EXPERIMENTAL TRANSMISSION OF CHIKUNGUNYA VIRUS BY *ANOPHELES STEPHENSI* MOSQUITOES

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Summary. – The *Aedes aegypti* mosquito has been considered the principal vector of Chikungunya (CHIK) virus. As CHIK epidemics usually occur in urban regions and *Anopheles stephensi* is another highly endophilic and anthropophilic mosquito, there is a very high probability of this mosquito to feed on CHIK virus-infected patients, to pick up and transmit the virus. Therefore the present study was conducted to test the CHIK virus transmission capability for the *A. stephensi* mosquito. The obtained results showed that this mosquito species is capable of transmitting CHIK virus. It is surmised that during any epidemic of febrile illness CHIK virus isolation attempts should also be made from this mosquito species.

Key words: Aedes aegypti; Anopheles stephensi; Chikungunya virus; mosquitoes; RT-PCR; suckling mice; susceptibility; transmission

CHIK virus (the species *Chikungunya virus*, the genus *Alphavirus*, the family *Togaviridae*) is prevalent throughout the Southeast Asia and Africa (Jupp and McIntosh, 1988). This virus has been found responsible for several febrile epidemics in India. Recent reports on isolation of CHIK virus from the Maharashtra State have suggested that this virus did not disappear from India but remained there at low level (Mourya *et al.*, 2002). The *Aedes aegypti* mosquito has been incriminated as the principal vector and during various epidemics frequent virus isolations have been obtained from this species (Rao, 1966; Mourya *et al*., 2002). *A. stephensi* is an important vector of malaria in urban regions in India. This mosquito species is also highly endophilic and anthropophilic. In the past, during the investigations of epidemics of a hemorrhagic febrile illness, all the efforts were directed towards the isolation of the virus from *A. aegypti* mosquitoes. However, during epidemics, there is possibility of the *A. stephensi* mosquito to feed on CHIK patients and pickup the virus. Therefore it was felt prudent to determine transmission capability of the *A. stephensi* mosquito for CHIK virus in the laboratory.

A. stephensi mosquitoes employed in this study were obtained from a laboratory colony maintained at the National Institute of Virology since 1995. At the insectary the temperature of 28 ± 2°C and the relative humidity of 80 ± 10% were maintained. Adults were held in plastic jars and fed on 10% glucose solution in soaked cotton pads. The CHIK virus strain NIV 634029 employed in this study was originally isolated from a febrile patient in Kolkata, India during the 1963 epidemic (Pavri *et al*., 1964). The virus stock employed was at the 13th mouse brain passage level. Mosquitoes were infected with the virus diluted in a defibrinated chicken (white leghorn) blood through an artificial membrane (Parafilm, American National Can Co. USA). Four-to-five-day-old female mosquitoes were fed on the infected blood as described by Mourya *et al.* (2000). The post-feeding virus titer of the blood was determined in mice by intracerebral (i.c.) route. The infected mosquitoes were maintained in the insectory until further use. Virus transmission was studied as follows. From the days 6–8 post infection (p.i.) batches of 10–15 mosquitoes were fed on

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Abbreviations: CHIK = Chikungunya; i.c. = intracerebral; IFA = immunofluorescence assay; p.i. = post infection; RH = relative humidity; RT-PCR = reverse transcription–polymerase chain reaction

individual suckling mice. The mice infected by the bite of mosquitoes were observed for development of the illness. The brains of sick mice were harvested and tested for the presence of CHIK virus by reverse transcription–polymerase chain reaction (RT-PCR). Presence of the CHIK viral antigen in head squashes of mosquitoes was tested on days 4 and 10 p.i. by use of an indirect immunofluorescence antibody (IFA) technique (Ilkal *et al.*, 1984). Detection of the CHIK virus in the brains of sick mice and thus confirmation of the virus transmission was done by RT-PCR (Powers *et al*., 2000). Total RNA was extracted from mouse brains using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The brains harvested from normal mice were used as negative controls while the brains of CHIK virus-infected mice were used as positive control. PCR products were identified by 1% agarose gel electrophoresis.

Mosquitoe head squashes were found positive for viral antigen by IFA as early as on day 4 post feeding on infective bloodmeal. The percent positivity was increasing with increased post-feeding days (Table 1). The mice on which transmission attempts were made showed sickness between days 3 and 4 post feeding. RT-PCR made on the brains harvested from sick mice showed the presence of the virus.

Table 1. Susceptibility and transmission potential of *A. stephensi* mosquitoes to CHIK virus

Day p.i.	Head squashes positive/examined (%)[a]	Virus transmission to suckling mice	
		No. of mice that received infective bite	No. of sick mice
4	6/24 (25.0)	ND	ND
6	9/24 (37.5)	ND	ND
8	12/24 (50.0)	8	1
10	13/24 (54.1)	8	1

[a]Post-feeding virus titer of 6.7 log ID_{50}/0.02 ml in mice by i.c. route.
ND = not done.

The PCR products obtained for CHIK virus had size of approximately 1200 bp; such a products were not obtained with non-infected mouse brains (negative controls) (Fig. 1).

The percent positivity was low in *A. stephensi* as compared to that from an earlier report on *A. aegypti* (Mourya *et al*., 1987). Earlier reports have shown that multiplication of CHIK virus in *A. aegypti* mosquitoes was rapid and that these mosquitoes were able even to transmit the virus from day 4 post feeding (Mourya and Banerjee, 1987). Our present experiments (data not shown) revealed that *A. stephensi* mosquitoes could pick up this virus and transmit it successfully to suckling mice. It is surmised that these mosquitoes are highly anthropophilic and in the case of an opportunity to feed on a viremic host, they will be capable of transmitting the virus to humans. The *A. stephensi* mosquito species is often found sharing breeding places with *A. aegypti* mosquitoes. Therefore, this species might play a role in natural transmission of CHIK virus.

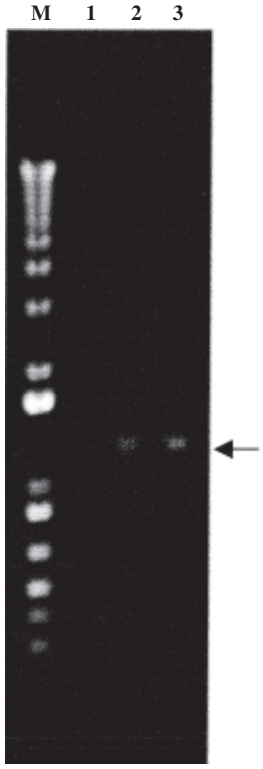

Fig. 1
Agarose gel electrophoresis of PCR products

1 kb DNA ladder as size marker (lane M); non-infected mouse brain, CHIK virus-specific primers (negative control, lane 1); virus-infected mouse brain, CHIK virus-specific primers (positive control, lane 2); a sick mouse brain after infective mosquito bite, virus-specific primers (lane 3).

CHIK virus has a wider host range as compared to flaviviruses. *A. aegypti* mosquitoes are day biters hence the disturbed biting is considered to be one of the factors of higher transmission rate caused by mechanical transmission (Jupp and McIntosh, 1988). Due to this behavior *A. aegypti* is a more efficient vector of CHIK virus than *A. stephansi*. Earlier studies have shown that *A. stephensi* is susceptible to CHIK virus infection (Rao *et al.,* 1964).

This is the first report from India showing that a virus belonging to alphaviruses (members of the genus *Alphavirus*) is transmitted by *A. stephensi* mosquitoes. Among alphaviruses, only the O'nyong-nyong (ONN) virus is transmitted by *Anopheline* mosquitoes. This virus has never been reported from India and recent phylogenetic studies have also shown that ONN virus is different then CHIK virus (Powers *et al.*, 2000).

Though in the present study the CHIK virus multiplication in *A. stephensi* mosquitoes was found lower then in its principal vector *A. aegypti* mosquito, further detailed studies will be needed to understand the vector competence for this

virus. This further characterization of other mosquito species present in the endemic/epidemic areas and their vector competence for CHIK virus could also provide valuable information on the potential of this virus to re-emerge in human populations.

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