Acta virologica 45: 305 – 309, 2001

ISOLATION OF CHIKUNGUNYA VIRUS FROM AEDES AEGYPTI MOSQUITOES COLLECTED IN THE TOWN OF YAWAT, PUNE DISTRICT, MAHARASHTRA STATE, INDIA

D.T. MOURYA[1*], J.P. THAKARE[1], M.D. GOKHALE[1], A.M. POWERS[2], S.L. HUNDEKAR[1], P.C. JAYAKUMAR[3], V.P. BONDRE[1], Y.S. SHOUCHE[3], V.S. PADBIDRI[1]

[1]National Institute of Virology, Pune, India; [2]Division of Vector Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, USA; [3]National Centre for Cell Science, Pune University Campus, Ganeshkhind, Pune, India

Received December 4, 2001; accepted February 24, 2002

Summary. – Chikungunya (CHIK) virus is prevalent throughout Southeast Asia and Africa. It has caused numerous large outbreaks in India. No active or passive surveillance has been carried out since the last epidemic occurring in 1971. During a recent outbreak of Dengue (DEN)-like illness in eastern India, *Aedes aegypti* mosquitoes collected from the affected area were positive for CHIK virus. Evidence of dual infection with CHIK and DEN type1 virus was also obtained. A widely circulating low-virulent CHIK virus is a possible explanation for the epidemiological pattern of the CHIK virus disease in this region.

Key words: Aedes aegypti; Chikungunya virus; Dengue virus

Introduction

CHIK virus belongs to the species *Chikungunya virus*, genus *Alphavirus*, family *Togaviridae*. It has been found responsible for several febrile epidemics in India. The *Aedes aegypti* mosquito has been incriminated as the principal vector of this virus since it has been frequently isolated from this vector during various epidemics (Rao, 1966). Earlier studies conducted on different geographical *Ae. aegypti* strains have shown significant differences with respect to the susceptibility to and transmissibility of this virus (Banerjee *et al.*, 1988). Similarly, differences in the susceptibility and transmissibility have also been recorded for different strains of CHIK virus (Mourya *et al.*, 1987). Recent molecular epidemiological studies on CHIK virus have shown that CHIK virus strains form two distinct lineages; the Asian strains form a lineage (genotype) that is distinct from the African lineage (Powers *et al.*, 2000). In Africa, transmission of this virus involves a sylvatic cycle while no such cycle has been detected in India; it is presumed that the virus transmission is maintained through an urban cycle involving humans and *Ae. aegypti* mosquitoes (Jupp and McIntosh, 1988). In India, CHIK virus has caused various large-scale epidemics and disappeared abruptly from the area for long periods of time. It is surmised that during the periods of silence the virus might be maintained in nature in a sylvatic cycle or by transovarial (t.o.) transmission, although there is no such evidence for Asia (Jupp and McIntosh, 1988). DEN virus (the species *Dengue virus*, genus *Flavivirus*, family *Togaviridae*) is a major cause of febrile illness in India and is also transmitted by *Ae. aegypti*.

*Present address: Microbial Containment Complex, Indian Council of Medical Research, Sus Road, Pashan, Pune 411021, India. E-mail: mouryadt@vsnl.net; fax: +9120-5883595.

Abbreviations: BAPS = 0.75% bovine albumin phosphate saline; CHIK = Chikungunya; DEN = Dengue; ELISA = enzyme-linked immunosorbent assay; i.c. = intracerebral; IF = immunofluorescence; i.t. = intrathoracic; MAb = monoclonal antibody; p.i. = post infection; P/N = ratio of absorbance of positive to negative sample; RH = relative humidity; RT = room temperature; RT-PCR = reverse transcription–polymerase chain reaction; t.o. = transovarial

However, it has been postulated that CHIK virus appears to be maintained in nature at a low level due to misdiagnosis of many cases of CHIK virus infections as DEN virus infections (Carey, 1971).

In India, no active or passive surveillance has been carried out since the last epidemic occurring in 1971 (Padbidri *et al.*, 1973). In August 2000, cases of pyrexia have been reported from the town of Yawat; some of these cases also had hemorrhagic manifestations. Acute phase sera from several individuals were sent to this laboratory for diagnosis. Serological tests did not show any evidence of recent DEN infection. A survey of *Ae. aegypti* mosquitoes was therefore conducted in this town to monitor CHIK virus, since it also causes clinical symptoms similar to the DEN hemorrhagic fever. The results of this survey are reported here.

Materials and Methods

Viruses. The following viruses and strains were used in this study. CHIK virus Kolkatta strain isolated from a patient during the 1963 epidemic at Kolkatta, India, was used at the 7th mouse passage.

DEN type 2 virus isolated in 1990 from a patient suffering from DEN hemorrhagic fever during an epidemic in Jammu, India. A stock virus was prepared from the 8th passage in infant mice.

Mosquito collection. Daytime-indoor mosquito collections were carried out in October 2000 in Yawat, a small town 40 km away from the city of Pune (latitude 18–19°; longitude 74–75°). Six of the eighty houses searched were positive for *Ae. aegypti*. A total of 48 female mosquitoes were collected by means of mouth-operated aspirators. The live mosquitoes were brought to the laboratory and held in cages in the insectary (28 ± 2°C, 85 ± 5% relative humidity (RH)) for at least 48 hrs. Wet filter papers were provided to gravid females for egg deposition. These mosquitoes were identified under a stereoscopic microscope after anaesthetizing on ice bags.

Detection of CHIK and DEN virus antigens in mosquitoes. Heads of field-collected mosquitoes were squashed between two slides so that double impressions could be obtained and screened for both DEN and CHIK virus antigens by indirect immunofluorescence (IF) test and polyclonal antisera to each virus antigen (Mourya and Mishra, 2000). The bodies of these mosquitoes were stored at -70°C until processed further.

Detection of vertical virus transmission in mosquitoes by enzyme-linked immunosorbent assay (ELISA). The pools of 100 3rd to 4th instar field-collected larvae were triturated in phosphate-buffered saline (PBS) containing 0.1% CHAPSO and centrifuged at 10,000 x g for 30 mins at 4°C. The supernatants were tested for DEN and CHIK virus antigens by an antigen capture ELISA using a flavivirus cross-reactive monoclonal antibody (MAb Hx-2) and CHIK MAb as a capturing and probing antibody, respectively, as described by Joshi *et al.* (1997). Similarly, a pool of 3rd to 4th instar F1-larvae obtained from field-caught mosquitoes was processed to detect vertical transmission of these viruses.

Virus isolation. The bodies of mosquitoes whose head squashes were positive by IF test were triturated in BAPS and centrifuged at 10,000 x g for 1 hr at 4°C. The supernatant was inoculated intrathoracically (i.t.) into 20–25 female *Ae. aegypti* mosquitoes according to the method of Rosen and Gubler (1974). The mosquitoes used for the inoculation were obtained from a laboratory breeding. The inoculated mosquitoes were incubated at 28±2°C and 85±5% RH for 10 days. After inoculation, their heads were squashed and the IF test was performed to detect CHIK antigen. The mosquito bodies in which the antigen was detected in the head squashes were subjected to virus isolation. Homogenates of these mosquito bodies were further inoculated into clean mosquitoes in three successive passages. The suspension obtained from the second mosquito passage was also inoculated into infant mice. A complement fixation test was performed on brain suspension from sick mice by the method of Pavri and Shaikh (1966). Attempts were also made to isolate the virus from Vero cells inoculated with homogenates.

Detection of CHIK and DEN virus antigens in mosquitoes and mouse brain suspensions by ELISA was done by use of MAbs as described above.

Confirmation of virus isolates by reverse transcription–polymerase chain reaction (RT-PCR). In order to support the IF test and antigen capture ELISA results, RT-PCRs was carried out on the mosquitoes and brains of sick mice inoculated with a third passage field material. Total RNA was extracted from mouse brains and mosquitoes using TRIZOL reagent (Gibco BRL) according to the manufacturer's instructions. The primers and PCR conditions have been described by Morita *et al.* (1991) and Powers *et al.* (2000). β-actin primers were used as control in the RT-PCR to check the quality of RNA extracted from mouse brains.

Results

The study area has rural characteristics and pipe supply water. The information obtained from the local residents revealed that the water supply was erratic and inadequate during the time of the outbreak of illness. There were also two tube wells in the area and prolific breeding of *Ae. aegypti* was noticed in some cement tanks.

Forty-eight female *Ae. aegypti* mosquitoes collected from houses were screened by IF test for the presence of CHIK and DEN virus antigen by head squash examination. Four head squashes were CHIK virus antigen-positive (Table 1). Supernatant from the bodies of positive mosquitoes was passaged in clean mosquitoes. These mosquitoes were again found positive for CHIK virus antigen on day 10 p.i. Similarly, the second and third passages were positive for CHIK but not DEN virus antigen. Based on the number of positive head squashes, the multiplication of virus observed in these mosquitoes was very low as compared to the CHIK virus strain used as positive control. The control mosquitoes infected i.t. with 1.2 log MID_{50}/0.2 µl Kolkatta strain showed 97.5% of head squashes positive by IF test on day 10 p.i., while only 33% of head squashes were positive when the mosquitoes were inoculated with the 3rd passage field isolate (Table 1).

The 3rd mosquito passage suspensions were inoculated into 2–3 day-old Swiss albino mice by intracerebral (i.c.) route. The inoculated mice showed a typical incapacitating type of illness. It was interesting to note that some of the mice exhibited these symptoms on days 3–4 p.i. while the rest of the mice became sick after day 8 p.i. These results indicated the possibility of dual infection with DEN and CHIK viruses or differences in virulence among various strains of CHIK virus. ELISA tests performed on the mouse brains and suspensions of mosquitoes inoculated with field material showed a weakly positive reaction for CHIK virus antigen (ratio of absorbances of positive to negative samples (P/N) was 2.0). ELISA with the flavivirus cross-reactive MAb Hx-2 gave negative results. Similarly, the attempts made to propagate field isolates in Vero cells did not lead to any cytopathic effect, and both ELISA and IF test applied to the supernatants harvested on the day 6 p.i. did not confirm the presence of an antigen. When brain suspensions from the third passage of sick mice were inoculated into clean mosquitoes, 20% of the head squashes were found positive for CHIK virus on day 10 p.i. (Table 1).

The F1-mosquitoes reared from field-collected adults as well as field-collected larvae were screened for the presence of both the viral antigens by ELISA. None of the pools was positive (Table 1).

RT-PCR assays performed to detect DEN virus type 1 and CHIK virus in the mosquitoes and brains of mice, which were inoculated with the 3rd passage field isolate, showed that the samples was positive for both viruses (Figs. 1 and 2). The PCR products obtained for CHIK virus and DEN virus type 1 had size of approximately 1200 bp and 490 bp, respectively. Such a PCR products were not obtained with clean colony mosquitoes and non-infected mouse brains used as negative controls. Similarly beta-actin primers used as controls to check the quality of mRNAs present in RNA extracted from mouse brains showed that mRNAs were not degraded during the extraction.

Table 1. Results of CHIK virus isolation experiments on head squashes from *Ae. aegypti* mosquitoes

	No. of positives/No. of total (%)	
	CHIK	DEN
Field material		
Field caught mosquitoes	4/48 (8.3)	0/48 (0.0)
Negative control	0/6 (0.0)	0/6 (0.0)
Positive control	6/6 (100.0)*	6/6 (100.0)**
Vertical transmission		
F1 progeny of field collected mosquitoes	0/1#	0/1#
Field collected larvae	0/6##	0/6##
First passage		
First passage of field material in mosquitoes	6/24 (25.0)	0/24 (0.0)
Negative control	0/6 (0.0)	0/6 (0.0)
Positive control	6/6 (100.0)*	6/6 (100.0)**
Second passage		
Second passage of field material mosquitoes	8/24 (33.3)	0/24 (0.0)
Negative control	0/6 (0.0)	0/6 (0.0)
Positive control	6/6 (100.0)*	6/6 (100.0)**
Third passage		
Third passage of field material in mosquitoes	9/30 (30.0)	0/30 (0.0)
Negative control	0/6 (0.0)	0/6 (0.0)
Positive control	6/6 (100.0)*	6/6 (100.0)**
Fourth passage		
10% mouse brain suspension***	5/25 (20.0)	0/25 (0.0)
Negative control	0/6 (0.0)	0/6 (0.0)
Positive control	39/40 (97.5)*	6/6 (100.0)**

*The dose of CHIK virus Kolkatta strain inoculated i.t. was 1.2 log MID_{50}/0.2 µl. Head squashes were prepared on day 10 p.i.
**The dose of DEN virus inoculated i.t. was 2.3 log MID_{50}/0.2 µl. Head squashes were prepared on day 10 p.i.
***10% suspensions of brains from suckling mice exhibiting signs of illness. Mice were inoculated with the 3rd passsage mosquito suspension.
#No. of positives/No. of total tested by ELISA. Each pool consisted of 50 F1 III-IV instar larvae.
##No. of positives/No. of total tested by ELISA. Each pool consisted of 100 field-collected III-IV instar larvae).

Discussion

CHIK virus causes a spectrum of clinical diseases ranging from a simple febrile illness to febrile illness with arthralgia and/or hemorrhage. It has caused major epidemics of DEN-like disease in adults and hemorrhagic fever in children in India, Cambodia, Thailand and Singapore (Deller and Russell, 1968). On several occasions, the identified CHIK virus infections have coincided with DEN epidemics (Rao, 1966; Halstead *et al.*, 1969a,b). In this communication, we report the occurrence of dual infection of CHIK and DEN type-1 in field-collected mosquitoes from an area where the presence of DEN was reported in November 1997. After CHIK virus epidemics in Thailand in 1960–1991, it was thought that CHIK virus had disappeared from this country. However, the reemergence of CHIK virus infections in 1997 led to public health concern. In India, a somewhat similar situation has been observed. After the CHIK virus epidemics in Barsi and Vellore, attention was not paid to CHIK virus despite reports of outbreaks of a febrile illness. It was therefore thought that CHIK virus infection was declining even from the areas where it had previously caused large-scale epidemics (Neogi *et al.*, 1995).

Interestingly, the pattern of CHIK (Yawat strain) virulence observed was somewhat unique in both mice and mosquitoes

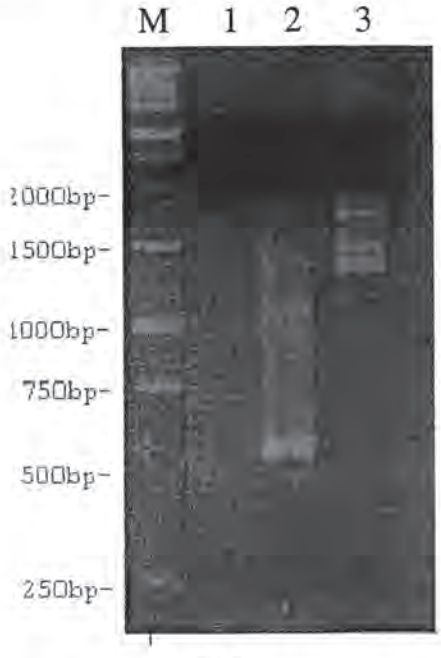

Fig. 1
Results of RT-PCR for CHIK virus
Size marker, a 250 bp ladder (lane M). Mouse brains, negative control (lane 1). β-actin control (lane 2). Brains of mice inoculated with a field material, CHIK virus-specific primers (lane 3).

as compared to the Kolkatta strain epidemic. The standard CHIK virus isolation is usually obtained within a short period of time with typically 100% mortality in mice on primary inoculation (Burke *et al.*, 1985). The CHIK virus isolate obtained in the present study showed low virulence based upon the symptoms of illness observed in mice. The isolate was identified as CHIK virus by employing assays such as IF, ELISA and RT-PCR. Furthermore, when mosquitoes were inoculated with the Kolkatta strain of CHIK virus as positive control, a high multiplication rate was observed. However,

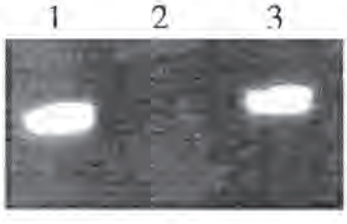

Fig. 2
Results of RT-PCR for DEN viruses
β-actin control (lane 1). Mouse brain negative control (lane 2). Brains of mice infected with a field material (lane 3).

the field isolate did not multiply as profusely as the Kolkatta strain that was isolated during an epidemic. This suggests that a strain isolated during an epidemic is virulent while strains of low virulence may be active during non-epidemic periods.

Based on the possibility that the virus is maintained in nature in a low-virulent form, we looked for evidence of vertical transmission of this strain. Data presented here showed that vertical transmission could not be detected in the progeny of field-caught infected mosquitoes although only a small number of mosquitoes were available for this analysis. This supports previous results demonstrating that vertical transmission of this virus has not been observed in nature or in the laboratory (Mourya, 1987; Jupp and McIntosh, 1990). Since there is no known vertebrate reservoir of CHIK virus in India, this further strengthens the hypothesis that the virus is maintained in nature at low levels in a mosquito-human-mosquito cycle.

During an epidemic at Vellore, dual infection with DEN and CHIK viruses in one of the cases has been reported (Myers and Carey, 1967). A report of CHIK virus infection during an epidemic of yellow fever has been also reported in Senegal (Diallo *et al.*, 1999). In the presented study, a striking feature was a dual infection with both DEN-1 and CHIK viruses in mosquitoes. The sickness of some of the mice on day 8 p.i. suggested the presence of a DEN virus, however it could not be detected by serological tests. The presence of a DEN virus could be confirmed only by RT-PCR. Since all the field-caught CHIK virus-positive mosquitoes were pooled for testing, it is unknown how many were dually infected.

In our earlier study, putative CHIK virus receptors have been found in the brush border membrane of mosquito midguts, and their abundance has been considered an important factor determining susceptibility of this mosquito species to the virus (Mourya *et al.*, 1998). Our recent studies have shown that DEN viruses also bind to one of these receptors (data not shown). At this stage it is difficult to postulate that one virus may suppress replication of the other in dually infected mosquitoes. It has been recently shown that Sindbis virus (the genus *Alphavirus*) suppresses the growth of a DEN virus (the genus *Flavivirus*) in mosquitoes (Adelman *et al.*, 2001). Our earlier studies have shown that the multiplication of certain CHIK virus strains in orally infected *Ae. aegypti* mosquitoes is robust and that the mosquitoes could transmit the virus on day 4 p.i. (Mourya *et al.*, 1987). In comparison to CHIK virus, the rate of multiplication of DEN viruses is slow in this vector species. There is no evidence suggesting that orally infected mosquitoes can transmit DEN virus on days 4–5 p.i. It may be assumed that if there is dual infection in *Ae. aegypti* mosquitoes, CHIK virus would undoubtedly replicate and disseminate more rapidly, generating a situation where the

detection of a DEN virus by conventional methods could be difficult.

The identification of a strain of CHIK virus with an apparently reduced virulence phenotype leads to a number of intriguing speculations. The CHIK virus infection is not easily noticeable due to factors as (i) generally, the battery of virological diagnostic tests does not routinely include CHIK virus, (ii) the possibility of continuous exposure of people to small amount of CHIK virus or to the virus of low virulence, leading to immunity, and (iii) the possibility of a persistent CHIK virus infection in invertebrate vectors and humans (Diallo *et al.*, 1999). To answer these questions, a continuous active and passive surveillance would be of great importance. The present communication should increase the awareness of the prevalence of CHIK virus in Maharashtra State, India.

References

Adelman ZN, Blair CD, Carlson JO, Beaty BJ, Olson KE (2001): Sindbis virus-induced silencing of dengue viruses in mosquitoes. *Insect Mol. Biol.* **10**, 265–273.

Banerjee K, Mourya DT, Malunjkar AS (1988): Susceptibility and transmissibility of different geographical strains of Aedes aegypti mosquitoes to Chikungunya virus. *Indian J. Med. Res.* **87**, 134–138.

Burke DS, Nisalak A, Nimmannitya S (1985): Disappearance of Chikungunya virus from Bangkok. *Tran. Royal Soc. Trop. Med. Hyg.* **79**, 419–420.

Carey DE (1971): Chikungunya and dengue: a case of mistaken identity? *J. History Med. Allied Sci.* **26**, 243–262.

Deller JJ Jr, Russell PK (1968): Chikungunya disease. *Am. J. Trop. Med. Hyg.* **17**, 107–111.

Diallo M, Thonnon J, TraoreLamizana M, Fontenille D (1999): Vectors of chikungunya virus in Senegal: current data and transmission cycles. *Am. J. Trop. Med. Hyg.* **60**, 281–286.

Halstead SB, Scanlon JE, Umpaivit P, Udomsakdi S (1969a): Dengue and chikungunya virus infection in man in Thailand, 1962–1964. IV. Epidemiologic studies in the Bangkok metropolitan area. *Am. J. Trop. Med. Hyg.* **18**, 997–1021.

Halstead SB, Udomsakdi S, Scanlon JE, Rohitayodhin S (1969b): Dengue and chikungunya virus infection in man in Thailand, 1962–1964. V. Epidemiologic observations outside Bangkok. *Am. J. Trop. Med. Hyg.* **18**, 1022–1033.

Joshi AM, Walimbe AM, Banerjee K (1997): Potency testing of inactivated tissue culture vaccine against Japanese encephalitis using antigen capture ELISA. *Acta Virol.* **41**, 157–159.

Jupp PG, McIntosh BM (1988): Chikungunya virus disease. In TP Monath TP (Ed.): *The Arbovirus: Epidemiology and Ecology*. Vol. II, CRC Press, Boca Raton, Florida, pp. 137–157.

Jupp PG, McIntosh BM (1990): Aedes furcifer and other mosquitoes as vectors of chikungunya virus at Mica, northeastern Transvaal, South Africa. *J. Am. Mosq. Control Assoc.* **6**, 415–420.

Morita K, Tanaka M, Igarashi A (1991): Rapid identification of dengue virus serotypes by using polymerase chain reaction. *J. Clinic. Microbiol.* **29**, 2107–2110.

Mourya DT, Ranadive SN, Gokhale MD, Barde PV, Padbidri VS, Banerjee K (1998): Putative chikungunya virus-specific receptor proteins on the midgut brush border membrane of Aedes aegypti mosquito. *Indian J. Med. Res.* **107**, 10–14.

Mourya DT (1987): Absence of transovarial transmission of Chikungunya virus in Aedes aegypti & Ae. albopictus mosquitoes. *Indian J. Med. Res.* **85**, 593–595.

Mourya DT, Mishra AC (2000): Antigen distribution pattern of Japanese encephalitis virus in Culex tritaeniorhynchus, C. vishnui & C. pseudovishnui. *Indian J. Med. Res.* **111**, 157–161.

Mourya DT, Malunjkar AS, Banerjee K (1987): Susceptibility & transmissibility of Aedes aegypti to four strains of chikungunya virus. *Indian J. Med. Res.* **86**, 185–190.

Myers RM, Carey DE (1967): Concurrent isolation from patient of two arboviruses, chikungunya and dengue type 2. *Science* **157**, 1307–1308.

Neogi DK, Bhattacharya N, Mukherjee KK, Chakraborty MS, Banerjee P, Mitra K, Lahiri M, Chakravarti SK (1995): Serosurvey of chikungunya antibody in Calcutta metropolis. *J. Com. Dis.* **27**, 19–22.

Padbidri VS, Dandawate CN, Goverdhan MK, Pavri KM (1973): Chikungunya epidemic at Barsi: I Preliminary epidemiological virological and serological findings. *Maharashtra Med. J.* **20**, 221–224.

Pavri KM, Shaikh BH (1966): A rapid method of specific identification of Japanese encephalitis – West Nile subgroup of arboviruses. *Current Science* **35**, 453–456.

Powers AM, Brault AC, Tesh RB, Weaver SC (2000): Re-emergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. *J. Gen. Virol.* **81**, 471–479.

Rao TR (1966): Recent epidemics caused by chikungunya virus in India, 1963–1965. *Scientific Culture* **32**, 215.

Rosen L, Gubler DJ (1974): The use of mosquitoes to detect and propagate dengue viruses. *Am. J. Trop. Med. Hyg.* **23**, 1153–1160.