*J Vector Borne Dis* 52, June 2015, pp. 153–158

# Vectorial capacity of *Culex gelidus* (Theobald) mosquitoes to certain viruses of public health importance in India

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## ABSTRACT

*Background & objectives: Culex gelidus*, a widely prevalent mosquito in India and Southeast Asia region, is an important vector of Japanese encephalitis virus (JEV). Experimental studies have shown its potential to transmit West Nile, Kunjin, Murray Valley encephalitis and Ross River viruses. An attempt was therefore made to study its susceptibility and vector competence to some of the arboviruses of public health importance in India.

*Methods:* Mosquitoes were infected with six viruses, *viz.* JEV, chikungunya (CHIKV), Chandipura (CHPV), Chittoor (CHITV), Ingwavuma (INGV) and Umbre (UMBV) by intra thoracic inoculation to determine virus susceptibility and vector competence. Growth kinetics of the viruses were studied by determining the titres of inoculated mosquitoes on different days post-infection by titration in Vero E6 cells. Vector competence was studied by detecting the presence of the viruses in saliva of infected mosquitoes.

*Results:* All the six viruses were replicated in *Cx. gelidus*. JEV, CHPV, CHIKV and CHITV yielded > 5 $\log_{10}TCID_{50}$/ml virus while UMBV and INGV yielded approx 4 $\log_{10}TCID_{50}$/ml virus. JEV, CHIKV and CHITV could be detected in the saliva of the infected mosquitoes, while CHPV, INGV and UMBV could not be detected in the saliva of the infected mosquitoes.

*Interpretation & conclusion:* Replication potential and vector competence of *Cx. gelidus* to some of the viruses of public health importance in India, *viz*. JEV, CHIKV, CHITV *etc*, pose a serious threat to general population, especially in the wake of spurt in its population in certain parts of India.

**Key words** Chikungunya virus, Chittoor virus, *Culex gelidus*, replication, Japanese encephalitis virus, vector competence

## INTRODUCTION

*Culex gelidus* Theobald (Diptera: Culicidae), a Southeast Asian native mosquito, has enhanced its geographic distribution covering the whole of Southeast Asia, Australia and several Pacific Islands during the last few decades[1-2]. In countries like India, Sri Lanka, Malaysia, *etc*. *Cx. gelidus* population has increased substantially replacing other dominant mosquitoes like *Cx. tritaeniorhynchus etc*[3-4]. Its ability to breed in polluted waters with high concentration of organic matter and to feed on a variety of animals could be the probable reasons for the rapid surge in its population. It is a prominent vector of Japanese encephalitis virus (JEV) in Malaysia and plays a secondary role in the transmission of JEV in India, Sri Lanka and other countries as observed by repeated virus isolations from wild caught mosquitoes. In addition to JEV, several other viruses of public health importance, *viz.* Ross River virus (RRV), Getah virus (GETV), Sindbis Virus (SINV), Tembusu, Ingwavuma virus (INGV), *etc.* have been isolated from the mosquito[2, 5-6]. Experimental studies have shown its competency to transmit West Nile virus (1999 New York isolate), Kunjin virus, Murray Valley encephalitis virus (MVEV) and RRV in the laboratory[7-9]. The rapid increase in population, enhanced geographic expansion and its competency to transmit a number of encephalitis causing viruses make this mosquito a threat to public health.

In India, *Cx. gelidus* has been found widely prevalent, though in a negligible proportion in almost all the states except the northern region, *i.e.* Jammu and Kashmir, Punjab, Haryana, *etc*[2, 9]. However, in recent years, a spurt in its population has been recorded in several states with percentages exceeding 50% of the total mosquito population[2-4]. Though, several arboviruses of public health importance have been isolated from the mosquito elsewhere, only isolation of JEV has been reported from India[10-11]. However, a recent study has demonstrated the potential of the mosquito to transmit WNV experimentally[9]. Considering the broad spectrum susceptibility and vector competence of the species to several viruses, a study was designed to determine the susceptibility and vector competence of the mosquito to some of the arboviruses of public health importance in India.

## MATERIAL & METHODS

*Study site*

The study was carried out at the Entomology Division of Microbial Containment Complex, National Institute of Virology (NIV), Pashan, Pune, India.

*Collection of mosquitoes*

A laboratory colony of the mosquito was established from adult mosquitoes collected from Pashan, Pune. The F1 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 commercially available fish food while adults were maintained on a diet of 10% glucose. Female mosquitoes were provided with 3–4 months 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.

*Viruses used*

All the strains of viruses were procured from the virus repository maintained at the National Institute of Virology, Pune. Virus stocks were prepared in Vero E6 cell line, aliquoted and used for the study. Details of virus used in the study are given in Table 1.

*Growth kinetics of different viruses in Cx. gelidus*

Two to three days old mosquitoes maintained on 10% glucose *ad libitum* were infected by intra-thoracic inoculation as described by Rosen and Gubler[12]. The infected mosquitoes were secured in plastic mosquito holding jars inside double walled mosquito cages and incubated for a period of 14 days as described by Sudeep *et al*[13]. About five mosquitoes were harvested on alternate days from Day 0 to 14 post-infection (PI) and titrated in Vero E6 cell line as described earlier[9, 13]. In brief, mosquitoes were triturated in 1 ml minimum essential medium (MEM) supplemented with 2% FBS (both procured from Invitrogen, USA) using a hand held battery operated homogenizer (Sigma, USA); cleared by centrifugation at 5000 rpm at 4°C (Hettich, Germany), Millipore filtered (pore size = 0.22 µm), diluted serially (ten-fold) and titrated on confluent monolayer of Vero E6 cells or Vero E6 cell line mosquito grown in 96-well tissue culture plates (Nunc, Denmark) in quadruplicate. The cultures were observed daily, readings (cells with cytopathic effects) were scored, stained with amido black and virus titre of each sample was determined as described by Reed and Muench[14]. All the experiments were carried out in triplicate and the data were analyzed to determine the growth kinetics of viruses in the mosquito and its vector competency.

*Determination of vector competence*

Saliva was extracted from infected mosquitoes following the technique described earlier[15] with slight modifications. In short, proboscis of infected mosquitoes was inserted into a capillary tube containing MEM supplemented with 2% FBS after clipping the wings and the mosquitoes were allowed to excite for 30 min. Contents of the capillary tube (saliva from 3–5 mosquitoes) were transferred to 0.5 ml chilled MEM; Millipore filtered, and virus titre was determined in Vero E6 cell line as described above.

*Determination of horizontal transmission*

Horizontal transmission studies were carried out only with INGV. Mosquitoes were infected by intra-thoracic inoculation, incubated at 28°C for 10 days and fed on Day 1 old infant mice as described earlier[9, 13]. Mice were observed for sickness for 7 days. Permission for animal experiments was taken from the Animal Ethics Committee of the Institute and the animals were maintained as per the guidelines.

Table 1. Details of viruses used in the study

| Name of virus | Family | Abbreviation | Strain No. | Accession number |
|---|---|---|---|---|
| Japanese encephalitis virus | *Flaviviridae* | JEV | P20778 | AF080251 |
| Chikungunya virus | *Togaviridae* | CHIKV | 061573 | EF027134 |
| Chandipura virus | *Rhabdoviridae* | CHPV | 125672 | KF570390 |
| Ingwavuma virus | *Bunyaviridae* | INGV | 86627-2 | JQ0299994 |
| Chittoor virus | *Bunyaviridae* | CHITV | 804992 | FJ436796 (L) |
| Umbre virus | *Bunyaviridae* | UMBV | 631308 | EU697944 |

## RESULTS

*Susceptibility and replication potential of the mosquito to different arboviruses*

*Cx. gelidus* was found susceptible to all the viruses as increase in virus titre was observed on progressive days post-infection (PI). Growth kinetic studies have shown that *Cx. gelidus* was highly susceptible to JEV, CHIKV, CHPV and CHITV yielding high titres while moderate replication was observed with INGV and UMBV (Fig. 1). CHIKV yielded the highest titre in the mosquito among the viruses screened in the study. A rapid increase in CHIKV titre was observed in the mosquito reaching a peak titre of 6.3 $\log_{10}$ $TCID_{50}$/ml on Day 4 PI followed by a plateau, maintaining a titre of ≥ 6 $\log_{10}$ up to 10 Days PI. Thereafter, a slight decline in titre was observed, reaching 5.3 $\log_{10}$ $TCID_{50}$/ml on Day 14 PI. Similarly, the mosquitoes were found highly susceptible to CHPV as progressive increase in virus titre was observed in the mosquito. A yield of 5.5 $\log_{10}$ $TCID_{50}$/ml was obtained from Day 4 to Day 10 PI with a subsequent increase to 6 $\log_{10}$ $TCID_{50}$/ml on Day 12 PI. JEV replication was characteristic as slow growth was observed initially up to Day 4 PI. Virus could not be detected in samples on Day 0 and Day 2. However, on Day 4 PI the mosquitoes yielded 4.7 $\log_{10}$ $TCID_{50}$/ml which increased to 5.7 $\log_{10}$ on Day 6 PI, which was maintained throughout the study period. CHITV has shown rapid multiplication in the mosquito, yielding 5.7 $\log_{10}$ $TCID_{50}$/ml on Day 6 PI and the titre was almost maintained up to Day 14 PI. INGV replication in the mosquito was also characteristic as presence of virus could not be detected in the Day 0 samples despite inoculating 2 log virus. However, samples of progressive days PI have shown replication of INGV as evidenced by a few plaques (10-15 cells). Virus replication was found slow as the number of plaques increased slowly up to Day 3 PI, while sharp increase in the number of plaques were found on Days 4 and 5 suggestive of a longer incubation period. Maximum virus yield was obtained on Day 12 PI (4.3 $\log_{10}$ $TCID_{50}$/ml). UMBV also showed a very slow rate of replication in the mosquito as a very few infected foci could be seen on Day 3 PI in cultures infected with Day 0 and Day 2 PI samples. However, on Day 5 PI, virus induced cytopathic effect could be seen quite clearly. An increase of 1 $\log_{10}$ $TCID_{50}$/ml on Day 4 demonstrated the susceptibility and replication potential of the mosquito. The mosquitoes maintained the virus till Day 14 PI (Fig. 1).

*Vector competence of Cx. gelidus to different arboviruses*

*Cx. gelidus* mosquito is found competent to transmit JEV, CHIKV and CHITV as these viruses were found in saliva of infected mosquitoes. JEV and CHITV could be detected on Day 10 and 14 PI in infected mosquitoes respectively while presence of CHIKV in saliva could be detected as early as Day 5 PI (Table 2). With all the three viruses, a titre of ≥ 2 $\log_{10}$ $TCID_{50}$/ml was detected in the saliva. CHPV, INGV and UMBV could not be detected in the saliva of mosquitoes infected with these viruses despite having high titres of virus in the whole body (Table 2).

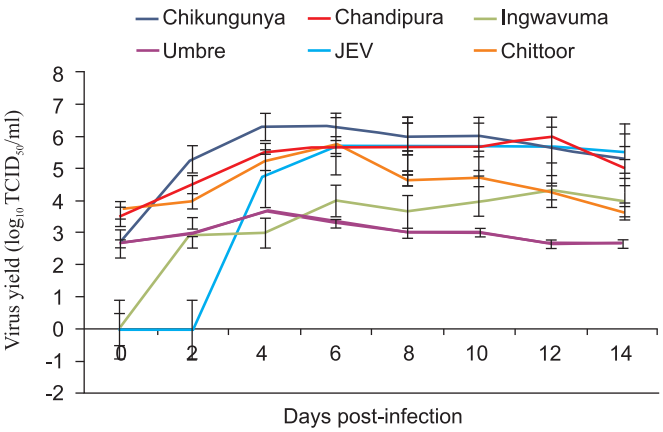


*Fig. 1:* Growth kinetics of certain arboviruses in *Culex gelidus* mosquitoes after intra-thoracic inoculation

*Experimental transmission of INGV to infant mice*

Horizontal transmission of INGV to infant mice could not be induced by the bite of infected mosquitoes. The mosquitoes were also found unable to pick up INGV from viraemic mice.

## DISCUSSION

*Cx. gelidus* is one of the important vectors of JEV and several strains of the virus were isolated from Malaysia, India and other Southeast Asian countries since 1950s[2]. In Malaysia, the mosquito is considered as the

Table 2. Vector competence of *Culex gelidus* mosquitoes to different arboviruses

| Virus used in the study | Virus titre on day of saliva extraction ($\log_{10}$ $TCID_{50}$/ml) | Virus titre in saliva ($\log_{10}$ $TCID_{50}$/ml) | Vector competence status |
|---|---|---|---|
| JEV | 5.7 | ≥2 | Yes |
| CHITV | 4.7 | ≈2 | Yes |
| CHIKV | 6.3 | ≤2 | Yes |
| INGV | 4.3 | Nil | No |
| UMBV | 3 | Nil | No |
| CHPV | 5.7 | Nil | No |

primary vector of JEV while in many other countries including India; it has emerged as the second important vector, next only to *Cx. tritaeniorhynchus* based on the number of virus isolations and ELISA based antigen detections[2-3, 16-20]. Even though the population density of the species is found much lower than *Cx. tritaeniorhynchus* in many places, the minimum infection rate to JEV was found comparable in both the species[21]. In India too, despite its meager population density, several strains of JEV have been isolated from Karnataka and Tamil Nadu[10-11]. In the present study also, we found rapid replication of JEV to yield 5.7 $\log_{10}$ virus on Day 6 PI, from a very low titre, which is below the detection levels in the cell culture. The mosquito was also found highly competent to transmit JEV as ≥2 $\log_{10}$ virus was detected in saliva of infected mosquitoes (Table 2).

Though no natural isolations of West Nile virus (WNV) have been reported so far from the species, the mosquito was found highly competent to transmit different strains of WNV experimentally, *i.e.* the New York strain of 1999; Kunjin virus, the Australian variant of WNV *etc*[7-8]. Earlier studies by Sudeep *et al*[9] with the prototype strain of WNV (Eg101) have also shown replication and successful horizontal transmission to infant mice by the mosquito. The mosquitoes could pick up infection while feeding on viraemic mice and transmit the virus to infant mice by bite on Day 8 PI. Experimental studies have also demonstrated vector competence of the mosquito to MVEV, another encephalitis causing virus prevalent in Australian region[8].

The present study has shown that the mosquito is competent to transmit only JEV, CHIKV and CHITV among the viruses included in the study. The mosquitoes were found highly competent to transmit CHIKV as a titre of ≥2 log TCID 50/ml could be detected in the saliva of infected mosquitoes on Days 5 and 6 PI. Though, no efforts to determine horizontal transmission to infant mice was undertaken, presence of virus in saliva with >2 $\log_{10}$ clearly demonstrates its potential to transmit the virus to susceptible hosts. Though, no natural isolation of CHIKV has been reported so far, several alphaviruses, *viz*. RRV, SINV and GETV were isolated from wild caught mosquitoes in Malaysia, Sri Lanka and Australia[2, 6]. Experimental studies have also shown the competence of the species to transmit RRV, which is an important arbovirus in Australia[8]. Recent studies have shown the establishment of *Cx. gelidus* in the Northern Territory and Queensland regions of Australia making it an important vector mosquito to be monitored critically for JEV and RRV[1].

CHPV, an important virus in Central India causing Chandipura virus encephalitis in children with high case fatality ratio has replicated in the mosquito to high titres. However, virus could not be detected in the saliva of infected mosquitoes. Earlier studies have shown that several mosquitoes, *viz. Aedes aegypti, Ae. albopictus, Cx. tritaeniorhynchus, Cx. quinquefasciatus, Anopheles stephensi*, *etc*, not only replicated the virus but also transmitted the virus to infant mice[22]. Despite the growth and transmission potential, CHPV has never been isolated from any of the mosquito species listed above. However, it is surprising to find that the species is not competent to transmit the virus despite replicating the virus to high titres.

Another interesting finding of the study was the replication potential and vector competence of the mosquito to CHITV, an arbovirus belonging to the Batai virus (BATV) group. BATV has a worldwide distribution and members of the group are known for genetic reassortments and cause severe hemorrhagic diseases in humans[23-24]. In India, CHITV has been isolated repeatedly from mosquitoes and pigs[23]. Though, no isolations from humans have been reported, antibodies were detected in human sera collected from Karnataka. Previous isolations of CHITV from mosquitoes in India have been made from *Anopheles barbirostris, An. subpictus, Culex pseudovishnui* and *Cx. tritaeniorhynchus* collected from Andhra Pradesh and Karnataka[23]. In the present study, *Cx. gelidus* is found to be competent to transmit CHITV as approx. 2 $\log_{10}$ virus was detected in the saliva on the Day 14 PI. Repeated isolations from different species of mosquitoes and pigs as well as detection of neutralizing antibodies in humans are suggestive of active circulation of the virus in India. Though, no outbreaks involving humans have been reported from the country yet, the prevalence of the virus, availability of vertebrate hosts for amplification, increasing populations of competent mosquitoes, pose a serious threat, should the virus form genetic reassortments with other bunyaviruses prevailing in this part of the country.

It has been emerged from the study that vector competence is a complex phenomenon involving several intrinsic and extrinsic factors. In the present study, we have observed that *Cx. gelidus* replicated CHPV and INGV to high titres. Despite, having high titres for a prolonged period in the body, saliva was found uninfected suggestive of non-infection of salivary glands. INGV is reported to have been isolated from *Cx. gelidus* in Indonesia[5]. However, results of the present study are not supporting the species as a vector of INGV. Experimental transmission studies with the virus also failed to infect infant mice which confirmed the absence of virus in saliva. Isolation of a virus alone is not enough to incriminate a mosquito as a vector. Probably, the mosquito could have been fed

on a viraemic animal and the mosquitoes might have been processed for virus isolation before the blood was digested. Infection of salivary glands and presence of virus in saliva is one of the pre-requisites for designating a mosquito as a vector. From the results of the present study, it is suggestive that *Cx. gelidus* may not be a vector of CHPV, INGV and UMBV. However, more precise studies with advanced tools are needed to confirm these findings as we have used exhibition of CPE by Vero E6 cells or Vero E6 cell line for detection of the viruses. It could also be possible that the viruses might be present in very low titres in saliva which was not sufficient to infect Vero E6 cells or Vero E6 cell line.

## CONCLUSION

The present study demonstrated susceptibility of *Cx. gelidus* mosquitoes to six arboviruses of public health importance in India. The species was found competent to transmit JEV, CHIKV and CHITV while failed to demonstrate vector competence to INGV, UMBV and CHPV despite replicating the viruses to high titres. The study, therefore, is significant to understand that vector competence is a unique phenomenon that involves several characteristics apart from virus replication. Earlier studies by the authors have also demonstrated the potential of the mosquito to transmit WNV horizontally to infant mice. WNV has already been held responsible for a few deaths in Assam and Kerala states recently. In both the places, the species is highly prevalent and could contribute to transmission of both JEV and WNV. Though, the role of the mosquito in the transmission of CHIKV and CHITV is not clear, it's potential to transmit JEV and WNV is a concern in this part of the world. The broad spectrum susceptibility of the mosquito and its competency to transmit JEV, CHITV and WNV pose a major threat to public health especially in the wake of recent surge in its population in certain parts of the country.

## ACKNOWLEDGEMENTS

The authors thank Dr D.T. Mourya, Director, NIV, Pune, India for constant support and encouragement; Dr Atanu Basu for critical reading of the manuscript and Mrs. M.S. Mavale for technical assistance.

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*Received:* 19 January 2015 *Accepted in revised form:* 6 March 2015