Review Article

J Vector Borne Dis 44, December 2007, pp. 233–240

Xenodiagnosis: use of mosquitoes for the diagnosis of arboviral infections

D.T. Mourya, M.D. Gokhale & R. Kumar

Microbial Containment Complex, National Institute of Virology, Pune, India

Abstract

The arboviruses have a worldwide distribution and, mosquitoes and ticks contribute principally in their transmission. In the last two decades, arboviral diseases have been recognised due to their resurgence and spread in newer geographic areas. Surveys to determine the prevalence of arboviruses in any region largely depend on the isolation attempts from the arthropods along with the sero-surveys. Xenodiagnosis means use of insects for the diagnosis of infectious diseases affecting human being. The present communication discusses the application of mosquitoes for propagation and assays of arboviruses, the technique of mosquito inoculation and importance of xenodiagnosis.

Key words Arboviruses – mosquitoes – xenodiagnosis

Introduction

Viruses transmitted by blood-feeding arthropods are called as arboviruses. The arboviruses have a worldwide distribution and, mosquitoes and ticks contribute principally in their transmission[1]. In the last two decades, arboviral diseases have been recognised due to their resurgence and spread to newer geographic areas. A large number of the arboviruses have been isolated from Africa, South America and Asia[2]. The geographic distribution of each arbovirus is restricted by the ecological conditions governing its transmission cycle, which is influenced by the distribution of the arthropod vectors and the vertebrate reservoir host required for virus maintenance. Arboviruses are maintained in complex natural cycles involving a vertebrate host and an arthropod vector. These cycles are usually silent and undetected in nature until some environmental change allows the virus to escape the primary cycle via a secondary vector or vertebrate host, or when humans invade or encroach on the nidus of infection. Epidemics in humans and domestic animals usually occur only after the virus is introduced into the peridomestic environment by a vector[1,3].

Viruses do not have an independent existence in nature. For their propagation, they must invade the cells of other living beings. Arboviruses are no exceptions and they utilise the cells of their vertebrate as well as arthropod hosts. When such viruses multiply in human cells they may cause disease. Recent scenario of arboviral diseases on the global scale shows that a large number of viruses, which were either confined to small geographical areas causing enzootics or present in the endemic form, have now spread and emerged in newer areas. The establishment of mosquito vectors in newer areas have helped the spread of such viruses[4]. Surveys to determine the prevalence of arboviruses in any region are largely depending on the isolation attempts from the arthropods along with

sero-surveys[5,6]. The present communication discusses the use of mosquitoes for propagation and assays of arboviruses and xenodiagnosis.

Xenodiagnosis

The term xenodiagnosis (XD)[7] was introduced in 1914 for the method of detecting trypanosomes in mammal hosts by feeding laboratory bred Reduviid bugs on the animal. Nymphs of triatomines used for this purpose were reared on chicken, refractory to this parasite. Since the 1930s, series of valuable contributions have stressed on different aspects of XD including generalities[8], quality and number of XD kits and species, instar and number of insects to be used[9], blood ingestion and mortality of insects utilised in XD[10]. The technique was further refined for obtaining the substratum to be examined[10–12], sensitivity of test[13], reading and interpretation of results[14–17].

Up to now, xenodiagnosis used in screening chagasic patients, confirming the diagnosis of seropositive individuals, treatment, control and stock isolation, is the most specific and sensitive technique currently available for diagnosis. However, XD using *Ixodes scapularis* (Acari: Ixodidae) ticks was employed to determine whether spirochetes persist in mice after one month of antibiotic therapy for vector-borne *Borrelia burgdorferi* infection[18]. *Aedes aegypti, Ae. albopictus* (Diptera: Culicidae) and *Toxorhynchites* have all been successfully used for isolation of dengue.

A simple protocol was described for indirect xenodiagnosis on peripheral blood samples tested by offering 50, 7-day old, laboratory-bred, female sandfly *Phlebotomus pernicosus* (Diptera: Psychodidae) on 1.5 ml sample, held at 37ºC for 1 h in sterile, membrane-feeding apparatus, using the skin of 3-day old chicken membrane[19]. This provided alternative to bone-marrow biopsies. Similarly, XD was used for Venezuelan cutaneous leishmaniasis with *Lutzomyia youngi*, before and after specific antileishmanial treatment[20].

In 1970s, the very first attempt of using mosquitoes for diagnosis of infectious diseases of man was made[21] and specific method of infecting mosquitoes with virus by intrathoracic inoculation was developed where a known quantum of virus could be inoculated. It was demonstrated that virus profusely multiplied in different organs of mosquito system, which was found to be more sensitive than the conventional method of inoculating mice[21]. Till today, this method is found suitable for isolation of arboviruses in several laboratories worldwide. Since then, the mosquito inoculation and immunofluorescence techniques for the detection of dengue viruses are being routinely used. This technique is relatively simple and has been used by a number of workers[22–27]. The use of non-biting mosquitoes like *Toxorhynchites* species[28] further refined this technique and eliminated the risk of laboratory infection through infective bite. The *Toxorhynchites* is a large mosquito and has the advantage of tolerating a much larger inoculum than does *Aedes*. Also *Toxorhynchites* produce large quantities of viruses, facilitating detection by fluorescence antibody (FA) and identification.

Advantages of XD

It is known that the arboviruses were originally viruses of arthropods and during evolution they get adapted to vertebrates. This is the reason, no arbovirus is known to harm the arthropod vector, which transmit the organism[1]. However, arboviruses are known to cause cytopathic effects in the cell lines derived from vertebrates but no such effect is known for cell lines derived from arthropods. It has been shown that even several arthropods when attempted to infect by feeding on virus infected material do not support multiplication but when infected material is inoculated in the body, they allow the multiplication of viruses. Most arboviruses are either mosquito-borne or tick-borne. Since it is difficult to maintain ticks and even difficult to inoculate them, mosquitoes have thus been preferred for this. Moreover, this system facilitates to work on very small quantity of

sample and has been found very suitable even when virus titre is low in the samples.

Research carried out during last two-three decades on the use of mosquitoes for the propagation and assay of viruses have shown that this method is gaining importance not only for the reason that it is sensitive and cheaper, but also for its rapid outcome[29]. Another reason is the increasing pressure of strict regulation of reducing use of animals for virus isolation work. In such situations, almost all the virological work like virus pools preparation, titration, diagnostic work, *etc*. can be carried out using mosquito inoculation system except neutralisation study, which requires either mice or tissue culture.

Mosquitoes launch a variety of cellular and humoral defence responses against bacteria and other metazoans[30,31]. Thus, even if field samples get contaminated with bacteria, mosquito survives through incubation period. Thus, confirmation and identification of arboviral isolate becomes possible.

Examples of XD

Besides the use of mosquito inoculation system for routine diagnosis works, it is also the method of choice used in the following situations:

1. Need for virus isolation when the sample is available in very small quantity or exhausted while performing routine test[21].
2. When the sample is to be processed with suspicion of arbovirus which is not reported earlier from that geographical region.
3. When surveillance for existence of newer arboviruses in an area is to be carried out[32].
4. Avoiding use of animals in the laboratory studies for determining virulence of arbovirus or other such virological work[33].
5. *In vivo* determining efficacy of antiviral compounds which are available in very small quantity[34].
6. Mosquito inoculation system has also been found to be rapid when mosquitoes are inoculated intracerebraly and in many situations almost at par with PCR. The advantage of PCR is that it can detect presence of virus at much lower quantum but cannot isolate the virus. However, XD can simultaneously isolate the virus in this system[25,35].
7. In case of many arboviruses when no animal model is available for their propagation, it is difficult to determine the virus transmission capabilities of any mosquito vector. Recently, it has been shown that even mosquito inoculation system can be used to determine virus transmission capabilities[36–39].
8. Besides arboviruses, this system is also used to propagate certain rickettsial agents and even the effects of antibiotics can be studied *in vivo*[34, 40].
9. This method is also used for the determination of efficacy of attenuated viral vaccines. It shows whether at a given dose mosquito can pick up virus and whether attenuated strain shows any reversion of the virus strain after it reaches natural host's body[41].
10. Similarly, this method is used in studies where possible genomic changes in virus are required to be known after the virus enters into vertebrate system. It requires initial passages at least three times in mosquitoes and then introduced in vertebrate system.
11. Using mosquito inoculation system, transmission potential of any arbovirus can be studied even if no animal model is available[36, 38, 42].
12. Now-a-days, attempts are being made to make transgenic mosquitoes for directly immunising people by the bite of mosquitoes. Similar attempts are being made to develop mosquito

strains resistant to virus multiplication. This system plays an important role in developing such strategies[43].

In any application of mosquito inoculation system, identification is required after propagation of the suspected etiological agent. This is normally done by coupling any of the following methods or some times more than one method is used, like fluorescent antibody technique, usually performed on the head squashes using either cross-reaction polyclonal or specific monoclonal antibodies and/or PCRs/RT-PCRs are performed on the mosquitoes after incubation period.

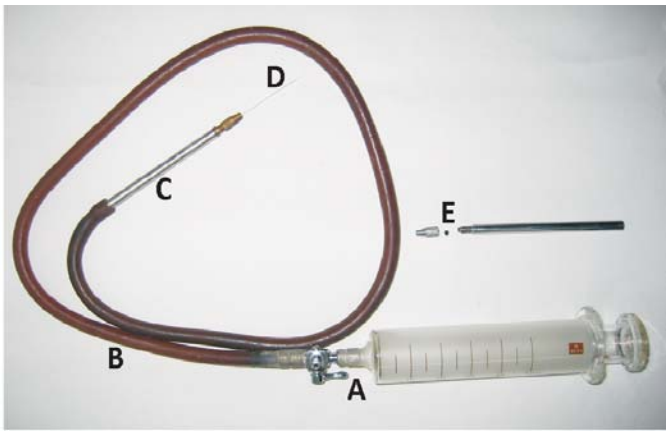

Fig. 1: Parts of inoculation apparatus (A—Three-way leur lock; B—Latex tubing; C— Metal needle holder; D—Glass capillary; and E—Rubber washer)

Infection by parenteral inoculation

Inoculation needle used for infecting mosquitoes is prepared from capillary tubing normally measuring about 60 mm in length with 0.5 mm inner and 1 mm outer diameter. Preparation of the inoculation needle involves heating at the middle of the capillary tube over a micro-burner and by drawing apart, the two inoculation needles are obtained. The tapering tip of each inoculation needle thus prepared is broken off at an appropriate point to leave a sharp tip with fine bore. To graduate the capillary for calculating the precise amount of inoculum, the untapered portion of the needle is marked at 1 mm distance with a rubber stamp specially prepared for the purpose or with any marking device/pen. The calibration permits the inoculation of the required volume of inoculum by observing the length through which the inoculum is moved. These capillaries are attached to a fabricated tubular metal needle holder through a rubber washer to prevent the leakage of air at the connecting points of the inoculation needle and needle holder. At the other end of the needle holder, latex tubing is slid over which is connected to a three-way leur lock fixed to the syringe (Fig. 1). The diameter of the needle and small tapering end make it difficult to draw the fluid in. This requires considerable pressure in order to overcome the resistance offered at the fine tip of the inoculation needle. The three-way leur lock is turned on so that the needle is connected to the syringe. The plunger of the syringe is then withdrawn. Care is taken to prevent the inoculum from traversing the entire length of the inoculation needle and entering the needle holder.

Before inoculation, the mosquitoes are immobilised by confining to glass test tubes on ice bath or smoke/CO_2. These are then transferred on a filter paper disc placed on the stage of the stereoscopic microscope. Mosquitoes remain immobile for about half a minute, which is sufficient to manipulate them for inoculation. The inoculation method employs compressed air by pushing the plunger of the syringe to force the inoculum through the glass inoculation needle. The inoculation needle is introduced inside the mosquito thorax by piercing the membranous area, just anterior to the mesenteron below the spiracle for female mosquitoes (Fig. 2). For male mosquitoes below the neck membrane is inoculated. After inoculation, mosquitoes are kept in the jars provided with cotton pad soaked in 10% sucrose solution to feed and held for incubation in the insectary at 28 ± 1^{o}C with high relative humidity (70–80%).

After completion of the incubation period, the mosquitoes infected through different methods, i.e. either

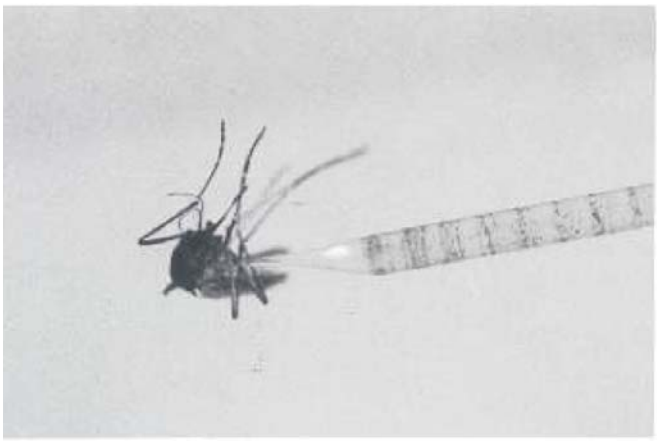

Fig. 2: Pierced capillary needle in the thorax of mosquito

by feeding[44] or by inoculation can be tested for presence of virus. This can be accomplished using various methods like tissue culture, complement fixation, inoculation of infant Swiss albino mice and immunofluorescence technique. Generally, out of these three systems, the indirect immunofluorescence technique (IIFT) is commonly used which gives quick results. The mosquitoes are tested individually by making head squashes and staining by IIFT using specific antibodies (Fig. 3).

Advantages of the use of mosquito system

The various merits of mosquito inoculation system are as follows.

1. It is cheaper since maintaining mosquito colony is easier and no sophisticated equipments are required.
2. It is more sensitive than the conventional methods like use of mice and many cell lines.
3. Both propagation and detection of virus is possible in certain situations where monoclonal antibodies are available.
4. Higher rate of replication in arthropods during the gradual processing is advantageous than in mice system where blind passages are some times required for isolation.
5. Mosquitoes launch very strong cellular and humoral defence responses against both gram positive and negative bacteria. Hence, even if samples get contaminated by bacteria, it does not kill the host which normally survives through the incubation period of viruses.
6. Use of male mosquitoes for propagation of virus provides safety and prevents accidental spread.

During the current decade, technology has emerged for developing transgenic mosquitoes and attempts are also being made to develop genetically engineered strains which may be resistant to particular arboviruses. In the present scenario, it is also felt that this technology may also be applied the other way to develop highly anthropophilic strains of mosquitoes to carry any particular arbovirus. Moreover, mosquito

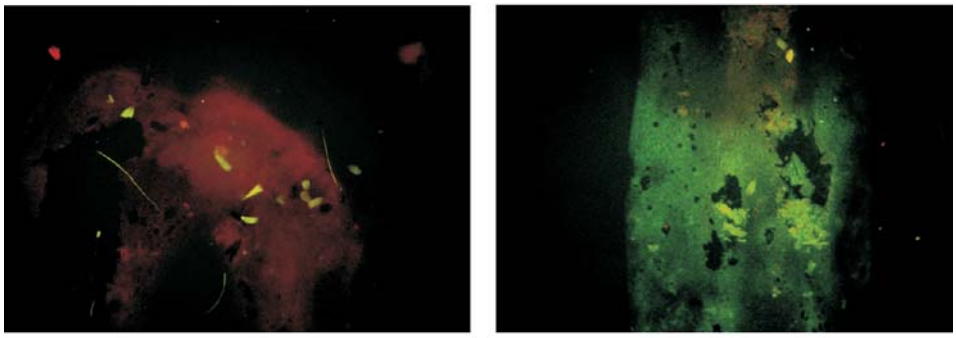

Fig. 3: Head squash of mosquito screened by fluorescence antibodies technique (A–Virus negative and B–Virus positive; Magnification–200X)

inoculation system is useful to detect arthropod-borne haemorrhagic fevers particularly when the sample volume is less. In such a situation, it further necessitates the requirement of an insectary and the availability of virus propagation system using mosquitoes. It will not only help in earliest detection of plausible use of mosquitoes in bioterrorism and biological warfare but also in formulating faster and effective control strategies.

References

1. Gubler DJ, Roehrig JT. Arboviruses (Togaviridae and Flaviviridae). In: Mahy BWJ, Collier L, editors. *Topley and Wilson's Microbiology and Microbial Infections*, v 1, edn. 29. Virology 1998. New York: Oxford University Press 1998; p. 579–600.

2. Karabatsos N. International catalogue of arboviruses, including certain other viruses of vertebrates. *Am Soc Trop Med Hyg*, San Antonio 1985; TX 2001 update.

3. Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Knipe DM, Howley PM *et al*, editors. *Fields Virology*, III edn. Philadelphia, PA: Lippincott-Raven Publishers 1996; p. 961–1034.

4. Gubler DJ. Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. In: Gubler DJ, Kuno G, editors. *Dengue and dengue hemorrhagic fever*. London, UK: CAB International 1997; p. 1–22.

5. Dhanda V, Ilkal MA. Mosquito inoculation and immunofluorescence technique for studies on dengue viruses. *Indian J Virol* 1985; *1:* 6–9.

6. Halstead SB. The XX century dengue pandemic: need for surveillance and research. *World Health Stat Q* 1992; *45:* 292–8.

7. Brumpt E. LE xenodiagnostic. Application au diagnostic de quelques infections parasitaires et en particular a la trypanosomose de Chagas. *Bull Soc Path Exot* 1914; *7:* 706–10.

8. Pereira VL, Marcos de AA, Boainain E. Xenodiagnóstico, hemocultura e teste de lise mediada pelo complemento, como critérios de seleção de pacientes chagásicos crônicos para quimioterapia. *Rev Inst Med Trop São Paulo* 1989; *31:* 301–7.

9. Schenone H. Xenodiagnosis. *Mem Inst Oswaldo Cruz, Rio de Janeiro* 1999; *94:* 289–94.

10. Bronfen E, Alvarenga NJ. O xenodiagnóstico e os critérios para avaliar o nível de parasitemia dopaciente chagásico crônico. *Rev Soc Bras Med Trop* 1991; *24:* 37–42.

11. Coura JR, Abreu LL, Willcox HPF, Petana W. Evaluation of the xenodiagnosis of chronic Chagas patients infected ten years or over in an area where transmission has been interrupted – Iguatama and Pains, West Minas Gerais State, Brazil. *Mem Inst Oswaldo Cruz* 1991; *86:* 395–8.

12. Silva IG, Silva HHG, Luquetti AO, Rezende JM. Positividade do xenodiagnóstico de acordo com a faixa etária, o sexo e a forma clínica da doença de Chagas. *Rev Pat Trop* 1995; *24:* 193.

13. Bronfen E, Rocha FSA, Machado GBN, Perillo MM, Romanha AJ, Chiari E. Isolamento de amostras do Trypanosoma cruzi por xenodiagnóstico e hemocultura de pacientes na fase crônica da doença de Chagas. *Mem Inst Oswaldo Cruz* 1989; *84:* 237–40.

14. Cerisola JA, Del Prado CE, Rohwedder R, Bozzini JP. Blastocrithidia triatomae n. sp. found in *Triatoma infestans* from Argentina. *J Protozool* 1971; *18:* 503–6.

15. Cedillos RA, Torrealba JW, Tonn RJ, Mosca W, Ortegón A. El xenodiagnóstico artificial en la enfermedad de Chagas. *Bol Sanit Panam* 1982; *93:* 240–9.

16. Freitas JLP. O diagnóstico de laboratorio da moléstia de Chagas. *Rev Clín S Paulo* 1952; *28:* 1–10.

17. Castro CN, Alves MT, Macedo VO. Importância da repetição do xenodiagnóstico para avaliação da parasitemia na fase crônica da doença de Chagas. *Rev Soc Bras Med Trop* 1983; *16:* 98–103.

18. Linda KB, Mao J, Hodzic E, Barthold SW, Fish D. Detection of attenuated, non-infectious spirochetes in *Borrelia burgdorferi*–infected mice after antibiotic treatment. *J Infect Dis* 2002; *186:* 1430–7.

19. Molina R, Alvar J. A simple protocol for the indirect xenodiagnosis of *Leishmania infantum* in the blood of HIV-infected patients. *Ann Trop Med Parasitol* 1996; *90* (6): 639–40.

20. Rojas E, Scorza E, Scorza JV. Xenodiagnostico con Lutzomyia Young en casos venezolanos de Leishmaniasis cutanea por *Leishmania braziliensis*. *Mem Inst Oswaldo Cruz, Rio de Janeiro* 1989; *84:* 29–34.

21. Rosen L, Gubler D. The use of mosquitoes to detect and propagate dengue viruses. *Am J Trop Med Hyg* 1974; *23*(6): 1153–60.

22. Kuberski TT, Rosen L. Simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence. *Am J Trop Med Hyg* 1977; *26*(3): 533–7.

23. Tesh RB. A method for the isolation and identification of dengue viruses using mosquito cell culture. *Am J Trop Med Hyg* 1979; *28*(6): 1053–9.

24. Henchal EA, Gentry MK, McCown JM, Brandt WE. Dengue virus specific and flavivirus group determinate identified with monoclonal antibodies by indirect immunofluorescence. *Am J Trop Med Hyg* 1982; *31*(4): 830–6.

25. Thet-win. Detection of dengue virus by immunofluorescence after intracerebral inoculation of mosquitoes. *Lancet* 1982; *1:* 53–4.

26. Waterman SH, Monath TP. Fluorescent antibody techniques applied to the identification of dengue virus in infected tissue. *Adv Virol* 1982; *26*(5): 376–81.

27. Ilkal MA, Dhanda V, Rodrigues JJ, Mohan Rao CV, Mourya DT. Xenodiagnosis of laboratory acquired dengue infection by mosquito inoculation and immunofluorescence. *Indian J Med Res* 1984; *79:* 587–90.

28. Watt DM, Harrison BA, Nisalak A, Scott R, Burke DS. Evaluation of *Toxorhynchites splendens* (Diptera: Culicidae) as a bioassay host for dengue viruses. *J Med Entomol* 1982; *19:* 54.

29. Dhanda V, Padbidri VS, Mourya DT. A rapid diagnostic test for 'Q' fever employing immunofluorescence and the mosquito inoculation techniques. *Curr Sci* 1985; *54* (15): 745–7.

30. Lowenberger C. Innate Immune response of *Aedes aegypti*. *Insect Bio Mol Biol* 2001; *31*(3): 219–29.

31. Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspectives in innate immunity. *Science* 1999; *284*(5418): 1313–8.

32. Arthropod-borne and Rodent-borne Viral Diseases. Geneva: World Health Organization 1985. *Tech Rep Ser* No. 719.

33. Mourya DT, Malunjkar AS, Banerjee K. Susceptibility and transmissibility of *Aedes aegypti* to four strains of Chikungunya virus. *Indian J Med Res* 1987; *86:* 185–90.

34. Mourya DT, Padbidri VS. The use of mosquito inoculation system for testing the efficacy of antirickettsial drugs. *Indian J Parasitol* 1987; *11:* 123–5.

35. Mourya DT. Rapid detection of Japanese encephalitis virus by immunofluorescence after intracerebral inoculation of mosquito larvae. *Tran R Soc Trop Med Hyg* 1990; *84*(4): 580.

36. Mourya DT, Banerjee K. Experimental transmission of Chikungunya virus by *Aedes vittatus* mosquitoes. *Indian J Med Res* 1987; *86:* 269–71.

37. Mourya DT, Raut CG, Deolanker RP, Goverdhan MK, Gokhale MD, Lande CB. Failure of *Aedes aegypti* mosquitoes to pickup infection when fed on dengue virus infected immunocompromised nude mice. *Acta Virol* 1997; *41:* 57–8.

38. Mourya DT, Gokhale MD, Basu A, Barde PV, Sapkal GN, Padbidri VS, Gore MM. Horizontal and vertical transmission of dengue-2 virus in highly and lowly susceptible strains of *Aedes aegypti* mosquitoes. *Acta Virol* 2001; *45:* 67–71.

39. Mourya DT, Thakare JP, Gokhale MD, Powers AM, Hundekar SL, Jayakumar PC, Bondre VP, Shouche YS, Padbidri VS. Isolation of chikungunya and dengue viruses from dually infected *Aedes aegypti* mosquitoes collected in nature from Yawat Town, Pune district, Maharashtra State, India. *Acta Virol* 2001; *45:* 305–9.

40. Padbidri VS, Mourya DT, Dhanda V. Inhibitory action of penicillin and streptomycin on the multiplication of *Coxiella burnetii* in *Aedes aegypti* infected by intrathoracic inoculation. *Proc III Oriental Entomological Symposium 1987; 3:* p. 39–42.

41. Turell MJ, Malinoski FJ. Limited potential for mosquito transmission of a live, attenuated chikungunya virus vaccine. *Am J Trop Med Hyg* 1992; *47*(2): 98–103.

42. Mourya DT, Ilkal MA, Mishra AC, George Jacob P, Pant U, Ramanujam S, Mavale MS, Bhat HR, Dhanda V.

Isolation of Japanese encephalitis virus from mosquitoes collected in Karnataka State, India from 1985 to 1987. *Trans R Soc Trop Med Hyg* 1989; *83:* 550–3.

43. Beerntsen BT, James AA, Christensen BM. Genetics of mosquito vector competence. *Microbiol Mol Biol Rev* 2000; *64*(1): 115–37.

44. Mourya DT, Gokhale MD, Barde PV, Padbidri VS. A simple artificial membrane feeding method for mosquitoes. *Tran R Soc Trop Med Hyg* 2000; *94:* 460.

Corresponding author: Dr. D.T. Mourya, Microbial Containment Complex, National Institute of Virology, Sus Road, Pashan, Pune–411 021, India.
E-mail: mouryadt@vsnl.net

Received: 3 September 2007 *Accepted in revised form:* 5 October 2007