Am. J. Trop. Med. Hyg., 83(6), 2010, pp. 1242–1244
doi:10.4269/ajtmh.2010.09-0577

Venereal Transmission of Chikungunya Virus by *Aedes aegypti* Mosquitoes (Diptera: Culicidae)

Mangala Mavale, Deepti Parashar, Anakkathil Sudeep,* Mangesh Gokhale, Youwaraj Ghodke, Geevarghese Geevarghese, Vidya Arankalle, and Akhilesh Chandra Mishra
National Institute of Virology, Pune, India

Abstract. Experiments were conducted to demonstrate the role of male *Aedes aegypti* mosquitoes in the maintenance and transmission of chikungunya virus (CHIKV) to female mosquitoes. We demonstrated that infected male mosquitoes are capable of infecting females during mating. The infection rate in female mosquitoes was 11% when virgin female mosquitoes were allowed to coinhabit with infected males. The body suspension of venereally infected female mosquitoes induced illness in infant Swiss albino mice, which demonstrated the infectivity of the venereally transmitted virus. The presence of CHIKV in the brains of the ill mice was confirmed by a reverse transcription–polymerase chain reaction specific for partial sequences of nonstructural protein 4 and envelope 1 genes. In the light of the recent report of transovarial transmission of CHIKV in mosquitoes, although at a lower level, this finding has significance because it may help in transmission of the virus to females venereally to start a new infection cycle.

INTRODUCTION

Chikungunya fever is one of the most important mosquito-borne viral diseases in countries in Africa and Southeast Asia, including India. The disease is caused by Chikungunya virus (CHIKV), an *Alphavirus* of the family *Togaviridae*, which was first isolated in Africa during a dengue like illness in 1952–1953.[1] The disease, although self-limiting, causes high morbidity and is characterized by sudden onset of high fever, polyarthralgia, and myalgia. *Aedes aegypti* mosquitoes are the principal vector of CHIKV and are involved virtually in all chikungunya epidemics reported so far from Africa, India, and other countries in Southeast Asia.[2–5] However, *Ae. albopictus*, the Asian tiger mosquito, has also played a prominent role in the transmission of CHIKV during the recent outbreaks in India, the Reunion Islands in the Indian Ocean, and Italy, as observed by high incidence in the outbreak area and virus isolation.[6,7]

In Africa, the virus is maintained in a sylvatic cycle that involves non-human primates and a number of forest-dwelling mosquitoes. However, it is not clear how the virus is maintained in Asia because there is no known animal reservoir and the virus is maintained between humans and mosquitoes. Chikungunya virus has a peculiar characteristic in that it is absent for a long period and then suddenly reappears in an explosive form.[8] Transovarial transmission of the virus in the mosquitoes might be a mechanism to maintain the virus during inter-epidemic periods because the virus has been detected in wild-caught male *Ae. aegypti* and *Ae. albopictus* mosquitoes.[9,10] In this context, we tested the possibility of maintenance of the virus in male *Ae. aegypti* mosquitoes and transmission to the females during mating. The present study describes the successful venereal transmission of CHIKV from experimentally infected male mosquitoes to females.

MATERIALS AND METHODS

Mosquitoes. *Aedes aegypti* mosquitoes were obtained from a laboratory colony maintained at the National Institute of Virology, Pune, India, for the past 17 years at a temperature of 28 ± 2°C and a relative humidity of 80 ± 5%.

Virus. We used chikungunya virus strain 061573, which was isolated from human serum sample obtained during the 2006 outbreak in Andhra Pradesh, India, and had undergone four passages in Vero E6 cells. The virus had a 50% tissue culture infectious dose titer of 7 $\log_{10}$/mL.

Mosquito infection and venereal transmission. Before conducting experiments, male and female mosquitoes were screened for CHIKV antigen. Emerged virgin males and females were kept in separate cages. One hundred male mosquitoes were inoculated intra-thoracically with 0.2 μL of virus suspension per mosquito, as described by Rosen and Gubler.[11] Inoculated mosquitoes were maintained on a 10% glucose solution. On day 4 post infection, 10 mosquitoes were removed randomly and head squashes were made and screened for CHIKV antigen by using an indirect immunofluorescent antibody technique (IFAT) as described by Dhanda and Ilkal[12] and CHIKV hyperimmune serum raised in rabbits.

Once the presence of CHIKV in all 10 infected males was confirmed, the rest of the mosquitoes were allowed to mate en masse with virgin females of the same age at a ratio of 1:2 in three cages. Eight days after cohabitation, 92 surviving females were removed from the cage and head squashes were made and screened for CHIKV antigen by IFAT. Bodies of decapitated mosquitoes were triturated in 0.75% bovine albumin in phosphate-buffered saline, centrifuged at 10,000 rpm for one hour at 4°C, and the supernatant was inoculated into 1–2-day-old suckling mice by the intracranial route. Inoculated mice were observed for illness, and brains were obtained and screened for viral RNA by using a reverse transcription–polymerase chain reaction (RT-PCR) as described.[4]

Transmission of CHIKV through cotton pads. To rule out CHIKV transmission through glucose-soaked cotton pads, 50 male mosquitoes were inoculated intra-thoracically as above, incubated for 4 days, and screened for virus antigen in 10 randomly selected males. After confirmation of 100% (10 of 10) positivity, the remaining males were allowed to feed on 10% glucose through cotton pads. The same glucose pads were used to feed uninfected females, who were incubated for eight days and screened for CHIKV antigen in head squashes by IFAT. Bodies were also processed and inoculated into infant mice as described above.

*Address correspondence to Anakkathil Sudeep, National Institute of Virology, (Indian Council of Medical Research), Dr. Ambedkar Road, Pune 411001, India. E-mails: sudeepmcc@yahoo.co.in or sudeepab@icmr.org.in

RESULTS

Detection of CHIKV in head squashes. The 10 inoculated male mosquitoes harvested on day 4 post-infection were positive for CHIKV antigen by IFAT, demonstrating 100% positivity in the inoculated mosquitoes. Head squashes of the females mosquitoes after coinhabitation for 8 days contained CHIKV antigen in 10 mosquitoes when screened by IFAT (11% positivity). Although the same ratio of male and female mosquitoes was maintained in all three cages, the percent positivity ranged from 6% to 17% per cage (Table 1).

Demonstration of infectivity of venereally transmitted CHIKV in infant mice. Swiss albino mice (1–2 days old) infected with the suspension of decapitated bodies of positive female mosquitoes became ill on the third and fourth days post infection in the second passage (8/8). Brains of the ill mice contained CHIKV RNA by CHIK-specific RT-PCR.

Absence of CHIKV transmission through cotton pads. Suckling mice did not show signs of infection after inoculation with mosquito body suspensions from female mosquitoes that fed on glucose pads. These findings clearly demonstrated that CHIKV was not transmitted through cotton pads while mosquitoes were feeding on these pads.

DISCUSSION

Chikungunya virus, which is endemic to countries in Africa and Asia, behaves differently in terms of virus maintenance and transmission in these two continents.[3,13] Because no sylvatic cycle is involved in Asia, it is useful to know how CHIKV exists in nature, especially during inter-epidemic periods in Asia. Recent studies have shown that vertical transmission or transovarial transmission of CHIKV occurs at a low level in *Ae. aegypti* and *Ae. albopictus* mosquitoes.[9,10] Because no vertebrate reservoir is known to harbor the virus, a low prevalence of transovarial transmission and venereal transmission might be responsible for the maintenance of the virus in nature.

If transovarial transmission of CHIKV occurs, the virus may be present in male mosquitoes. This possibility has been demonstrated in a recent study in Thailand, in which CHIKV was detected in *Ae. aegypti* and *Ae. albopictus* male mosquitoes.[9] Ratsitorahina and others[10] also reported CHIKV in larvae-reared pools of *Ae. albopictus* mosquitoes during their study in Toamasina, Madgascar. During a recent study carried out at the National Institute of Virology, CHIKV antigen was detected in wild-caught male *Ae. aegypti* mosquitoes obtained in Kerala State (National Institute of Virology, unpublished data). This state in India is unique because *Ae. albopictus* mosquitoes have been identified as the vector of CHIKV during the 2006–07 outbreak because of their high incidence in outbreak areas. The potential of this mosquito in transmission of CHIKV has already been demonstrated in Asia, Europe (Italy), and on islands in the Indian Ocean.[6,7] The presence of the virus in the male mosquitoes is suggestive that they can initiate a new cycle of infection by venereal transmission to female mosquitoes.

Venereal transmission of viruses by male mosquitoes is well documented and is considered as one of the modes of maintenance of virus in nature. This phenomenon has been studied in several vector species and demonstrated in mosquito vectors for bunyaviruses, alphaviruses, flaviviruses, and rhabdoviruses.[14–19] However, no information is available for CHIKV venereal transmission in mosquitoes. Our data demonstrating approximately 17% positivity in female mosquitoes when coinhabited with experimentally infected males is an important observation and may have immense epidemiologic significance. The present study also demonstrated that CHIKV-infected males are capable of transmitting the virus efficiently to females venereally during mating. Induction of illness in suckling mouse when inoculated with decapitated bodies of venereally infected females demonstrates the infectivity of the virus to vertebrate hosts. This finding is indicative of the transmission potential of venereally infected mosquitoes.

In the present study, we did not test experimental transmission of the virus through the bite of the mosquitoes. The presence of virus in the head is suggestive of the potential of the mosquitoes to transmit the virus by bite. Dubrulle and others showed in experiments with *Ae. aegypti* and *Ae. albopictus* mosquitoes that the salivary glands become infected as early as the second day post-infection when infected orally.[20] These investigators also observed that infective virus particles increased as the incubation progressed, and maximum virus titer was observed on days 6 and 7 for *Ae. aegypti* and *Ae. albopictus* mosquitoes, respectively.

We believe that infected males might be maintaining the virus at a low threshold without causing outbreaks in human population, and that venereally infected female *Ae. aegypti* mosquitoes may start a new infection cycle within the focus. It would be useful to study the actual mode of virus transmission from infected male mosquitoes to female mosquitoes. One probable explanation for venereal transmission could be through infected sperm to spermatheca or by seminal fluid. However, a more precise study is needed to understand the actual mechanism involved in this process and expand the existing realm of our understanding of virus–vector interaction

Table 1

Chikungunya virus positivity of female *Aedes aegypti* mosquitoes after co-inhabitation with infected males in different groups

Cage no.	No. positive/no. tested (%)
1	3/30 (10)
2	2/32 (6.25)
3	5/30 (16.66)
Total	10/92 (10.97)

Received September 23, 2009. Accepted for publication July 8, 2010.

Acknowledgments: We thank the staff of the Entomology Division of the National Institute of Virology, especially Prakash Salunke, Reuben George, and Pradeep Fulmali for excellent technical assistance, and Dr. P. V. M. Mahadev (Scientist Emeritus) for enthusiasm and encouragement during the study.

Authors' address: Mangala Mavale, Deepti Parashar, Anakkathil Sudeep, Mangesh Gokhale, Youwaraj Ghodke, Geevarghese Geevarghese, Vidya Arankalle, and Akhilesh Chandra Mishra, National Institute of Virology, (Indian Council of Medical Research), 20-A, Dr Ambedkar Road, Pune 411001, India, E-mails: niv@yahoo.co.in, deeptiparashar@scientist.com, sudeepmcc@yahoo.co.in, gokhal40@gmail.com, niv@yahoo.co.in, niv@yahoo.co.in, varankalle@yahoo.com, and acm1750@rediffmail.com.

REFERENCES

1. Ross RW, 1956. The Newala epidemic III. The virus: isolation, pathogenic properties and relationship to the epidemic. *J Hyg (Lond) 54:* 177–191.

2. Rao TR, 1966. Recent epidemics caused by chikungunya virus in India, 1963–1965. *Sci Cult 32:* 215–220.
3. Diallo M, Thonnon J, Traore Lamizana M, Fontenille D, 1999. Vectors of chikungunya virus in Senegal: current data and transmission cycles. *Am J Trop Med Hyg 60:* 281–286.
4. Yergolkar PN, Tandale BV, Arankalle VA, Sathe PS, Sudeep AB, Gandhe SS, Gokhale MD, Jacob GP, Hundekar SL, Mishra AC, 2006. Chikungunya outbreaks caused by African genotype, India. *Emerg Infect Dis 12:* 1580–1583.
5. Sang RC, Ahmed O, Faye O, Kelly CL, Yahaya AA, Mmadi I, Toilibou A, Sergon K, Brown J, Agata N, Yakouide A, Ball MD, Breiman RF, Miller BR, Powers AM, 2008. Entomologic investigations of a chikungunya virus epidemic in the Union of the Comoros, 2005. *Am J Trop Med Hyg 78:* 77–82.
6. de Lamballerie Xde Leroy E, Charrel RN, Ttsetsarkin K, Higgs S, Gould EA, 2008. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? *Virol J 5:* 33.
7. Bonilauri P, Bellini R, Calzolari M, Angelini R, Venturi L, Fallacara F, Cordioli P, Angelini P, Venturelli C, Merialdi G, Dottori M, 2008. Chikungunya virus in *Aedes albopictus*, Italy. *Emerg Infect Dis 14:* 852–853.
8. Pavri KM, 1986. Disappearance of chikungunya virus from India and Southeast Asia. *Trans R Soc Trop Med Hyg 80:* 491.
9. Thavara U, Tawatsin A, Pengsakul T, Bhakdeenuan P, Chanama S, Anantapreecha S, Molito C, Chompoorsri J, Thammapalo S, Sawaboabyakert Om Siriyasatien P, 2009. Outbreak of chikungunya fever in Thailand and virus detection in field population of vector mosquito *Aedes aegypti* and *Aedes albopictus*. *Southeast Asian J Trop Med Public Health 40:* 951–962.
10. Ratsitorahina M, Harisoa J, Ratovonjato J, Biacabe S, Reynes J-M, Zeller H, Raoelina Y, Talarmin A, Richard V, Soares JL, 2008. Outbreak of dengue and chikungunya fevers, Toamasina, Madagascar, 2006. *Emerg Infect Dis 14:* 1135–1137.
11. Rosen L, Gubler D, 1974. The use of mosquitoes to detect and propagate dengue viruses. *Am J Trop Med Hyg 23:* 1153–1160.
12. Dhanda V, Ilkal MA, 1985. Mosquito inoculation and immunofluorescence technique for studies on dengue viruses. *Indian J Virol 1:* 6–9.
13. McIntosh BM, Jupp PG, Dos SI, 1977. Rural epidemic of chikungunya in South Africa with involvement of *Aedes* (Diceromyia) *furcifer* (Edwards) and baboons. *S Afr J Sci 73:* 267–269.
14. Thompson WH, Beaty BJ, 1977. Venereal transmission of La Crosse (California encephalitis) arbovirus in *Aedes triseriatus* mosquitoes. *Science 196:* 530–531.
15. Ovenden JR, Mahon RJ, 1984. Venereal transmission of Sindbis virus between individuals of *Aedes australis* (Diptera: Culicidae). *J Med Entomol 21:* 292–295.
16. Shroyer DA, 1990. Venereal transmission of St. Louis encephalitis virus by *Culex quinquefasciatus* males (Diptera: Culicidae). *J Med Entomol 27:* 334–337.
17. Geevarghese G, Mavale MS, Ghodke YS, Kode SS, Ciceilia D, 2003. Venereal transmission of Japanese encephalitis virus in *Culex quinquifasciatus* and West Nile virus in *Cx. bitaeniorhynchus*. *Am J Trop Med Hyg 69:* 446–447.
18. Mavale MS, Geevarghese G, Ghodke YS, Fulmali PV, Singh A, Mishra AC, 2005. Vertical and venereal transmission of Chandipura virus by *Aedes aegypti* mosquito. *J Med Entomol 42:* 909–911.
19. Mavale MS, Fulmali PV, Geevarghese G, Arankalle VA, Ghodke YS, Kanogia PC, Mishra AC, 2006. Venereal transmission of Chandipura virus by *Phlebotomus papatasi* (Scopoli). *Am J Trop Med Hyg 75:* 1151–1152.
20. Dubrulle M, Mousson L, Moutailler S, Vazeille M, Failloux AB, 2009. Chikungunya virus and *Aedes* mosquitoes: saliva is infectious as soon as two days after oral infection. *PLoS ONE 4:* e5895.