Indian J Med Res 149, June 2019, pp 771-777
DOI: 10.4103/ijmr.IJMR_453_17

Quick Response Code:

Differential susceptibility & replication potential of Vero E6, BHK-21, RD, A-549, C6/36 cells & *Aedes aegypti* mosquitoes to three strains of chikungunya virus

Anakkathil Balan Sudeep[1], Pratik B. Vyas[1], Deepti Parashar[2] & Pratip Shil[3]

[1]Medical Entomology & Zoology, [2]Dengue & Chikungunya & [3]Bioinformatics Groups, ICMR-National Institute of Virology, Pune, India

Received March 15, 2017

***Background & objectives*: Chikungunya virus (CHIKV), a mosquito-borne arthritogenic virus causes infections ranging from febrile illness to debilitating polyarthralgia in humans. Re-emergence of the virus has affected millions of people in Africa and Asia since 2004. During the outbreak, a new lineage of the virus has evolved as an adaptation for enhanced replication and transmission by *Aedes albopictus* mosquito. A study was designed to compare the susceptibility of four vertebrate cell lines, namely Vero E6 (African green monkey kidney), BHK-21 (Baby hamster kidney), RD (human rhabdomyosarcoma), A-549 (human alveolar basal epithelial cell) and C6/36 (*Ae. albopictus*) to Asian genotype and two lineages of East, Central and South African (E1:A226 and E1:A226V) of CHIKV.**

***Methods*: One-step growth kinetics of different CHIKV strains was carried out in the above five cell lines to determine the growth kinetics and virus yield. Virus titre was determined by 50 per cent tissue culture infectious dose assay and titres were calculated by the Reed and Muench formula. Growth and virus yield of the three strains in *Ae. aegypti* mosquitoes was studied by intrathoracic inoculation and virus titration in Vero E6 cell line.**

***Results*: Virus titration showed Vero E6, C6/36 and BHK-21 cell lines are high virus yielding with all the three lineages while RD and A-549 yielded low virus titres. C6/36 cell line was the most sensitive and yielded the maximum titre. *Ae. aegypti* mosquitoes, when inoculated with high titre virus, yielded an almost equal growth with the three strains while rapid growth of E1:A226V and Asian strain was observed with 1 log virus.**

***Interpretation & conclusions*: C6/36 cell line was found to be the most sensitive and high yielding for CHIKV irrespective of lineages while Vero E6 and BHK-21 cell lines yielded high titres and may find application for vaccine/diagnostic development. Infection of *Ae. aegypti* mosquitoes with the three CHIKV strains gave almost identical pattern of growth.**

Key words *Aedes aegypti* - C6/36 cells - Chikungunya - growth kinetics - vertebrate cell lines

Chikungunya virus (CHIKV), an arbovirus belonging to the genus *alphavirus*, family *Togaviridae*, has become health concern in countries in Africa, Indian Ocean basin, India, Southeast Asian countries

© 2019 Indian Journal of Medical Research, published by Wolters Kluwer - Medknow for Director-General, Indian Council of Medical Research

and recently the Caribbean Islands and the Americas[1-5]. In the Americas, autochthonous transmission was reported first in 2013 in St. Martin followed by 26 Islands and 14 mainland countries with millions of cases[5]. CHIKV infection is generally self-limiting, but in a small percentage of cases, persistence of arthralgia for years has been reported. Although the exact mechanism of persistence of the manifestations is not clearly understood, it has been postulated that viral antigen persists in the skeletal muscle progenitor cells and passes on to muscle cells during development, causing recurrent myalgia/arthralgia[6]. The re-emergence was characterized by debilitating polyarthralgia associated with a number of unusual clinical manifestations either singly or in combination[3]. The outbreak which commenced in 2004 in the Lamu Island, Kenya had shown several clinical complications which are unheard of CHIKV infection *i.e.* swelling of limbs, periventricular and meningoencephalitis in neonates, hepatic and renal dysfunctions, hypokalemic paralysis, hearing loss, ophthalmic involvement, acute flaccid paralysis and Guillain-Barre Syndrome[7-15]. Mother to child transmission and CHIKV-associated mortality were also reported in La Reunion Islands as well as in India for the first time[12,14,15]. Although rare, mortality has been reported from La Reunion Islands, India, Malaysia, Brazil, *etc*[13,16-18].

The re-emergence of CHIKV in India after a gap of 32 years has been explosive and affected more than six million people in 29 States/Union Territories[1,19,20]. Though the high incidence declined after 2007, the virus is still prevalent in the country with sporadic outbreaks[4,5,20]. Introduction of the African lineage, the East Central South African (ECSA) for the first time, wide prevalence of both the vectors (*Aedes aegypti* and *Ae. albopictus)* and a naïve population were attributed to the plausible reasons for the massive outbreak. India also witnessed introduction of the mutated strain of CHIKV *i.e.* ECSA (E1:A226V) in Kerala and Karnataka, where enhanced transmission of the virus by *Ae. albopictus* was observed[21,22].

Since no vaccines or antivirals are available commercially, early diagnosis has been the mainstay in outbreak management. Although several techniques are available for diagnosis, virus isolation has remained the gold standard as it provides the most conclusive evidence of the aetiological agent. The availability of well-characterized cell lines, modern infrastructural facilities and trained personnel have enhanced the rate of virus isolations from clinical samples[18,23]. The cell culture system has replaced infant mice and other systems for virus isolation due to their high sensitivity and virus yield. Well-characterized cell lines, namely Vero, BHK-21 (Baby hamster kidney) and MRC-5 have been employed successfully not only for virus isolation but also for the development of attenuated and inactivated vaccines globally. The advancements made in the development and maintenance of cell lines have made their management easier than other systems, namely infant mouse inoculation and egg inoculation.

CHIKV replicated in a broad spectrum of cell lines originated from monkeys, humans, mosquitoes, *etc* and has been used routinely for virus isolation and propagation[18,23]. A comparative analysis was, therefore, made in this study to determine the differential susceptibility of five cell lines originating from different hosts [Vero E6, BHK-21, RD (human rhabdomyosarcoma), A-549 (human alveolar basal epithelial cells) and C6/36 (*Ae. albopictus*)] and one strain of *Ae. aegypti* mosquitoes to three different lineages of CHIKV including the virus with changed genome, for susceptibility and virus yield.

Material & Methods

Virus: CHIKV strains 634029 (Asian, Acc. No. EF027140), 061573 (E1:A226, Acc. No. EF027134) and 074831 (E1:A226V, Acc. No. FJ000069) were obtained from the virus repository maintained at the National Institute of Virology (NIV), Pune, India, in the lyophilized form. All the viruses were screened by the NIV before lyophilization. CHIKV strain 634029 was isolated during the 1963 outbreak in Calcutta (now Kolkata), West Bengal, from human serum while 061573 and 074831 were isolated from human serum samples during 2006 and 2007 CHIKV outbreaks in Andhra Pradesh and Kerala, respectively. Strain No. 634029 has undergone 12 passages in infant mouse and two passages in Vero E6 cell line while 061573 and 074831 have undergone one passage in C6/36 cell line (*Ae. albopictus*) followed by four passages in Vero E6 cell line.

Cell lines: Five cell lines of different hosts of origin *i.e.* Vero E6 (African green monkey kidney), Passage (P) No. 220-225, BHK-21 (Baby hamster kidney), P. No. 72-78, RD (human rhabdomyosarcoma, received from WHO), P. No. 120-130, A-549 (human alveolar basal epithelial), P. No. 88-94 and C6/36, P-130-134 were used in the study. Vertebrate cell lines were maintained in minimum essential medium (MEM, HiMedia, Mumbai) supplemented with 10 per cent foetal bovine

serum (FBS, Invitrogen, USA) at 37°C with five per cent CO_2 and 75 per cent humidity while C6/36 cells were maintained in Mitsuhashi Maramorosch medium (HiMedia, Mumbai) supplemented with 10 per cent FBS at 28°C.

Mosquitoes: *Ae. aegypti* mosquitoes were obtained from the insectary maintained at the Entomology Division of NIV, Pune. The mosquito larvae were fed on commercially available fish food (Aakar, Mumbai) while adults were maintained on 10 per cent glucose solution. For oviposition, adults were fed occasionally on fowl blood. The mosquito colony was maintained at 28±2°C with relative humidity of 80±5 per cent and 12:12 h dark:light cycle.

Infection of cell lines: Growth kinetic study of the three strains were carried out in VeroE6, BHK-21, RD and A-549 and C6/36 cell lines as described earlier[23]. Cells were grown to 90-95 per cent confluence in 24-well plates (Nunc, Denmark) and were infected independently with the three strains of CHIKV at 10 MOI (multiplicity of infection). Cell supernatants were collected at daily intervals for six days and stored at −80°C (New Brunswick, USA) until further analysis.

Infection of mosquitoes: Intrathoracic inoculation of mosquitoes was carried out in a biosafety level 2 arthropod containment facility maintained at NIV, Pune, as described previously[24]. Three to four days old female *Ae. aegypti* mosquitoes (n=60) were inoculated intrathoracically with individual CHIKV strains at the rate 0.2 µl virus suspension per mosquito. After infection, the mosquitoes were maintained on 10 per cent glucose solution and incubated at 28°C for 15 days. Five mosquitoes each were harvested on '0' day, day 1 post infection (PI) and on every alternate days thereafter till day 15 PI, stored at −80°C till completion of the experiment and titrated in Vero E6 cells as described below. Pools of five mosquitoes collected on the particular day PI were processed as single sample (not individual mosquitoes).

Virus quantification by 50% tissue culture infectious dose ($TCID_{50}$) assay: Virus quantitation was carried out using 50 per cent tissue culture infectious dose ($TCID_{50}$/ml) method as described earlier[24]. Frozen samples were retrieved, thawed quickly, spun at 2790×g for 20 min at 4°C and the supernatant was diluted serially (10-fold) in MEM supplemented with 2 per cent FBS. The serially diluted virus was inoculated over Vero E6 cell line grown to confluent monolayer in 96 well plates (Nunc, Denmark) in quadruplicate. Each plate had 12 wells as negative controls which were inoculated only with culture medium. Cytopathic changes in the infected wells were compared with the negative controls during scoring of infection. The cultures were incubated at 37°C for 96 h, scored cytopathic effect (CPE) under an inverted microscope, stained with amido black and determined virus titre as described by Reed and Muench[25]. Mosquito samples were triturated using a battery-operated hand-held homogenizer (Sigma, USA) with sterile disposable pestles in 1 ml chilled MEM supplemented with 2 per cent FBS, Millipore filtered (0.22 µm), diluted serially and determined $TCID_{50}$/ml in Vero E6 cell line.

Results

Growth kinetics of CHIKV strain 634029 in different cell lines: The Asian strain replicated in all the five cell lines used in the study with differential virus yields (Fig. 1). Although higher virus yield (~8 log $TCID_{50}$/ml) was obtained in Vero E6, C6/36 and BHK-21 cell lines, C6/36 cell line maintained the titre consistently throughout the study period. Vero E6 and BHK-21 cell lines have maintained the titre of 8 log till the day 4 PI, but declined subsequently. Virus growth in RD cell line was comparatively low and the maximum titre was achieved on day 3 PI yielding almost 8 log, but a rapid decline in virus yield was observed thereafter. A-549 cell line, though susceptible, was the least productive as maximum virus yield obtained was just above 4 log during 1st to 5th day PI.

Growth kinetics of ECSA A226 strain (African) in different cell lines: All the cell lines replicated the

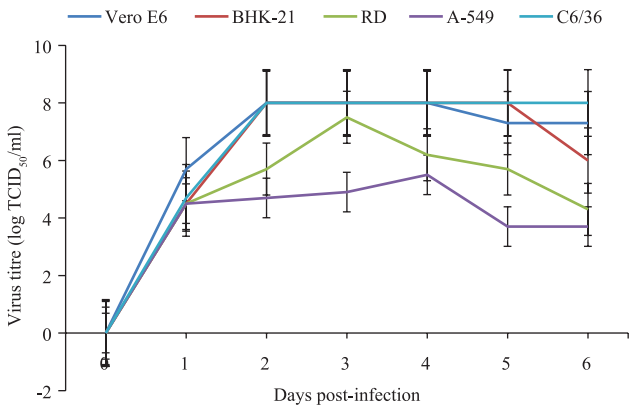

Fig. 1. Growth kinetics of chikungunya virus strain 634029 (Asian strain) in five cell lines. Each experiment was performed in triplicate. Values are provided as mean±SD.

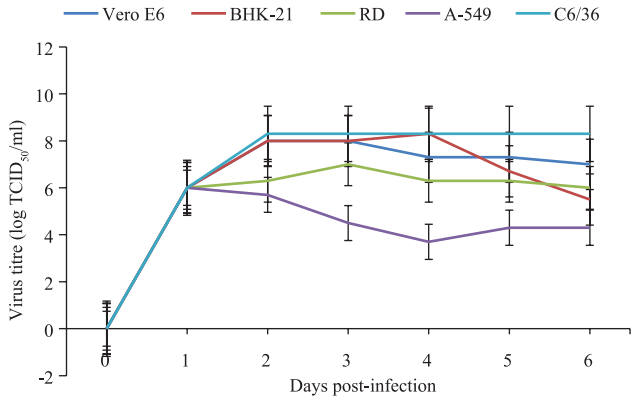

Fig. 2. Growth kinetics of chikungunya virus strain E1:A226 in five cell lines. Each experiment was performed in triplicate. Values are provided as mean±SD.

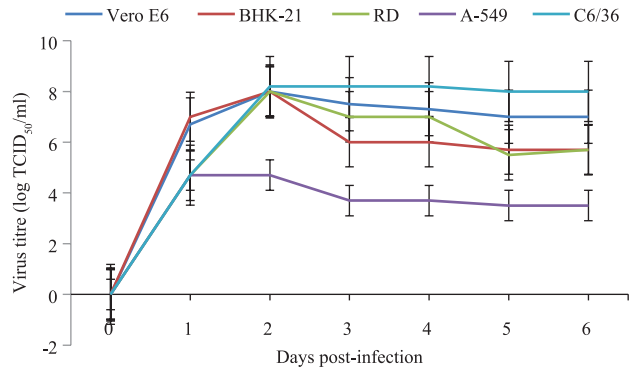

Fig. 3. Growth kinetics of chikungunya virus (E1:A226V strain) in five cell lines. Each experiment was performed in triplicate. Values are provided as mean±SD.

strain, and the replication kinetics was found almost similar to that of the Asian strain (Fig. 2). Maximum virus yield was in C6/36 cell line which showed a consistent yield of virus till day 6 PI. Vero E6 cell line also yielded the maximum titre of 8 log but only till day three PI and thereafter showed a slight decline in virus titre. Growth kinetics of the strain in BHK-21 cell line was equivalent to that of Asian strain and maintained a titre of 8 log till day 4 PI. However, a sharp decline in virus titre was seen yielding 3 log on day 6 PI. RD and A-549 cell lines though replicated the strain were found less virus yielding as the maximum yield was 6.5 and 6 log, respectively. The latter has showed a sharp decline after the 1st day PI and maintained approximately 4 log till day 6 PI.

Growth kinetics of CHIKV (E1:A226V) strain in different cell lines: The replication profile of the strain in the cell lines was almost similar to that of the other two strains discussed earlier (Fig. 3). The strain showed rapid replication in C6/36, Vero E6, BHK-21 and RD cells yielding ≥8 log virus yield on day 2 PI. However, except for C6/36 cell line, virus yield declined on subsequent days PI. Virus yield in Vero E6 cell line was consistent though loss of 1 log in virus titre was observed from day 3 PI. BHK-21 and RD cell lines showed a sudden decline in virus yield after day 2 PI but maintained a titre of approximately 6 log throughout the study period. A-549 cell line was the least productive. Except for the initial spurt on day 1 PI reaching >4 log, the virus yield declined to <4 log on subsequent days PI.

Replication kinetics of three strains of CHIKV in Aedes aegypti mosquitoes: *Ae. aegypti* mosquitoes showed replication of the three CHIKV strains with different virus yields (Fig. 4A & B). When infected with a high titre (approximately 3 log), growth kinetics of Asian and ECSA (E1:A226) were found almost identical with an initial spurt in virus titre yielding 5.7 log on day 1 PI. The latter, however, maintained the virus titre till day 7 PI while a rapid decline in virus titre was detected in the former (Fig. 4A). In comparison to the growth kinetics of the other two strains, the growth of ECSA (E1:A226V) was slow with a maximum yield of 4.5 log. However, the mosquito maintained the titre for 15 days without showing drastic decline. Despite the difference in the virus yield in the initial stages by the three strains, the mosquitoes maintained the virus with a titre approximately equal to 3 log throughout the study period.

When the mosquitoes were infected with a low titre of virus (1 log), rapid increase in virus replication was observed in Asian and ECSA (E1:A226V) strains on day 1 PI with a four-fold increase in virus titre (Fig. 4B). In the other African strain (E1:A226), however, the yield was only 2 log (2-fold) during the same period. Although all the three strains showed almost equal virus yield, the Asian strain showed slightly higher yield on day 3 PI. As far as the maintenance of the virus in the inoculated mosquitoes was concerned, the mosquito maintained all the strains throughout the study period. Viral growth kinetics of Asian and the mutated strain of ECSA (E1:A226V) had similar pattern though virus yield varied after day 7 PI.

Discussion

CHIKV induced distinct cytopathic effects in vertebrate cell lines and showed high level of apoptosis within 2-3 days of infection[18]. The virus infects macrophages, fibroblasts, endothelial, epithelial

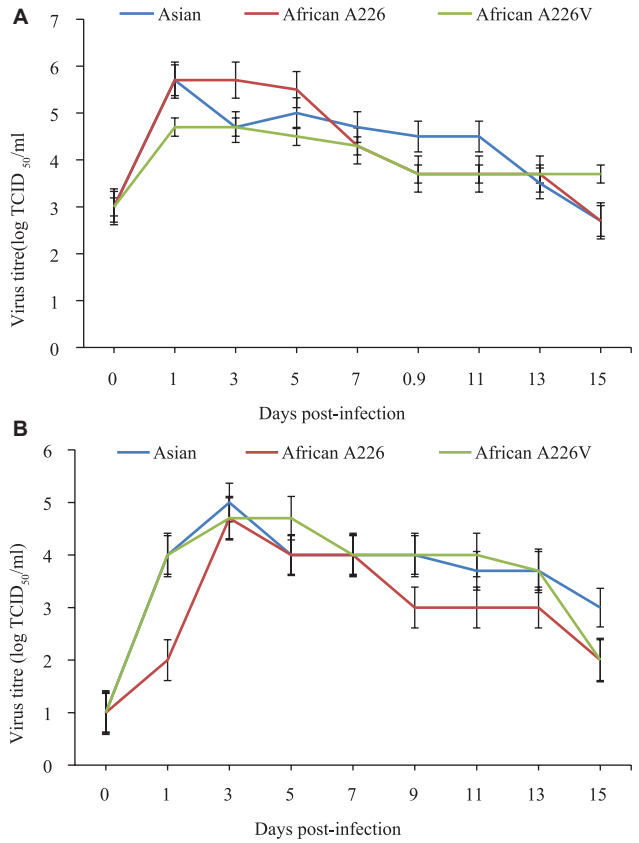

Fig. 4. Replication kinetics of three chikungunya virus strains in *Aedes aegypti*. with high (**A**) and low (**B**) dose of virus. Each experiment was performed in triplicate. Values are provided as mean±SD.

cells, *etc* in humans but not dendritic cells or T and B lymphocytes[26]. It has also been reported that fibroblast cells are the targets of CHIKV replication *in vivo*. In the present study, it was, therefore, decided to compare the potential of fibroblast-like and epithelial-like cells to replicate different strains of CHIKV *in vitro*. C6/36 cells developed from *Ae. albopictus* mosquitoes were found to be the most permissible cell culture system for all the three strains of CHIKV. The cells not only yielded high virus titre but also maintained the titre throughout the study period irrespective of virus strain. However, distinct CPE could not be seen in the cells with any of the virus strains. The ECSA (E1:226V) strain, a mutant of the African strain, however, had an edge over the other strains in virus yield in the cell. It is expected as CHIKV got mutated during the latter half of 2005 as an adaptation to grow in *Ae. albopictus* mosquitoes during the outbreak in La Reunion Island[3-5,11]. The strain is a recent mutant of the ECSA strain with a mutation in the E1 protein at position 226 replacing the amino acid alanine with valine, which has enabled the virus to adapt for enhanced virus transmission by *Ae. albopictus* mosquitoes[4,27]. Similar results were reported by Wikan *et al*[18] when they conducted a comparative growth study with three strains of CHIKV, and observed higher level of infectivity by the ECSA E:A226 and ECSA E:A226V strains, in comparison to Ross strain, the original ECSA strain. They opined that higher infectivity by the new strains could be due to genetic changes occurred to the original strain during the passage of time. This has been substantiated experimentally by Tsetsarkin *et al*[27] as they demonstrated several changes in the virus genome, especially in the E2 protein during the recent mutation of ECSA strain. Wikan *et al*[18] also observed significant change in viral infectivity of the three strains of CHIKV in an *Ae. aegypti* cell line (CCL-125). They demonstrated higher infectivity of the mutated strain (E1:A226V), substantiating the earlier reports of higher infectivity by the strain in *Ae. aegypti* mosquitoes[27,28]. In our study with *Ae. aegypti* mosquitoes higher replication of the strain was observed as mosquitoes infected with 1 log $TCID_{50}$/ml virus yielded a four-fold increase in virus titre at 24 h PI. This has also been observed with Asian strain probably due to laboratory adaptation after serial passages in mice (12 passages). In mosquitoes infected with higher dose of virus, no difference in virus yield was observed among the three strains. When the dose of virus infection was compared, no change in virus growth pattern was observed except for virus yield. With high dose of infection, the peak virus yield was almost 1 log more than the low dose of infection. It was interesting to note that irrespective of the titres used for infection of ECSA (A226V), virus yield in *Ae. aegypti* mosquitoes was equal (4.5 log $TCID_{50}$/ml) and maintained the titre without much change throughout the study period. The mode of infection in the present study was parenteral inoculation which is not the natural route. Therefore, more systematic studies with natural route of infection are needed to make any conclusive statements.

Comparing other cell lines for virus susceptibility and yield, Vero E6 was found highly consistent as the virus yield was high with the three strains. Vero E6, which has a broad spectrum susceptibility to a large number of viruses, is used globally for virus isolation[23]. It is also approved by the WHO for the production of vaccines of human use and has been used to develop a range of vaccines, namely influenza, rabies, and Japanese encephalitis (JEV)[29-31]. A comparable yield of CHIKV was obtained in BHK-21 cell line irrespective of the CHIKV strains. In comparison, the other two cell

lines employed in the study, namely RD and A-549, CHIKV yield was comparatively low. In the present study, virus yield in vertebrate cell lines declined faster than that of C6/36, which remained viable for 7-10 days without showing CPE. Hence, all the experiments were terminated at day 6 PI.

In the present study, $TCID_{50}$ method was the only assay used to quantitate the virus yield. Since our objective was to determine the viable virus to determine growth, other assays, namely RT-PCR or qPCR were not used.

In conclusion, five cell lines (Vero E6, BHK-21, RD, C6/36 and A-549) were compared for their susceptibility and virus yield to the three lineages of chikungunya virus. Vero E6, BHK-21 and C6/36 cell lines yielded high titres to the three lineages of the virus while RD and A-549 cells were found low virus yielding. The *Ae. aegypti* mosquitoes showed an identical pattern of virus growth despite infection with two different doses of virus.

***Financial support & sponsorship*:** Authors thank the Director for supporting the study from the intramural funding of ICMR-NIV.

***Conflicts of Interest*:** None.

References

1. Ang LW, Kam YW, Lin C, Krishnan PU, Tay J, Ng LC, *et al.* Seroprevalence of antibodies against chikungunya virus in Singapore resident adult population. *PLoS Negl Trop Dis* 2017; *11* : e0006163.
2. Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, de Lamballerie X. Chikungunya in the Americas. *Lancet* 2014; *383* : 514.
3. Weaver SC. Arrival of chikungunya virus in the new world: Prospects for spread and impact on public health. *PLoS Negl Trop Dis* 2014; *8* : e2921.
4. Weaver SC, Lecuit M. Chikungunya virus and the global spread of a mosquito-borne disease. *N Engl J Med* 2015; *372* : 1231-9.
5. Petersen LR, Powers AM. Chikungunya: Epidemiology. *F1000Res* 2016; *5*. pii: F1000 Faculty Rev-82.
6. Ozden S, Huerre M, Riviere JP, Coffey LL, Afonso PV, Mouly V, *et al.* Human muscle satellite cells as targets of chikungunya virus infection. *PLoS One* 2007; *2* : e527.
7. Acevedo N, Waggoner J, Rodriguez M, Rivera L, Landivar J, Pinsky B, *et al.* Zika virus, chikungunya virus, and dengue virus in cerebrospinal fluid from adults with neurological manifestations, Guayaquil, Ecuador. *Front Microbiol* 2017; *8* : 42.
8. Foissac M, Javelle E, Ray S, Guérin B, Simon F. Post-chikungunya rheumatoid arthritis, saint martin. *Emerg Infect Dis* 2015; *21* : 530-2.
9. Powers AM, Logue CH. Changing patterns of chikungunya virus: Re-emergence of a zoonotic arbovirus. *J Gen Virol* 2007; *88* : 2363-77.
10. Mahendradas P, Avadhani K, Shetty R. Chikungunya and the eye: A review. *J Ophthalmic Inflamm Infect* 2013; *3* : 35.
11. Tsetsarkin KA, Weaver SC. Sequential adaptive mutations enhance efficient vector switching by chikungunya virus and its epidemic emergence. *PLoS Pathog* 2011; *7* : e1002412.
12. Chopra A, Anuradha V, Ghorpade R, Saluja M. Acute chikungunya and persistent musculoskeletal pain following the 2006 Indian epidemic: A 2-year prospective rural community study. *Epidemiol Infect* 2012; *140* : 842-50.
13. Brito CAA. Alert: Severe cases and deaths associated with chikungunya in Brazil. *Rev Soc Bras Med Trop* 2017; *50* : 585-9.
14. Pialoux G, Gaüzère BA, Jauréguiberry S, Strobel M. Chikungunya, an epidemic arbovirosis. *Lancet Infect Dis* 2007; *7* : 319-27.
15. Couderc T, Chrétien F, Schilte C, Disson O, Brigitte M, Guivel-Benhassine F, *et al.* A mouse model for chikungunya: Young age and inefficient type-I interferon signaling are risk factors for severe disease. *PLoS Pathog* 2008; *4* : e29.
16. Mavalankar D, Shastri P, Bandyopadhyay T, Parmar J, Ramani KV. Increased mortality rate associated with chikungunya epidemic, Ahmedabad, India. *Emerg Infect Dis* 2008; *14* : 412-5.
17. Manimunda SP, Singh SS, Sugunan AP, Singh O, Roy S, Shriram AN, *et al.* Chikungunya fever, Andaman and Nicobar Islands, India. *Emerg Infect Dis* 2007; *13* : 1259-60.
18. Wikan N, Sakoonwatanyoo P, Ubol S, Yoksan S, Smith DR. Chikungunya virus infection of cell lines: Analysis of the east, central and South African lineage. *PLoS One* 2012; *7* : e31102.
19. Das T, Jaffar-Bandjee MC, Hoarau JJ, Krejbich Trotot P, Denizot M, Lee-Pat-Yuen G, *et al.* Chikungunya fever: CNS infection and pathologies of a re-emerging arbovirus. *Prog Neurobiol* 2010; *91* : 121-9.
20. Mourya DT, Sudeep AB. Arboviral infections: A threat for 21st century. In: Kumar A, Rodrigues S, Dias A, editors. *Major tropical diseases: Public health perspective*. Panjim, Goa, India: Broadway Publishing House; 2014. p. 211-39.
21. Kumar NP, Joseph R, Kamaraj T, Jambulingam P. A226V mutation in virus during the 2007 chikungunya outbreak in Kerala, India. *J Gen Virol* 2008; *89* : 1945-8.
22. Santhosh SR, Dash PK, Parida MM, Khan M, Tiwari M, Lakshmana Rao PV, *et al.* Comparative full genome analysis revealed E1: A226V shift in 2007 Indian chikungunya virus isolates. *Virus Res* 2008; *135* : 36-41.
23. Jadi RS, Sudeep AB, Kumar S, Arankalle VA, Mishra AC. Chandipura virus growth kinetics in vertebrate cell lines, insect cell lines & embryonated eggs. *Indian J Med Res* 2010; *132* : 155-9.
24. Sudeep AB, Ghodke YS, Gokhale MD, George RP, Dhaigude SD, Bondre VP, *et al.* Replication potential and different modes of transmission of West Nile virus in an Indian strain of *Culex gelidus* Theobald (Diptera: Culicidae) mosquitoes. *J Vector Borne Dis* 2014; *51* : 333-8.

25. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938; *27* : 493-7.
26. Schilte C, Staikowsky F, Couderc T, Madec Y, Carpentier F, Kassab S, *et al.* Chikungunya virus-associated long-term arthralgia: A 36-month prospective longitudinal study. *PLoS Negl Trop Dis* 2013; *7* : e2137.
27. Tsetsarkin KA, McGee CE, Volk SM, Vanlandingham DL, Weaver SC, Higgs S, *et al.* Epistatic roles of E2 glycoprotein mutations in adaption of chikungunya virus to *Aedes albopictus* and *Ae. aegypti* mosquitoes. *PLoS One* 2009; *4* : e6835.
28. Vazeille M, Moutailler S, Coudrier D, Rousseaux C, Khun H, Huerre M, *et al.* Two chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. *PLoS One* 2007; *2* : e1168.
29. Barrett PN, Mundt W, Kistner O, Howard MK. Vero cell platform in vaccine production: Moving towards cell culture-based viral vaccines. *Expert Rev Vaccines* 2009; *8* : 607-18.
30. Kulkarni PS, Sahai A, Gunale B, Dhere RM. Development of a new purified vero cell rabies vaccine (Rabivax-S) at the serum institute of India Pvt Ltd. *Expert Rev Vaccines* 2017; *16* : 303-11.
31. Chen HL, Chang JK, Tang RB. Current recommendations for the Japanese encephalitis vaccine. *J Chin Med Assoc* 2015; *78* : 271-5.

For correspondence: Dr Anakkathil Balan Sudeep, ICMR-National Institute of Virology, Microbial Containment Complex, Sus Road, Pashan, Pune 411 021, Maharashtra, India
e-mail: sudeepmcc@yahoo.co.in