mosquito populations transmitted the virus to suckling mice, early mortality was found in the mice inoculated with Kerala population than the Pune population (Fig. 3). The difference in transmission rate between the two populations could be probably due to long-term colonization of the latter. However, more studies are needed to confirm whether long-term colonization plays any role in vector competency. Richards *et al*[4] recently demonstrated different attributes responsible for transmission of virus by mosquitoes.

Vertical transmission of WNV has been demonstrated earlier in more than eight species of mosquitoes[22, 25]. However, in the present study with three strains of WNV, we could not demonstrate vertical transmission by the two Indian populations of *Cx. quinquefasciatus* despite the parent mosquitoes had very high titers at the time of oviposition. This observation supports the results of the study conducted by Eastwood *et al*[11] in Galapagos Islands in *Cx. quinquefasciatus*. Geographic variation has played an important role in the vertical transmission of WNV as successful vertical transmission of WNV was detected by Goddard *et al*[25] during their studies with *Cx. quinquefasciatus* mosquitoes collected from California. Though *Cx. pipiens*, the incriminated vector of WNV have shown vertical transmission on several occasions[23-24], Goddard *et al*[25], however, could not detect WNV either in wild caught mosquitoes or in the F_1 generation of experimentally infected *Cx. pipiens* collected from Shasta County in California. Similarly, the investigators failed to demonstrate vertical transmission of WNV in *Cx. tarsalis* mosquitoes collected from Riverside County in California, though vertical transmission of WNV has been demonstrated in the species repeatedly[22].

Vertical transmission of WNV in mosquitoes also corresponds to incubation period. Anderson *et al*[22] demonstrated vertical transmission of WNV by *Cx. tarsalis* mosquitoes on eggs laid on Day 5 PI. However, in *Cx. pipiens*, vertical transmission was observed only in eggs laid on Day 13 PI or more. In the present study, however, we could not demonstrate vertical transmission despite using eggs laid during 11–17 days PI. Earlier studies have shown that ovarian calyx of *Cx. quinquefasciatus* gets WNV infection after Days 8 PI while ovarian follicles become positive subsequently[27]. Our studies with the same species with oviposition on Days 11 to 17 PI gives sufficient incubation period for WNV infection of ovaries. However, we have observed WNV positivity of ovarian calyx on Day 13 PI by IFA. Probably, progeny of F_2 generation could demonstrate presence of the virus.

Trans-stadial transmission of WNV from infected larvae to adult was also demonstrated during the present study by both the populations by parenteral inoculation of IV instar larvae with Eg101 strain. However, our studies failed to demonstrate trans-stadial transmission of WNV when larvae were fed on fragments of infected larvae. The study was initiated to determine larval susceptibility to WNV, while feeding on infected larvae or other tissues and to determine their ability to transmit the virus

to adults, probably as a mechanism to maintain the virus in nature. Larvae getting infected with WNV by oral route in nature are remote, however, in larval habitats, dead larvae or adults are a potential nutrient source and there exists a remote possibility of consuming remnants of infected larvae/adults. In the study, however, the sample size was low and probably with high numbers of mosquitoes the results would have been different.

Present study demonstrated efficient horizontal transmission of WNV by two Indian populations of *Cx. quinquefasciatus* mosquitoes, which is incriminated as one of the primary vectors for WNV[4, 25]. The preliminary observations demonstrated high potential of the mosquitoes to pick up infection from viraemic host, replicate and transmit the virus to infant mouse. WNV isolation from *Cx. quinquefasciatus* mosquitoes has already been reported from India from field collected mosquitoes[28]. Though earlier infections were nonfatal, re-emergence of WNV could be catastrophic especially when virulent strains are circulating in certain parts of the country[17-18]. Therefore, WNV activity in India needs to be monitored carefully as *Cx. quinquefasciatus* and other ornithophilic mosquitoes are abundant in almost all parts of the country especially in the light of the recent reports of WNV associated deaths in humans in Kerala and Assam.

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