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Replication potential and different modes of transmission of West Nile virus in an Indian strain of *Culex gelidus* Theobald (Diptera: Culicidae) mosquitoes

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ABSTRACT

Background & objectives: Culex gelidus mosquito, an important vector of Japanese encephalitis virus, has shown to transmit West Nile virus (WNV), Kunjin and Murray Valley encephalitis viruses experimentally. An attempt was, therefore, made to study the replication kinetics and vector competence of an Indian strain of *Cx. gelidus* to WNV.

Methods: Mosquitoes were infected by both intrathoracic inoculation and oral feeding and studied the growth kinetics by determining the virus titre on different days post-infection (PI). Vector competence was studied by determining the presence of WNV in saliva on subsequent days PI. Horizontal transmission was determined by demonstrating infection in infant mice by bite of mosquitoes that were fed on viraemic mice previously. Vertical transmission was studied by screening progeny derived from infected mosquitoes. Trans-stadial transmission was determined by screening adult mosquitoes emerged from parenterally inoculated IV instar larvae.

Results: The mosquito replicated WNV to $7\log_{10}$ $TCID_{50}$/ml on Day 8 PI and maintained the titre for 14 days. Virus dissemination to legs and salivary glands could be detected, but not to ovaries up to Day 10 PI. The mosquitoes picked up infection from viraemic blood and transmitted successfully to infant mice on subsequent feeding. Trans-stadial transmission also could be demonstrated. However, vertical transmission could not be demonstrated.

Interpretation & conclusion: The replication potential, maintenance of WNV for prolonged periods and ability to transmit WNV experimentally makes the mosquito a serious threat to public health especially in the wake of active WNV activity in certain parts of India.

Key words *Culex gelidus*, horizontal transmission, vector competence, West Nile virus

INTRODUCTION

Culex gelidus Theobald (Diptera: Culicidae) has emerged as a dominant mosquito species in India, Southeast Asian countries and Australia in recent years. Native to Southeast Asia, the species has enhanced its geographical range starting from Pakistan and India in the west to the whole of Southeast Asian countries, China, Korea and Japan in the northeast, and new guinea and tropical regions of Australia in the east[1–3]. Recent studies have shown a surge in the population of *Cx. gelidus* replacing other dominant mosquitoes like *Cx. tritaeniorhynchus* etc to second position in India, Sri Lanka, Malaysia, *etc*[4–5]. The species is indicted as one of the prominent vectors of Japanese encephalitis virus (JEV) as evidenced by repeated isolations from different countries[1–2, 6–7]. A few other viruses of public health importance such as Ross River virus, Getah virus, Tembusu virus and Sindbis virus have also been isolated from wild caught *Cx. gelidus*[8–10]. Competency of the mosquito species to transmit West Nile virus (WNV), Kunjin virus (KUNV) and Murray Valley encephalitis virus (MVEV) have also been shown experimentally[11–12].

In India, *Cx. gelidus* has been prevalent in many of the states, viz. Maharashtra, Goa, Rajasthan, Karnataka , Kerala, Tamil Nadu, Andhra Pradesh, Assam, Uttar Pradesh, *etc*[13–20]. However, its population was negligible in comparison to other *Culex* species[15, 21]. Recent studies have shown a tremendous increase in *Cx. gelidus* population in south Indian states with percentages reaching >50%. During long-term studies carried out in Arcot district, Tamil Nadu, Kurnool district, Andhra Pradesh and Alappuzha district, Kerala, a surge in the mosquito population was recorded reaching 49.7, 68.05 and 57.9% of the total collection, respectively[4, 17–18, 22–23]. In Kurnool district, increased population of *Cx. gelidus* was observed in peri-urban areas, while in rural areas its presence was negligible[4]. In Alappuzha district, Kerala, the increase in

Cx. gelidus population showed an inversely proportional growth of *Cx. tritaeniorhynchus,* from 67% in 2009 to <22% in 2012.

In India, *Cx. tritaeniorhynchus* is playing the primary role in the transmission of JEV in both rural and peri-urban areas followed by other members of *Cx. vishnui* group. However, recent studies in south India have shown an unprecedented increase in the population of *Cx. gelidus* which might play an important role in JEV transmission especially in urban and peri-urban areas[4, 18]. Though JEV isolations from the species were less in number compared to *Cx. tritaeniorhynchus*, the minimum infection rate of both the species was found comparable[4]. *Culex gelidus* has already accounted for >10 JEV isolations in India despite its meagre population[7, 17, 24].

WNV has not been isolated naturally from *Cx. gelidus* either from India or elsewhere. However, recent studies in Australia have shown infection and transmission rates ranging between 84 and >50%, respectively in the mosquito to the New York (1999) strain of WNV[11]. Subsequent studies with KUNV virus, a variant of WNV in Australia has also shown 95% transmission rate in the mosquitoes[12]. In India, though several strains of WNV have been isolated from man, mosquitoes, bats, *etc*, the virus has not accounted for major outbreaks with case fatalities unlike in the US or Europe. However, re-emergence of WNV after a gap of nearly three decades with virulent strains has been reported from Assam and Kerala recently, causing case fatalities in both the places[25–26]. In view of the increasing population of the mosquito, its potential to transmit WNV experimentally and evidence of recent activity of WNV in India, a study was initiated to determine the replication potential and vector competence of an Indian strain of *Cx. gelidus* to WNV.

MATERIAL & METHODS

Study site

The study was conducted in a Bio-safety level-2 laboratory with adequate containment facilities at the Microbial Containment Complex, National Institute of Virology (NIV), Pune, India.

Mosquitoes

A laboratory colony of the mosquito was established from adult mosquitoes collected from Pashan, Pune. The F_1 generation mosquitoes were screened for the presence of arboviruses by inoculating random samples in Vero E6 cell line for two consecutive passages and were found to be devoid of any infection. Mosquito larvae were fed on a mixture of yeast powder and dog biscuit (3:1) while adults were maintained on a diet of 10% glucose. Female mosquitoes were provided with 5–6 wk old fowls for blood meal on alternate days. Infected mosquitoes were kept in plastic jars inside mosquito cages and all the experiments were carried out inside a bio-safety level-2 laboratory, which has containment facility to prevent escape/entry of mosquitoes. Normal as well as infected mosquitoes were maintained at 28 ± 2°C with 80 ± 5% relative humidity and 12 : 12 h light : dark regime.

WNV

The prototype strain of WNV (Eg101) was used in the study. The strain had undergone several mouse brain passages before commencement of the study. A stock of the virus was prepared in Vero E6 cell line.

Growth kinetics of WNV in Cx. gelidus

Mosquitoes were infected by oral feeding as described by Sudeep *et al*[27]. In brief, mosquitoes were starved for 12 h and allowed to feed on blood virus mixture through a chicken membrane as previously described. Fully engorged mosquitoes were separated and used for the study. The infected mosquitoes were secured in plastic mosquito holding jars inside double walled mosquito cages and incubated as described earlier. Five mosquitoes were harvested on alternate days and stored at –80°C. After completion of the experiment, mosquitoes of each day post-infection (PI) were triturated in 1 ml minimum essential medium (Invitrogen, USA) containing 2% FBS using a chilled mortar and pestle. The mosquito suspension was centrifuged; Millipore filtered (pore size = 0.22 µm), diluted serially (10-fold) and titrated in Vero E6 cells in quadruplicate. The cultures were observed daily, readings (cells with cytopathic effects) were scored, stained with amido black and virus titre of every alternate day PI sample was determined as described by Reed and Muench[28]. All the experiments were carried out in triplicate and the data were analyzed to determine the growth kinetics of WNV in the mosquitoes.

Virus dissemination to salivary glands and ovaries

Mosquitoes were infected orally with WNV as described earlier and incubated at 28°C in the insectary. Salivary glands and ovaries from five mosquitoes were removed gently on every alternate day from Day 6 PI onwards and screened for WNV by immunofluorescent antibody technique as described earlier[27].

Reverse transcriptase (RT)-PCR for WNV

RNA was extracted from samples using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) as per

manufacturer's instructions and conducted reverse transcriptase (RT)-PCR targeting a 558 nucleotide fragment of E-gene as described earlier[29]. Cycling conditions used were one cycle at 94°C for 5 min; 35 cycles each of 94°C (1 min), 50°C (1 min), and 68°C (1.5 min); followed by final extension of 7 min at 68°C. Amplified fragments were visualized by ethidium bromide-agarose gel staining.

Determination of horizontal and vertical transmission of WNV

Horizontal and vertical transmission of WNV in the mosquito was carried out as described earlier[27]. For horizontal transmission, mosquitoes were either fed on viraemic mice or inoculated intra-thoracically as described earlier, and incubated at 28°C for 10 days (only fully engorged mosquitoes were used in the oral route of infection). After incubation, batches of five mosquitoes each were allowed to feed on a 2-day old infant mouse. Eight mice were used per experiment and the mice were observed for sickness. Brains of sick mice were harvested and RT-PCR targeting E-gene of WNV (558 bp) was conducted for confirmation. To determine vertical transmission, mosquitoes were fed on viraemic mice and fully engorged mosquitoes were separated and allowed to oviposit after a period of 10 days. Parent females after oviposition, eggs (approx 2000), IV instar larvae, pupae and adults of F_1 generation were processed to determine the presence of WNV by RT-PCR and cell culture as described earlier[27, 29]. The maintenance and care of the experimental animals is as per the guidelines for use of laboratory animals in research specified by the Animal Ethics Committee of the Institute.

Determination of trans-stadial transmission (infection by parenteral inoculation)

Inoculation of IV instar larvae was carried out as below and grown to adults. In brief, larvae were immobilized by keeping them on a Whatman filter paper above a block of wet ice and 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 female mosquitoes (n = 50) were harvested on Day 8 of emergence, head squashes were stained and determined the presence of virus.

RESULTS

Growth kinetics of WNV in Cx. gelidus

Culex gelidus replicated WNV to very high titres and maintained the virus for 14 days without much change in virus titre (Fig. 1). Maximum virus yield was same despite change in the mode of infection, *i.e.* by intra-thoracic inoculation and oral feeding though the initial intake varied in two methods. Maximum titre was obtained on 8th day PI ($7\log_{10}$ $TCID_{50}$/ml), followed by a slight decrease to approximately $6\log_{10}$ $TCID_{50}$/ml.

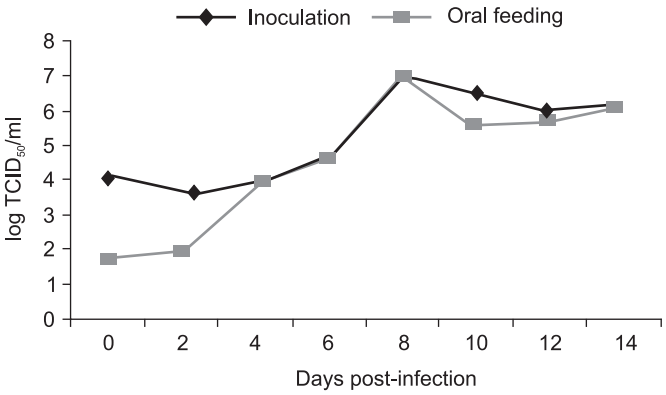

Fig. 1: Growth kinetics of West Nile virus in *Cx. gelidus* mosquito.

Virus dissemination to different organs

Virus in the whole body had titres in the range of 2–$3\log_{10}$ $TCID_{50}$/ml on Day 2 PI which showed a progressive increase as days post-infection progressed (Fig. 1). Virus dissemination to different organs, however, showed variation. Virus in legs was detected from Day 4 PI while virus presence in wings was detected only at 10 days PI (Table 1). Salivary gland infection was observed from Day 6 PI by IFA. Infection of ovaries could not be detected up to 10 days PI using IFA.

Horizontal transmission

Horizontal transmission of WNV by *Cx. gelidus* was established during the present study by both intra-thoracic inoculation of mosquitoes as well as orally infected mosquitoes. In the former, 100% infectivity was observed after an incubation period of eight days while only 56% infectivity was observed in orally fed mosquitoes. The mosquitoes were able to pick up infection while feeding

Table 1. WNV detection in different organs of infected *Cx. gelidus* on different days PI

Organ	Infectivity status on different days PI				
	2	4	6	8	10
Legs	–ve	+ve	+ve	+ve	+ve
Wings	–ve	–ve	–ve	–ve	+ve
Ovary	ND	ND	–ve	–ve	–ve
Salivary glands	ND	ND	+ve	+ve	+ve

ND: Not done.

on viraemic mice and could transmit the virus to fresh sibling mice on a subsequent feeding after the incubation period. The mosquitoes after blood feeding, had a titre in the range of 1.7 to 3.3 $\log_{10}$ $TCID_{50}$/ml, and replicated to 5–7 $\log_{10}$ $TCID_{50}$/ml on Day 8 PI. Intra-thoracically infected mosquitoes had titres in the range of 6.2–7.7 $\log_{10}TCID_{50}$/ml on Day 8 PI (immediately after feeding on baby mice). Mouse mortality (100%) was observed on Day 4 PI after showing sickness from Day 3 PI onwards. RT-PCR studies targeting E-gene demonstrated the presence of WNV in mice brain, thereby confirming horizontal transmission (Fig. 2). Transmission rates could not be determined as groups of five infected mosquitoes were fed on individual mice.

Vertical transmission

Vertical transmission of WNV by *Cx. gelidus* could not be demonstrated in the present study despite conducting repeated experiments. Virus could not be detected in eggs laid by infected mosquitoes or in larvae, pupae and adults of F_1 generation by RT-PCR targeting envelope gene (Fig. 2). It has been also seen that oviposition was affected by virus infection as only a few infected mosquitoes had oviposited. However, IFA studies could not detect virus infection of ovaries up to 10 days PI.

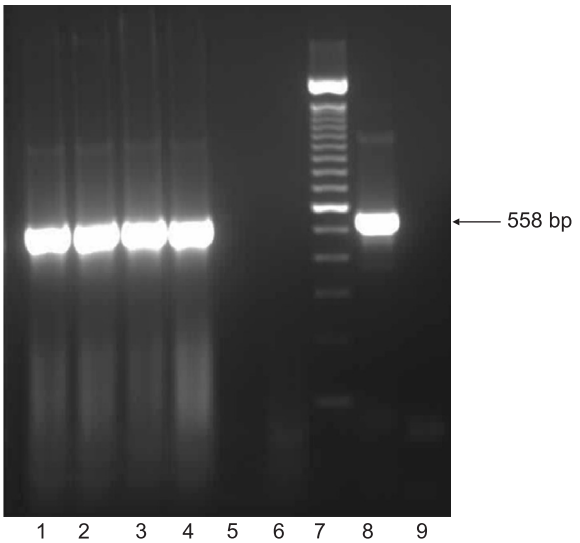

Fig. 2: WNV RT-PCR amplified product of 558 bp. Lane 1: WNV-infected mouse brain on which mosquitoes were fed; Lane 2: Suspension of mosquitoes-fed on viraemic mouse; Lanes 3 and 4: Brains of sick mice-infected by mosquito bite demonstrating horizontal transmission; Lane 5: Suspension of eggs laid by WNV-infected mosquitoes; Lane 6: F1 generation of adult mosquitoes emerged from eggs laid by WNV-infected mosquitoes; Lane 7: Marker; Lane 8: Positive control; and Lane 9: Negative control.

Trans-stadial transmission

Adults developed from parenterally infected larvae showed presence of virus. Though mortality was observed in a few larvae, infectivity rate was approx. 100% by IFA.

DISCUSSION

The unprecedented increase in *Cx. gelidus* population in India and Southeast Asian countries is a matter of concern due to its potential to transmit a number of encephalitis causing viruses. In the present study, the species has been found not only to replicate WNV to high titres but also transmitted the virus horizontally to susceptible hosts by bite after an incubation period of eight-days. Post-infection analysis of the mosquitoes demonstrated 100% infection rate in the parenterally infected mosquitoes and >50% in oral fed mosquitoes. Transmission rate could not be determined in the present study due to clubbing of infected mosquitoes for feeding on infant mouse. The rapid replication of the virus to 5–7 $\log_{10}$ $TCID_{50}$/ml, transmitting the virus to infant mice while feeding to cause 100% mortality, demonstrates the vector potential of the mosquito. This has been substantiated by detectable levels of WNV in the saliva seen from Day 6 PI onwards (Table 1). Should it get an access to feed on viraemic host; it could definitely cause focal outbreaks due to high replication potential and vector competence.

Vertical transmission, one of the methods of maintenance of the virus in nature, could not be demonstrated experimentally with the virus strain in the present study. Virus was not detected either in eggs laid by infected females or F_1 generation adults even by RT-PCR (Fig. 2). WNV is a unique arbovirus which exhibits vertical transmission inconsistently even with known vectors. Important vector mosquitoes such as *Cx. pipiens* and *Cx. quinquefasciatus* also yielded inconsistent results in the laboratory as well as in the field despite isolation of WNV from wild caught mosquitoes belonging to the species[30–32]. Our previous studies with *Cx. quinquefasciatus* mosquitoes also did not yield the virus in progeny despite repeated experiments with different strains of mosquitoes and viruses (Sudeep, unpublished data). Documented reports demonstrated that infection of ovaries commences very late, *i.e.* after 13 days post-infection and vertical transmission could be detected in the 2nd gonadotropic cycle[33]. Our results in the present study also substantiated this finding as we could not detect virus in ovaries up to 10 days PI by IFA. We could not conduct experiments to screen mosquitoes of second gonadotropic cycle and more studies are needed to confirm the absence of vertical transmission of WNV in the mosquitoes.

WNV, a highly pathogenic virus is known for its potential to cause neurotropic disease in humans[34]. In India, though WNV activity has been reported since 1952, no major outbreak similar to that of Europe or USA was reported[29]. However, >10 isolations from mosquitoes and a few from people with febrile illness and horses were made mainly from south India from 1957 to 1982[29]. After 1982, the country remained free from WNV activity for more than two decades until WNV activity has been reported from Assam during an investigation of acute encephalitis syndrome which is a major cause of mortality among children in the state[25]. During 2011–12, a few cases of WNV infection were also reported from Kerala state in Alappuzha district[26]. The exact route of entry to both the places could not be ascertained, however, no onward transmission leading to outbreaks could occur in either places due to active surveillance and timely intervention. It is, therefore, needed to monitor competent vector mosquitoes such as *Cx. gelidus*, which has the potential to transmit a number of encephalitis causing viruses such as JEV, MVEV, WNV, *etc* especially in the wake of WNV activity in certain parts of the country.

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